Molecular diagnostic markers for blood immune-metabolic indices and oxidative stress in production diseases during transition period and its management in buffaloes

By

Savleen Kour

(J-18-MV-538)

Thesis submitted to
Faculty of Postgraduate Studies
in partial fulfillment of requirements
for the degree of

MASTER OF VETERINARY SCIENCE
IN
VETERINARY MEDICINE

Division of Veterinary Medicine

Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu
Main Campus, Chatha, Jammu- 180009

2021

CERTIFICATE-I

This is to certify that the thesis entitled "**Molecular diagnostic markers for blood immune-metabolic indices and oxidative stress in production diseases during transition period and its management in buffaloes"** submitted in partial fulfillment of the requirements for the degree of **Master of Veterinary Sciences in subject of Veterinary Medicine** is a record of bonafide research, carried out by **Ms. Savleen kour** Registration No. **J-18-MV-538** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. It is further certified that help and assistance received during the course of thesis investigation have been duly acknowledged.

Dr. Neelesh Sharma
(Major Advisor)

Place: R. S. Pura

Date: 18/03/2021

Endorsed :

Dr. Rajiv Singh
Professor and Head
Division of Veterinary Medicine

Date: 18/03/2021

CERTIFICATE-II

We, the members of Advisory Committee of **Ms. Savleen Kour**, Registration No. **J-18-MV-538,** a candidate for the degree of **Master of Veterinary Sciences in subject of Veterinary Medicine** have gone through the manuscript of the thesis entitled "**Molecular diagnostic markers for blood immune-metabolic indices and oxidative stress in production diseases during transition period and its management in buffaloes**" and recommend that it may be submitted by the student in partial fulfillment of the requirements for the degree.

Dr. Neelesh Sharma
Major Advisor & Chairman
Advisory Committee

Place: R.S. Pura

Date: 18/03/2021

Advisory Committee Members

Dr. S.K. Gupta
Professor
Division of Veterinary Medicine

Dr. A.K. Pathak
Assistant Professor (SS)
Division of Animal Nutrition

Dr. N.K. Pankaj
Assistant Professor (SS)
Division of Veterinary Pharmacology
(Dean Nominee)

CERTIFICATE–III

This is to certify that the thesis entitled "**Molecular diagnostic markers for blood immune-metabolic indices and oxidative stress in production diseases during transition period and its management in buffaloes**" submitted by **Ms. Savleen Kour**, Registration No. **J-18-MV-538,** to the Faculty of Post Graduate Studies, Sher-e-Kashmir University of Agricultural Sciences and Technology-Jammu, in partial fulfillment of the requirement for the degree of **Master of Veterinary Sciences in subject of Veterinary Medicine** was examined and approved by the advisory committee and external examiner on 25-03-2021.

25.03.2021

External Examiner
(Dr. V.S. Wazir)
Retd. Professor
Division of Veterinary Medicine
F.V.Sc. & A.H., R.S. Pura, Jammu, J&K

Dr. Neelesh Sharma
Major Advisor

Dr. Rajiv Singh
Professor& Head
Division of Veterinary Medicine

Dr. M.S Bhadwal
Dean
F.V.Sc & A.H, R.S. Pura

ACKNOWLEDGMENT

"In the name of Waheguru Ji the most Beneficient, the most Merciful"

First and foremost, I wish to express my gratitude towards God who gave me the strength and courage to fulfill my dreams and has showered me with his choicest blessings always.

At the outset, I really feel the need to thank all the people around me who gave me support in one or the other way in completing my work. A true guide is a person who makes us to create our own milestones, rather than follow footsteps of others. I feel obliged and greatly privileged to express my gratitude towards my respected Major advisor ***Dr. Neelesh Sharma,*** *Assistant Professor (SS), Division of Veterinary Medicine, F.V.Sc and A.H. SKUAST-J for his unparalleled and excellent guidance, continuous encouragement and support in completion of my research successfully. His discipline, principles and fearless work environment was cherished during the course. I feel so lucky to have an advisor who always finds the time for listening to even little problems unavoidably faced in the course of performing research.*

I owe special gratitude and feel highly esteemed to thank members of my Advisory committee, ***Dr. S.K Gupta*** *, Professor VMD,* ***Dr A.K Pathak,*** *Asstt. Professor, Division of Animal nutrition and* ***Dr. N.K. Pankaj****, Asstt Professor, Division of Veterinary pharmacology for their suggestions and unconditional support to complete my work.*

I express my appreciation and thanks to ***Dr. Rajiv singh****, Professor and Head (VMD), for providing help, cooperation, valuable guidance and facilities at various stage of my study. I also owe my sincere thanks to other faculty members of VMD namely,* ***Dr. J.S. Soodan****, Professor and Head (TVCC),* ***Dr. Rajesh Agrawal****, Associate Professor (VMD),* ***Dr. Abha Tikoo****, Assistant Professor (VMD),* ***Dr. S.R. Upadhyay****, Assistant Professor (VMD) and* ***Dr. R.K. Bhardwaj****, Assistant Professor (VMD), for their genuine interest, guidance and conceptual technical ideas during the course of study.*

I am highly thankful to ***Prof. J.P Sharma****, Hon'ble Vice Chancellor of SKUAST-Jammu,* ***Dr. S.K Gupta*** *Director Education for being kind enough to provide me all the necessary facilities to carry out my research programme.*

Thanks are duly acknowledged to the official staff of Division of Veterinary Medicine specially ***Sh. Rajesh Ganjoo, Sh. Yash Pal Singh, Sh. Rakesh Bharti*** *and* ***Sh. Vinod Kumar*** *for their cooperation during my entire post graduate programme.*

I am also very much thankful to my seniors ***Dr. Zul-i-Huma, Dr Touqeer Ahmed and Mrs. Sapna sahni*** *for their wholehearted support, constant inspiration during the research.*

My time in faculty would have been futile and less productive without the support of my batchmate ***Dr. Fariha saleem*** *for their wholehearted support, constant inspiration, sharing the moments of laughter, sorrow during this investigation and serving as the pillars of my college life.*

Having friend is a blessing one can only hope for, friend is one to share all our ups and downs as we go through this roller coaster we call life, words are often inadequate to express my feelings but from core of my heart I would like to express my special thanks to ***Karman Kour, Anna Singh, Surbhi Gupta, Abhishek Mahajan and Anuj Mahajan*** *for their extreme psychological and moral support during the times when I required it the most.*

Cousins are special friends from the same family tree that love you unconditionally forever. A special appreciation for my cousins ***Miss Chanan deep kour, Ajay pratap singh and Avninder singh*** *for acting as my stress busters and putting a constant smile on my face.*

It is equally imperative for me to mention my deepest gratitude to my family members who have been constant source of inspiration to me to make me overcome my exhaustion and enable me to renew my efforts for completion of my research work. These words can hardly express my feelings for inspiration, encouragement, blessing, moral, emotional support of my grandparents ***Late Nanak singh and Smt. Inder kour*** *and adorable parents* ***Mr. Dilbagh Singh and Mrs. Jagjeet kour*** *Special thanks to my Brother* ***Kamal preet singh*** *for their love and help throughout the course.*

During the course of present study, I have received help from many persons in some way or the other whom I could not mention here individually by name. The short coming may please be pardoned.

Finally once again and above all I express my gratitude to Almighty Waheguru ji for giving me this life for His obedience and to serve other mankind. It is His name which will last forever.

Needless to say, all omissions and errors are mine.

Place: R. S. Pura

Dated:

Savleen Kour

ABSTRACT

Title of Thesis	:	**Molecular diagnostic markers for blood immune-metabolic indices and oxidative stress in production diseases during transition period and its management in buffaloes**
Name of Student	:	**Savleen Kour**
Admission no.	:	**J-18-MV-538**
Major Subject	:	**Veterinary Medicine**
Name and Designation of Major Advisor	:	**Dr. Neelesh Sharma, Assistant Professor, Division of Veterinary Medicine, F.V.Sc & A.H, R.S. Pura, Jammu**
Degree to be awarded	:	**Masters in Veterinary Sciences, (Veterinary Medicine).**
Year of award of degree	:	**2021**
Name of University	:	**Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Jammu (J&K)**

Abstract

A total number of 210 buffaloes, divided in three groups (n=70; each) during transition period were used for the study. Three groups viz Group I (-30 days), Group II (0 days) and Group III (+30 days) were subjected for diagnosis of milk fever, ketosis and mastitis by various tests. Only 37% of milk samples showed MCMT (T/+) with SCC of 278± 0.67(10^3/ml). No case of milk fever and ketosis were found on the basis of biochemical analysis. On the basis of in-silico analysis disease specific genes were selected for milk fever (NUAK1 and NESP55) , ketosis (CPT1A and IGF-1) and mastitis (TNF-α and INF-γ) showed positive results. Significant increase ($p<0.05$) in the expression level of NUAKI for milk fever/hypocalcemia, CPT1A for ketosis/negative energy balance, TNF-α and INF-γ for mastitis/inflammatory conditions from -30 to +30 days were observed. On the basis of expression of genes of NUAK1 and NESP55, 17.14% buffaloes in Group-I, 38% in Group-II and 45% in Group-III were found to have hypocalcemic condition. Buffaloes that showed positive result for genes of negative energy balance were 24%, 47% and 62% in group I, II and III. Hemato-biochemicaly parameters viz WBC, granulocytes, BHBA, SGOT, total cholesterol, GGT, BUN showed significant ($p<0.05$) increase from Gp-I to Gp-III. The level of cytokines; TNF-α and INF-γ showed significant increase ($p<0.05$) from -30 to +30 days. Oxidative stress markers; LPO showed significant increase ($p<0.05$) in Gp-II and anti-oxidants showed significant decrease from -30 to +30 days. Effect of transition feed was evaluated by various parameters and found significant effect on buffaloes. It was concluded that molecular markers are more specific for early diagnosis of production diseases in the buffaloes. It was also recommended that transition feed reduce the probability of occurrence of production diseases and increase the milk production after calving.

Keywords: Transition period, milk fever, ketosis, mastitis, Rother's test, RNA isolation, qRT-PCR, cytokines, Hemato-biochemical, oxidative stress markers.

Signature of the Major Advisor

Signature of the Student

TABLE OF CONTENTS

CHAPTER NO.	PARTICULARS	PAGE NO.
I.	INTRODUCTION	1-5
II.	REVIEW OF LITERATURE	6-32
III.	MATERIALS AND METHODS	33-55
IV.	RESULTS	56-110
V.	DISCUSSION	111-124
VI.	SUMMARY AND CONCLUSIONS	125-129
	REFERENCES	130-153

LIST OF TABLES

Table No.	Title	Page No.
3.1	PCR cyclic conditions for GAPDH primer for validation	39
3.2	Quantitative Real-time PCR cyclic condition	40
3.3	Primer sequences and annealing temperatures of genes of interest.	46
4.1	Top 10 up- and down-regulated genes in milk fever cows	59
4.2	Potential marker genes for detection of milk fever in cows	62
4.3	Top 10 up- and down-regulated genes in bovine ketosis.	65
4.4	Potential marker genes for detection of bovine ketosis	68
4.5	Top 10 up- and downregulated genes in mastitis mammary gland.	71
4.6	Potential marker genes for detection of mastitis in mammary gland	77
4.7	Microarray data of Hypocalcemia/milk fever genes expression from PBMC of buffaloes during the transition period.	78
4.8	Microarray data of genes expression for ketotic or negative balance energy from PBMC of buffaloes during the transition period.	79
4.9	Range BCS during transition period in buffaloes	80
4.10	Estimated Mean±SE values of TNF-alpha and interferon gamma via ELISA kit during the transition period in buffaloes.	81
4.11	Data of fold change in the expression level of selected genes quantitatively for inflammatory conditions/mastitis in buffaloes during transition period	82
4.12	Mean±SE values of different hematological indices in different stages of transition buffaloes.	84
4.13	Biochemical changes during transition period in buffaloes	87
4.14	Oxidative biomarker and anti-oxidants level during transition period in buffaloes	89
4.15	Profile of body condition score during transition period at different time interval in buffaloes.	91

4.16	Mean±SE values of hematological parameters within time period (-30, -15, 0, 15, 30 days) and between the groups (Transition feed group and control group)	92
4.17	Mean±SE values of Biochemical parameters within time period (-30, -15, 0, 15, 30 days) and between the groups (Transition feed group and control group)	96
4.18	Mean±SE values of oxidative biomarkers in transition feed group and control group at different time intervals of study period.	102
4.19	Difference in the fold of expression level of NUAKI gene in feed and control group at different time intervals in buffaloes	104
4.20	Difference in the fold of expression level of NESP55 gene in feed and control group	105
4.21	Difference in the fold of expression level of CPT1A gene in feed and control group at different time intervals in the buffaloes.	106
4.22	Difference in the fold of expression level of IGF-1 gene in feed and control group	107
4.23	Difference in the fold of expression level of TNF-α gene in feed and control group at different time intervals in buffaloes.	108
4.24	Difference in the fold of expression level of INF-gamma gene in feed and control group at different time intervals in buffaloes.	109

LIST OF FIGURES

Figure No.	Title	Page No.
3.1	Measurement of body score in buffaloes	33
4.1	Figure depicting negative results of Rothera's test with positive control	57
4.2	Negative result of ketone bodies detection in urine strips with positive control	57
4.3	Distribution of microarray probe intensities in milk fever and normal samples	58
4.4	Heatmap showing the log-fold changes of genes and hierarchal clustering of various milk fever and normal samples	60
4.5	Principal component analysis (PCA) of milk fever and normal samples sharing different principal components. Red spheres represent milk fever samples whereas blue spheres represent normal samples.	60
4.6	ICGS analysis of transcriptomes for finding markers of milk fever and normal samples.	61
4.7	Distribution of microarray probe intensities in ketosis and healthy samples.	63
4.8	Heatmap showing the log-fold changes of genes and hierarchal clustering of various Ketosis and Healthy samples.	64
4.9	Principal component analysis (PCA) of ketosis and healthy samples sharing different principal components. Red spheres represent ketosis samples whereas blue spheres represent healthy samples	64
4.10	Participation of up-regulated genes in cytokine receptor signaling pathways. Genes in star marked were found to be up-regulated in ketosis samples.	66
4.11	Participation of down-regulated genes in Glycosphingolipid biosynthesis. Genes in star marked were found to be down-regulated in ketosis samples.	66

4.12	Participation of down-regulated genes in fat digestion and absorption. Genes in star marked were found to be down-regulated in ketosis samples.	67
4.13	ICGS analysis of transcriptomes for finding markers of ketosis and healthy.	67
4.14	Distribution of microarray probe intensities in mastitis and healthy samples.	69
4.15	Signal intensity robust multi-array analysis algorithm analysis in mastitis and healthy samples for normalizing the distribution	70
4.16	Heatmap showing the log-fold changes of genes, and hierarchal clustering of various mastitis and healthy samples	72
4.17	Principal component analysis of mastitis and healthy samples sharing different principal components. Red spheres represent mastitis samples, whereas blue spheres represent healthy samples.	72
4.18	Participation of upregulated genes in bovine toll like receptor signaling pathway. Genes highlighted in star symbol were found to be upregulated in mastitis samples	73
4.19	Participation of upregulated genes in bovine TNF signalling pathways. Genes highlighted in star symbol were found to be upregulated in mastitis samples.	74
4.20	Participation of upregulated genes in bovine Chemokine signalling pathway. Genes highlighted in star symbol were found to be upregulated in mastitis samples	74
4.21	Lineage correlation analysis of genes, expressed in mastitis and healthy samples, with other cell and tissue types.	75
4.22	ICGS analysis of transcriptomes for finding markers of mastitis and healthy samples.	76
4.23	Percentage of buffaloes with hypocalcemic condition in three groups of study	78

4.24	Figure showing representation of threshold curve in qRT-PCR for TNF-α and IFN- γ**.** Sample with lowest Cq value is having highest curve in the graph (red line) and those with high Cq value are having lowest threshold curve (green and blue).	82
4.25	Representation of Cq values of selected genes in qRT-PCR software (Mygo mini)after the completion of cyclic conditions. The samples were selected against the target SYBR green dye which binds with the template and increases its florescence during cyclic condition until it reaches to its threshold cure (Cq value). SYBR green in qPCR tracks linearity.	83
4.26	Graphical representation of hematological parameters changes occur during transition period	85
4.27	Graphical representation of changes in biochemical parameters in all three different groups during different time intervals of transition period	88
4.28	Graphical representation of changes in hematological parameters in transition feed and control group at different time interval	94
4.29	Graphical representation of biochemical changes during transition period in feed and control groups.	99
4.30	Graphical representation of changes in oxidative stress markers during transition period in feed and control group at different time intervals	103
4.31	Change in the expression level of NUAKI gene between feed and control group during transition period at different time intervals in buffaloes.	105
4.32	Change in the expression level of NESP55 gene between feed and control group during transition period at time intervals in buffaloes.	106
4.33	Change in the expression level of CPT1A gene between feed and control group during transition period at different intervals in buffaloes	107

4.34	Change in the expression level of IGF-1 gene between feed and control group during transition period at different intervals in buffaloes.	108
4.35	Change in the expression level of TNF-α gene between feed and control group during transition period at different intervals in buffaloes.	109
4.36	Change in the expression level of INF-γ gene between feed and control group during transition period at different intervals in buffaloes.	109

ABBREVIATIONS

%	Percentage
∝	Alpha
≥	greater than or equal to
A: G	Albumin Globulin ratio
Alb	Albumin
ALT	Alanine transaminase
APPs	Acute Phase Proteins
AST	Aspartate transaminase
BCS	Body condition score
BHBA	Beta hydroxy butyric acid
Bp	Base pair
BUN	Biliary urinary nitrogen
℃	Degree Celsius
Ca	Calcium
CAT	Catalase
Cat	Catalogue
Cat.	Catalogue no.
cDNA	Complementary DNA
CF	Crude fibre
Cl	Chloride
CM	Clinical mastitis
CMT	California Mastitis Test
CP	Crude protein
CPT1A	Carnitine palmitoyl transferase 1A
Cq	Quantification cycle

DDH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
DF	Dilution factor
DM	Dry matter
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotide triphosphate
DP	Dry period
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid)
EE	Ether extract
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetate
ELISA	Enzyme linked immunosorbent assay
et al	And his associates
EtBr	Ethidium bromide
g/dL	Gram per decilitre
GAPDH	Glyceraldehydes 3-phosphate dehydrogenase
GGT	Gamma glutamyl transaminase
GHR	Growth hormone receptor
GH	Growth hormone
Glb	Globulin
GM	Group mean
GPx	Glutathione peroxidase
GSH	Glutathione
GSH-Px	Glutathione peroxidase
h	Hour
H_2O_2	Hydrogen peroxide

Hb	Hemoglobin
HC	Housekeeping control gene
HCl	Hydrochloric acid
HDL	High density lipoprotein
HE	Housekeeping experimental gene
HRP	Horse radish peroxidase
IFG-1	Insulin growth factor
IL-6	Interleukin-6
IL-8	Interleukin-8
INF-γ	Interferon gamma
IU/L	International unit per litre
K	Potassium
LPO	Lipid peroxidase
MDA	Malondialdehyde
mEq/L	Milliequivalent per litre
Mg	Magnesium
$MgCl_2$	Magnesium chloride
NEFA	Non esterfied fatty acid
NESP55	Neuroendocrine secretory protein 55
NUAKI	AMP-protein kinase family
min	Minute
ml	Millilitre
mM	milimoles
mm	Millimetre
mM	Millimolar
mmol/L	Millimole per litre

Na	Sodium
Na_2HPO_4	Disodium hydrogen phosphate
$NaHPO_4$	Disodium phosphate
NaN_3	Sodium azide
NFW	Nuclease free water
ng	Nanogram
NH_4OH	Ammonium hydroxide
nm	Nanometer
OD	Optical density
P	Phosphorus
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
Pg	Picogram
pH	Power of hydrogen
PM	Period mean
RBC	Red blood cells
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT-buffer	Reverse transcriptase buffer
qRT-PCR	Quantitative Real time polymerase chain reaction
S. aureus	*Staphylococcus aureus*
SCC	Somatic cell count
SCM	Sub -clinical mastitis
SE	Standard error
sec	Seconds

SGOT	Serum glutamic oxaloactetate transaminase
SGPT	Serum glutamic pyruvic transaminase
SOD	Superoxide dismutase
spp	Species
TAE	Tris-acetate-EDTA
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TC	Test control gene
TCA	Trichloro acetic acid
TE	Test experimental gene
TLC	Total leukocyte count
TM	Trade mark
TMB	3,3,5,5’-tetra methyl benzidine
TNF-∝	Tumor necrosis factor alpha
TNF-α	Tumor necrosis factor
TP	Total protein
V/cm	Volt per centimetre
v/v	Volume by volume
w/v	Weight by volume
xg	Acceleration due to gravity
$ZnSO_4$	Zinc sulphate
β	Beta
µL	Microlitre

Chapter-I

Introduction

CHAPTER-1

INTRODUCTION

Transition period is described as period from 3 weeks pre-calving to 3 weeks post-calving (Grummer, 1995). This period is the most critical period for dairy cows and buffaloes as there is economic loss to the farmers due to drop in milk production and high culling rates (El-Deeb and El-Bahr, 2017). During the critical prepartum period, feed intake is at the lowest point of the lactation–gestation cycle. This period is characterized by negative energy balance (NEB), fat mobilization, and elevation of circulating non-esterified fatty acids and ketone bodies. Major metabolic transitions occur in a dairy animal during this period as she converts from a non-lactating to a lactating state and undergoes through the stress of parturition (Pande *et al.,* 2016).In this period animals are affected with metabolic and infectious diseases for e.g. ketosis, milk fever, mastitis (El-Deeb and El-Bahr, 2017). There are marked changes in endocrine status, experience a period of insulin resistance, reduced feed intake, negative energy balance, hypocalcemia, reduced immune function, and bacterial contamination of the uterus soon before, or in the weeks after calving (Leblanc,2010).Nutrition requirement of foetus reaches to its maximum level (3 weeks pre-partum) but dry matter intake decreases by 10-30% (Bell, 1995). There is increase in milk yield, milk protein, fat, within three weeks of onset of lactation which rapidly exceed feed intake (Bertoni*et al.*, 2009). Feeding during transition period determines the cow productivity during the preceding lactation period. Diet of dairy cows changes from forage to concentrate rich, sharply at calving (Bertoni*et al.*, 2009). Providing the right nutrition during this period greatly improve the calving ease, cow and calf welfare, milk production and reproductive performance (Praveen and Dhaarani, 2018)

Requisite nutritional adaptation and milk production during post partum period induces a state of negative energy imbalances. Dairy cattle during lactation period depend upon body reserve to meet their demands, but prolong negative energy imbalance (NEB) is associated with the development of ketosis (Ingvartsen, 2006). Subclinical form of ketosis is common condition seen in high producing dairy cattle (SCK; 1.2–2.9 mmol of

β-hydroxybutyrate (BHBA)/L of serum) (Vanholder*et al.*, 2014). Decreased glucose concentration at parturition and lactation have to be considered as a result of constant energy loss with increased utilization of glucose for milk lactose synthesis and the low intake of nutrients i.e. negative energy balance (Antunovic*et al.*, 2017). NEB cause release of hormones lipases, which further triggers the process of lipid mobilisation and release of non-esterified fatty acids (NEFA) into blood streams from adipose tissues (El-Deeb and El-Bahr,2017). During the period of negative energy balance key hormone expression and tissue responsiveness alter to increase lipolysis and decrease lipogenesis, causing an increase in blood levels of non-esterified fatty acids (NEFA) and beta hydroxybutyrate (BHBA) (Holcomb *et al*., 2001; Fiore *et al*., 2014).Failure to adapt response for negative energy imbalance and lipolysis results in hyperketonemia, characterised by overproduction of NEFA, inadequate hepatic metabolism which leads to accumulation of ketone bodies (BHBA, acetoacetate, and acetone) (Drackley, 1999). Overall prevalence of ketosis in cows is 9.38% and in buffaloes is 2.92% in Tamil nadu(Thirunavukkarasu*et al.,* 2010). Ketosis increases the incidence of premature culling by affecting milk production and reproductive performances (Koller*et al.*, 2003). Risk factors responsible for ketosis development is high body condition score (BCS), lower transition period, dry period feed intake (Grummer*et al*., 1995), increased parity, dry period length and transition cow feeding managment (Gustafsson*et al.*, 1995). Biohemical markers used to diagnose ketosis in dairy cattle are NEFA(non esterfied fatty acid), BHBA(beta hydroxy butyric acid), Glucose level, insulin and liver enzymes (Aspartate transaminase,gammaglutamyltransferase) and oxidative stress markers (Nitric oxide, superoxide desmutase, Glutathione peroxidase). There is significant increase in NEFA, BHBA and lipid profiles and decrease in level of glucose, total cholesterol and anti oxidant molecules (peroxides) (El-Deeb and El-Bahr**,** 2017). However, the blood glucose level was significantly higher in dry buffaloes (52.72±4.22 mg/dl) than the early and late lactating buffaloes (48.23±3.44 mg/dl) (Hagawane*et al*., 2009). The serum total cholesterol concentration, total anti-oxidant capacity shows significant increase in early lactation period than late pregnancy in buffaloes (Ghada A.E *et al.,* 2015). The higher level of cholesterol with advancement of lactation was a physiological adjustment to meet the lactation requirements (Hagawane*et al*., 2009). The clinical cases of bubaline ketosis

shows marked hypoglycemia, slight hypocalcemia and significant increase in inorganic phosphorus, serum cholesterol, triglycerides (Rautmare and Anantwar, 1993).

The transition period involves various physiological and metabolic changes that increase the risk of hypocalcemia condition (milk fever) (Goff and Horst, 1997). It occurs immediately after or close to calving and has been linked to calving problems (retained placenta, uterine prolapse, metritis, mastitis, depression of the immune system, reduced in reproductive performances).

Hypocalcemia occurs due to loss of calcium through colostrum beyond its capacity to get absorbed from intestine and insufficiency of calcium mobilization from storage to skeleton (Fikadu, 2016). Risk factors responsible for milk fever are age (the risk increases with increasing age), pre partum diet (high dietary cation and anion difference increases the risk), breed (increased risk for Jersey and Guernsey breeds), milk production (susceptibility increases with herd production), presence of other diseases, parity, previous dry period length(long length increases the incidence), previous corrected milk yield, previous milk fever case, and month of calving (July and May; December was the least risky month to develop MF) (Erb and Gröhn, 1988; Oetzel, 1991; Enevoldsen, 1993). The calcium level within cells starts depleting several days before calving, but serum calcium concentration reduction occurs12 to 24 hrs after calving (Fikadu*et al.*, 2016).There is drop in calcium level during early stage of lactation (8.19±0.83 mg/dl) than the normal healthy buffaloes (11.21±0.19 mg/dl)(Hagawane*et al*.,2009). Milk fever condition is likely to occur when 50% of blood circulating calcium is lost (Fikadu*et al.*, 2016). Adaptation to calving starts with parathyroid hormone and vitamin D3 at the beginning of hypocalcemia. Vitamin D3 increases after 24 hrs of stimulation, with increase in transport of intestinal calcium and bone resorption of calcium by PTH hormone is not stimulated until 48hrs.

Dairy cattle faces more oxidative stress during early lactation or just after parturition than advanced pregnant cattle, and this appears to be the reason for their increased susceptibility to production diseases (e.g. mastitis, metritis, retention of fetal membranes etc.) (Sharma *et al.,* 2011).With the progress of pregnancy, lipid peroxidation level(oxidative stress) increases slowly and it increases marginally during 15 days of post

partum period, then sharply declined at 45 days of post partum (Dimri*et al.,* 2009). Level of superoxide dismutase and catalase(anti-oxidant enzymes) show gradual decrease in buffalo's erythrocytes as pregnancy progresses and level is maintained upto 45 days of post partum period(Dimri*et al.,* 2009).There is an important role of trace minerals in oxidative defense mechanism, example: copper and manganese dependent SOD, selenium dependent selenoprotein (GPx), zinc dependent catalase enzyme. Among the non enzymaticanti oxidants, vitamin E (alpha-tocopherol) is the major membrane bound antioxidant in the cell which triggers the apoptosis of reactive oxidative species (ROS) (Gonzalez *et al*. 2018). Supplementation of Vitamin E and selenium along with other trace minerals during the transition period has significantly decreased the level of circulating reactive oxidative species in the blood of the dairy cattle(Dimri*et al.,* 2009). Metabolic demands during transition period increase the concentration of reactive oxidative species (ROS) and immune cells are very sensitive to peroxidation (Spears and Weiss, 2008). Natural immunosuppression occurs in most cattle during transition period, exaggerated by the factors like negative energy balance, hypocalcemia and increased cortisol level around calving (Miltenburg*et al*., 2015). There are significant changes in the level of INF-gamma, TNF-alpha, IL-1(Pro-inflammatory), IL-10(anti-inflammatory) around calving. Pro-inflammatory cytokines have a crucial role in innate and adaptive immunity, development of cell mediated immune response(Kumar *et al*., 2015). In transition period, level of pro-inflammatory markers decreases from -14 to +14 days of post partum and anti- inflammatory cytokines (IL-10) increases as parturition progresses; maximum at day of calving as dairy cattle experience period of inflammation (Kumar *et al*., 2015).

Evaluation of body condition score (BCS) is a useful management tool to assess body fat. Roche *et al.* (2009) noted that the BCS of cattle at calving, and the post-partum BCS loss are associated with differences in milk production, reproduction, and health. Over conditioned cows with a BCS greater than 4.0 at calving had higher circulating concentrations of NEFA in early lactation until 7 week postpartum compared with cattle with moderate or low BCS (Barletta *et al.,* 2017). Cattle with greater BCS prior to expected calving will experience, on average, a greater magnitude of BCS loss during the 21 d before calving as well as the 21 d after calving (Barletta *et al.,* 2017). Nutritional

management in buffaloes is significant during transitionperiod as over conditioning leads to greater level of BCS and circulating fatty acidsand poor nutrition leads to peri-parturient complications. Buffaloes that are maintained on basal diet, along with 2kgs of concentrates (22% CP) and 50g of mineral mixture (essential trace minerals)likely to show early uterine involution, early postpartum estrus, low incidence of ROP and metritis (Vala*et al.,* 2018). The most underlying cause of hypocalcemia is metabolic alkalosis, majorly contributed by the cations(Na^{+1}, Ca^{+2}, Mg^{+2}) present in the forage whereas, anions (Sulphur and chloride) reduces the risk (Gobiraju*et al.,* 2017). Common anionic salts used are magnesium sulphate and calcium chloride, best to mix with corn silage (Gobiraju*et al.,* 2017). Monitoring of the urinary pH has the most direct and useful approach to establish the balance of cationic and anionic salts in feed of the animal and therefore can be used as an important parameter for the formulation of feed (Gobiraju*et al.,* 2017).

A molecular diagnostic assay using clinically accessible tissue, such as blood, would facilitate evaluation of disease conditions of animals (Almeida *et al.*, 2007). Many immune responses are seen in production diseases in cows and there is activation of local and systemic host defence mechanism that induces inflammation. There is release of immune signalling molecules in response to inflammation for examples cytokines (TNF alpha, IL-1 and IL-6) (Bionaz*et al.*, 2007).

In view of such considerations, the aim of the study was to evaluate the changes in metabolic parameter during the transition period in buffaloes, and nutritional management of occurrence of production diseases during this period. The study was planned for the following objectives:

1. To study the prevalence and associated risk factors of production diseases during transition period in buffaloes.
2. To evaluate the expression level of cytokines in the transition period of buffaloes.
3. To study the haemato-biochemical and oxidative markers during transition period.
4. To analyse the impact of nutritional intervention on the occurrence of production diseases during transition period.

Chapter-II

Review of Literature

CHAPTER-2

REVIEW OF LITERATURE

2.1 Prevalence and risk factors

Amongst the production diseases in livestock, ketosis, milk fever and mastitis hold an important place as it mostly affects the high yielding animals. The prevalence of these production diseases is more in cows than in buffaloes, although not much evidence is available for buffaloes. Thus, it is important to assess the prevalence and risk factors of economically important disease to prevent severe economic losses to farmer. Various studies have been conducted to determine the prevalence and risk factors of production diseases of cattle.

Rautmare and Anantwar (1993) conducted the study of 3,395 adult female buffaloes for ketosis by the Rothera's test in Maharashtra (India), revealed an incidence of 2.85%. The highest incidence of ketosis was seen in 8-9 yrs old buffaloes during fourth lactation.

Singh *et al.* (1994) conducted a survey of 9,432 buffaloes having calved at private and organized farms in the state of Punjab and revealed an incidence of parturient paresis as 3.1%, with 80% of cases occurring between July and December. A high milk fever incidence was recorded in adult buffaloes and was more common in dry areas where straws and stovers were the staple roughage. The incidence of milk fever observed during the 2nd to 7th lactations in buffaloes was 5.1%, 18%, 33.3%, 25.6%, 10.3%, and 7.7; majorly in 4^{th} and 5^{th} lactation.

Annen*et al*. (2004) studied the effect of shortened dry period by comparing milk yield, BW, and circulating concentrations of glucose and NEFA in cows given a 30-d dry period compared with cows given a 60-d dry period (controls). Results demonstrated that cows given a 30-d dry period had 305-d fat corrected milk yield equal to the controls. The 30-d dry group produced less milk during the first 2 months of lactation and lost less BW than 60-d dry cows during the first 3 month of lactation. Continuous milking has

been shown to reduce milk yield in subsequent lactations. A shortened dry period has been shown to cause both reductions and no change in subsequent milk yields.

Watters *et al.* (2008) conducted a study in which cows were randomly assigned to a traditional 55 d (T) or shortened 34 d (S) dry period (DP). Cows assigned to traditional dry period were fed a low-energy diet until 34 d before expected calving at which time all cows were fed a moderate-energy transition diet until calving. There was a tendency for prepartumnon-esterified fatty acid (NEFA) to be lower for cows assigned to traditional compared with shortened dry period. However, postpartum cows assigned to short dry period had significantly lower NEFA concentrations than those assigned to traditional dry period. The incidences of ketosis, retained placenta, displaced abomasum, and metritis did not differ between treatments. Postpartum energy balance, as indicated by plasma NEFA, may have been improved for cows assigned to shortened dry period.

Randhawa*et al*. (2009) conducted a survey for baseline concentration of plasma minerals in buffaloes. Buffaloes with plasma calcium concentration below 2.0 mmol/l were 78.2% and 87.3% at the organized and rural dairy units, respectively. Hypophosphataemia was detected in 26.7% of animals in the rural dairy units compared to 11.1% in the organized farm. The prevalence rate of hypophosphataemia was highest (31.2%) in mature buffaloes (>6 years).

Ghadge*et al*. (2010) did a study on sixty healthy lactating Marathwadi buffaloes yielding 1.75 to 3.5 litres/day, 1.5 to 3.5 months post-partum and maintained solely on dry fodder with no/ small quantity of concentrate and mineral supplementation were subjected to serum mineral analysis. The serum macro-mineral analysis results revealed maximum occurrence of phosphorus deficiency in 51.67% animals followed by magnesium 28.33%, calcium 21.67% and chloride deficiencies in 15% animals.

Thirunavukkarasu*et al*. (2010) studied the total of 3,774 cows. 354 cows were found to be affected by ketosis. Of 342 buffaloes observed, 10 were affected by ketosis. That is, the overall prevalence of ketosis was 9.38 per cent in cows and 2.92 per cent in buffaloes. The prevalence of ketosis in buffaloes was not associated with the districts ($P > 0.05$).

Youssef *et al.* (2010) mentioned that clinical and biochemical investigations were carried out for 61 suspected ketotic buffalo. Based on clinical findings and the level of beta-hydroxybutyrate (BHB), buffalo were allocated into ketotic(42) and sublinical cases (19). Clinically, there was an association between clinical ketosis and anorexia (*p*<0.001), constipation (p<0.001), decreased milk yield (p<0.001), ruminal stasis (p<0.001), and loss of body condition (p<0.01).

Anitha*et al.* (2011) studied the influence of body condition score of Murrah buffaloes on productive performance and showed that for every one unit increase in BCS, an increase of 395.27 kg, 795.55 kg and 4.57 kg was observed in the 18 weeks lactation yield, 305 day predicted lactation yield, and peak milk yield, respectively.The reproductive performance and milk production increased with BCS up to a score of 3.99, but beyond this there was a decline. The present study suggested that a BCS of 3.5-3.99 was ideal for better reproductive and productive performance of Murrah buffaloes.

Mushtaq*et al*. (2012) did a study on 154 animals comprising of Nili-Ravi (NR) buffaloes, Holstein Friesian (HF), Jersey (JC), Sahiwal (SW), Achai (AC) and crossbreds (XB) cattle and Beetal (BT) goats that were selected from various public and private farms. Milk yield and body condition score (BCS) were recorded weekly and milk samples were collected for analysis. The experiment continued for 6 months postpartum in buffaloes and cattle. Highest yield was recorded with moderate BCS in buffaloes. BCS correlated positively with fat and protein and negatively with lactose contents. MY decreased while BCS increased with advancing lactation.

Elrehman (2013) studied that in buffaloes, *S. aureus* and *E. coli* were the most severe cause of clinical mastitis in the form of fatal peracute and acute with systemic reaction. 1st and 2nd month post calving reported the highest incidence of mastitis with percentage of 51.1 and 17.7 as clinical mastitis respectively, and 38.1% and 19.8% as subclinical mastitis; respectively.

Purohit*et al*. (2013) did ten years case analysis of clinical referral records and found that 729 buffaloes were presented for different types of therapies, amongst them 39.23% buffaloes accounted for parturition related metabolic disorders only (2.60% milk

fever, 16.59% ketosis and 20.02% parturient hemoglobinuria). Most buffaloes (93.1%) suffering from different metabolic disorders were beyond their 3rd parity and above 7 years of age and only 1, 8 and 11 numbers of buffaloes that suffered from milk fever, ketosis and parturient hemoglobinuria, respectively were less than 7 years and below 3rd parity .

Kumar *et al.* (2015a) studied that incidence of the disease was found to be 16.55% and all the cattle had calved recently (mean: 1.42±0.14 month), on an average all the cattle were in their third lactation (mean: 2.38±0.30) and showed signs such as selective anorexia, reduction in milk yield (mean: 64.4±5.35%), ketotic odour from urine, breath, and milk and drastic loss of body condition. All the clinical vital parameters in ketotic buffaloes (body temperature, heart rate, respiration rate, and rumen movements) were within normal range.

Kumar *et al.* (2015b) did a study on 24 clinical cases (selected out of 145 screened) of primary ketosis in buffaloes which were reported at Teaching Veterinary Clinical Complex (TVCC), LUVAS, Hisar and from adjoining villages of the Hisar district. The incidence was seen highest in age between 3-5yrs followed by 6-9 years. In the lactation stage, maximum number of cases were recorded during 0-2nd month post-partum (91.68%) followed by 3rd-5th month post-partum (4.16%) while only one case occurred pre-partum (4.16%).

Hassan *et al*. (2017) to elucidate the economic impacts of calving season and parity on reproduction and production indices of Egyptian dairy buffaloes (1180) that were reared under subtropical environmental conditions.The buffaloes calving for the first time had the longest days open, calving interval, and number of services per conception among different parities (176.21 days, 490.05 days, and 2.18, respectively), then decreased thereafter. The total milk yield, lactation period, profit, and profit/cost ratio were increased to reach the peak values in the fourth parity (2051.5 kg, 252.44 days, 674.8 $, and 0.48, respectively), then decreased thereafter.

Hoeji*et al*. (2017) studied Holstein-Friesian dairy cows (n = 123), that were grouped to 1 of 2 dry period lengths: 0-d DP or 30-d DP. A 0-d dry period have reduced

lactation yield and improved the energy and metabolic status of cows in early lactation compared with a 30 day dry period length.

Nava Trujillo *et al*. (2018) did a study to determine the effect of the parity on productive performance (lactation length, total milk yield and milk yield by day of calving interval) and calving interval in water buffaloes. For this purpose, records of 663 lactations from 248 buffaloes were evaluated. Total milk yield was 1344.91 litres, lactation length was 291.20 days, calving interval was 453.55 days and milk by day of calving was 2.77 litres. Parity did not significantly affect total milk yield, but had a significant effect on lactation length, calving interval and milk by day of calving interval. First calving buffaloes had a longer lactation, a longer calving interval and in consequence lower productivity than buffaloes with two and three or more calving. Second calving buffaloes had intermediate and significantly different values than buffaloes with three or more calving. Calving interval was positively correlated with total milk yield ($r = 0.34983$, $p < 0.0001$) and length of lactation ($r = 0.67408$, $p = <0.0001$); and negatively with milk by day of calving ($r = -0.41263$, $p<0.0001$).

O'Hara *et al.* (2018) did a study on cows that were assigned according to breed and parity and then randomly assigned to one of two treatments; short DP of 4 weeks (4W, n = 43) or conventional DP of 8 weeks (8W, n = 34). Milk yield was reduced by 6.75 kg/day during the first 12 weeks postpartum ($P< 0.001$) for the 4W cows compared with 8W cows, but there was no significant difference in total MY (3724 kg compared with 3684 kg, $P = 0.7$) when the milk produced prepartum was included.

Ruprechter*et al.* (2018) conducted a study on Holstein dairy cows (n=126; primiparous and n=182; multiparous) from a 700-cow herd were selected, with an approximate milk yield of 8000 kg per lactation. Cows were evaluated from 3 weeks before calving, until 4 weeks after calving. Cows were classified as healthy one, cows with one occurrence, or cows with two clinical occurrence. All multiparous cows increased their NEFA concentrations from week -1 to week + 1, but sick MP cows presented higher NEFA concentrations than healthy MP cows at calving ($P<0.05$). In addition, healthy MP cows decreased their NEFA concentrations by week +3, while sick MP cows maintained higher NEFA concentrations ($P<0.05$). In PP cows, although NEFA

concentrations increased from week - 1 to week +1, healthy PP cows presented the greatest NEFA concentrations at week +1 in comparison to sick PP (P<0.05). Healthy cows (MP and PP) presented higher cholesterol concentrations than sick cows (MP and PP) at week +2 and +3 (P< 0.05). Healthy MP cows had higher albumin concentrations than sick MP cows during close-up and fresh period (P<0.05).Subclinical hypocalcemia was highest in multiparous cows than primiparous (44% and 9.5%) respectively.

Kabir*et al.* (2019) studied 120 quarter samples from suspected buffaloes in selected areas. California Mastitis Test was performed for screening of positive samples. Afterward, the bacterial profile was confirmed through biochemical testing. The quarter wise prevalence of subclinical mastitis was 25%. Within this, contribution of Gram-negative bacteria was 68% and that of Gram-positive bacteria was 32%. Among 30 positive samples, percentage prevalence of different bacterial species was: *E.coli* (37%), *S. aureus*(23%), Pseudomonas (20%), Streptococcus (10%), Proteus (7%) and Salmonella (3%).

Nava Trujillo *et al*. (2020) did a retrospective analysis of milk production at first lactation, season of calving, parity and length of calving interval (<400 days). Milk yield was classified as group I (<1090kg), group II(1090-1377kg), group III(1377-1684kg), group IV(>1684kg). Parity was classified as one, two and three. Seasons were assigned into 3; December to March, April to July and August to November. Shorter calving interval with <400 days were related with reduced milk production at first lactation, higher parity >three and calving between august to November. Increased lactation yield at first lactation negatively affected the production performance effectively at parity one and two.

2.2. Molecular Diagnostic Markers

Molecular markers include acute phase proteins, cytokines etc. Cytokines are of two types; pro and anti-inflammatory molecules. Pro-inflammatory cytokines are IL-1, IL-6, TNF-alpha, INF-gamma and anti-inflammatory are IL-4, IL-11, IL-10 etc. During postpartum period, there is increase in NEFA which induces inflammation cascade with subsequent release of pro-inflammatory cytokines. The plasma interleukins are correlated

with inflammation, worse health status and low milk production during early lactation. Even though there are several biochemical parameters that are positively related to the occurrence of production disease but cytokines detection helps in building the role of functional neutrophils and lymphocytes in the transition period along with susceptibility to inflammation. Thus, these cytokines can act as biomarker for postpartum diseases in buffaloes. Molecular biomarkers are an essential step toward the control of production and metabolic diseases.

Radcliff *et al.* (2003) generated a daily profile for total GHR (GHR tot; all GHR transcripts), GHR 1A, and IGF-I mRNA expression in liver of peri-parturient Holstein cows and evaluate these daily mRNA profiles relative to daily profiles for peri-parturient hormones and metabolites. Liver biopsies and blood samples (n = 139) were collected from 65 Holstein cows. Total cellular RNA was isolated and reverse transcribed to cDNA. Target cDNA were measured by quantitative real-time polymerase chain reaction.The IGF-I mRNA declined 1 d after parturition, was lowest 2 to 5 d after parturition and then increased.

Ishikawa *et al*. (2004) did a study to demonstrate a change in peripheral blood picture of IL-6 produced by Th2 cells before parturition, to investigate the correlation between concentration of IL-6 and post-partum disorders. The study showed that IL-6 level was higher before parturition than post-partum period and Th2 cell functioning is dominant during pregnancy of the animal.

Ametaj*et al*. (2005) investigated the relationship between activation of acute phase response and fatty liver in transition dairy cows. Liver and blood samples were obtained at days -4, 3, 8, 12, 14, 22, 27, and 36 postpartum. Cows with fatty liver had greater plasma tumor necrosis factor-alpha (TNF-α), non-esterified fatty acids (NEFA), and lower lactate concentrations than did control cows at day -4.After parturition, the concentration of plasma TNF-α in both groups peaked at day 3 postpartum but then fell sharply in fatty-liver cows.

Jonsson*et al*. (2013) conducted a study on twenty-two female Holstein-Friesian animals (12 cows that had calved at least once before and 10 heifers that had not

previously calved) were used for this study. Differences were seen between the 2 groups with respect to cytokine and cytokine-receptor mRNA expression, IL-1β was significantly higher at the time of parturition in heifers. Gene expression for IL-4 and IL-6 appeared to be higher in heifers (P = 0.091 and P = 0.073, respectively). The expression of IL-2 (P = 0.012) and IFN-γ (P = 0.013) was significantly higher in cows 3 weeks postpartum

Weber *et al.* (2013) did a study on German Holstein cows that were categorised according to mean total liver fat content on day 1, 14, and 28 after calving as low [300 mg of total fat/g of DM; n = 7), indicating fat mobilization during postpartum period. Liver biopsies were taken at day 56 and 15 before and day 1, 14, 28, and 49 after calving to measure mRNA expressions of carnitine palmitoyl-transferase 1A (CPT1A) The expression level of CPT1A mRNA increased (P < 0.01; entire study and transition period) after calving in all liver fat content groups, and the increase was highest in HI cows and lowest in LO cows (P < 0.01; LFC × time interactions during the entire study and the transition period .

Patra*et al*. (2013) conducted a study to correlate the peri-parturient immune status in terms of neutrophil functions and cytokine expression in peripheral blood mononuclear cell culture in buffaloes. Forty pregnant buffaloes were observed for occurrence of postpartum reproductive disorders (PRD) during one week pre-partum to four weeks post-partum period. The blood samples were collected at weekly interval from one week pre-partum to four weeks postpartum period considering the day of calving as ‘d 0’. Differential leucocytes counts, superoxide and hydrogen peroxide production activity in isolated neutrophils and the mRNA expression profile of cytokines i.e., IL-2, IL-4 and IFN-γ in PBMC culture were studied. A lower expression of IL-2, IFN-γ and IL-4 mRNA in PBMC culture was observed at calving in buffaloes that subsequently developed PRD at later post-partum. Thus, suppression in neutrophil function and cytokine expression at pre-partum to early post-partum period predisposes the buffaloes to develop postpartum reproductive disorders.

Sordillo*et al*. (2013) studied that during infectious disease like coliform mastitis, there is increase in pro-inflammatory cytokines like, TNF-alpha, IL-1 and 6 contribute to

inflammatory response. TNF stimulates lipolysis and causes breakdown of fat stores therefore increases the NEFA concentration during transition period.

Sasaki *et al.* (2014) selected 5 genes as being strongly related to both experimental hypocalcemia and milk fever: protein kinase (cAMP-dependent, catalytic) inhibitor β (PKIB); DNA-damage-inducible transcript 4 (DDIT4); period homolog 1 (PER1); NUAK family, SNF1-like kinase, 1 (NUAK1); and expressed sequence tag (BI537947). Another gene (neuroendocrine secretory protein 55, NESP55) was also determined to be specific for milk fever, independently of hypocalcemia.. The mRNA expression of these 6 genes in milk fever cases was verified by quantitative real-time reverse-transcription PCR and was significantly different compared with their expression in healthy parturient cows. In the present study, the selected genes appeared to be candidate biomarkers of milk fever.

Heiser*et al.* (2015) did a study on cytokine profiles which indicated decreased expression of IFNγ, tumor necrosis factor, and IL-17 and increased expression of IL-10 wk 1 after calving, which later al returned to pre-calving values (n = 39) . A combination of in vitro stimulation and quantitative PCR for cytokines was validated as a quantifiable immunocompetence assay in 29 cattle and a correlation of quantitative PCR and ELISA demonstrated.

Lange *et al.* (2016) did a study on the effect of pre-calving body condition score (BCS) and feeding level on immune- functioning during the peri-partum period. Twenty-three weeks before parturition, 78 cows were grouped to 1 of 6 treatment groups Blood was sampled pre-calving and at 1, 2 and 4 weeks after calving. Expression of the cytokines IFNG, TNF, IL17, and IL10 was not constant between individual cows. All groups showed with an increase in expression level of 4 cytokines in the week after parturition, although this was significant only for IFNG in the body condition score 4 groups ($P < 0.05$). Blood cells from dairy cows from all BCS and feeding level groups and from 4 time points were stimulated in vitro with SEB/LPS and mRNA expression for IFNG, TNF, IL17, and IL10 was analyzed by qPCR and compared with expression in unstimulated cells. Some samples presented with very low or absent cytokine expression in unstimulated samples.

Mili*et al*. (2015) did a study on Murrah buffaloes (12) during late gestation period in region of Haryana, dividing into two groups (6 each). Buffaloes of group 1 were given the control diet, while group 2 was given 2,000 IU/day/head vitamin E plus control feed. Blood sample was taken from each buffalo at weekly interval from 3 weeks prepartum to 3 weeks postpartum relative to parturition. There was significantly elevation of IL-6 levels ($P<0.05$) in vitamin E supplemented buffaloes throughout the experimental period. The level enhanced significantly around calving in relation to pre-partum level in both the groups ($P<0.05$) of buffaloes, declining during post-partum period .

Trevisi*et al*. (2015) did a study on twenty-one multiparous Holstein dairy cows (parity 2.5±1.0) in transition period [from -35 d to +28 d from parturition (DFP)], to determine the relevant potential of pro-inflammatory cytokine (PIC) with peri-parturient performances, the changes in the level of plasma interleukin-1beta (IL-1β) and interleukin-6 (IL-6). Cows were divided in 3 groups according to concentration curve of IL1β concentrations from 4 weeks before to the day of calving and designated as up (UPIL1), intermediate (INIL1) and low (LOIL1) IL-1β group. After calving the (UPIL1) cows did have increase level of acute phase reaction (APR), based on the marked increase of haptoglobin and the lower albumin concentrations during the first week of postpartum, and higher reactive oxygen species. Results showed that cows with the highest PIC concentrations in the 4 weeks prepartum showed reduced health status in early lactation.

El-Deeb and El-Bahr (2017) conducted a study on 25 cows suffering from ketosis in the post parturient period, together with 20 healthy cows who served as a control. Blood samples were collected from diseased and healthy animals. The results showed that there was a significant ($P\leq0.05$) increase in the levels of β-Hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA) and pro-inflammatory cytokines, namely; (IL-1β, IL-6, IL-8, IL-12), Tumor necrosis factor-alpha (TNF-α), interferon gamma (IFN-γ). A positive correlation was seen among traditional biomarkers (BHBA, NEFA) and suggested biomarkers (APP and cytokines) in cows affected with negative energy balance /Ketosis.

Dang*et al. (*2017) conducted a study on 10 healthy Murrah buffaloes approaching parturition were selected from the experimental herd of National Dairy Research Institute (NDRI), Karnal. Blood samples were collected from them starting from 15 days pre calving, -7 day, on the day of calving, +7 and on 15 days post calving. There were higher expression of TLR-2, TLR-4 and IL-8 on 15 day before and 15 day after parturition as compared to the day of calving. IL-8 regulates the recruitment of neutrophils as well as T lymphocytes to the site of infection. This indicates that the ability of neutrophils to migrate to the site of infection was lower during calving. Down regulation of these receptors as observed in our study may make the animal more susceptible to disease around calving.

Pacheco *et al.* (2018) studied the genetic basis underlying milk fever incidence in Holstein cattle. Total of eight different genomic regions, located on chromosomes BTA2, BTA3, BTA5, BTA6, BTA7, BTA14, BTA16, and BTA23, explain more than 0.45% of the additive genetic variance for milk fever incidence. Five of these regions harbor genes, namely *CYP27A1* (BTA2, 107–109 Mb), *CYP2J2* (BTA3, 86–88 Mb), *GC* (BTA6, 87–89 Mb), *SNAI2* (BTA14, 19.5–21.5 Mb), and *PIM1* (BTA23, 10.5–12.5 Mb), that play critical roles in vitamin D metabolism and signalling. Polymorphisms in CYP2J2 and CYP27A1 lead to reduced serum 25-(OH)D3 and ultimately are associated with incidence of milk fever. Whole-genome scan detected *CYP27A1* and *CYP2J2* as two putative genes affecting milk fever incidence.

Kulkarni*et al.* (2019) did a study on eighteen apparently healthy pregnant buffaloes from Instructional Livestock Farm Complex. The experimental animals were divided into three groups according to the stages of gestation, each consisting of six animals. The three groups were early gestation (0 to 100 days), mid gestation (101 to 200 days) and late gestation (201 to 310 days) stages, respectively. The mean concentrations of serum IL1β during early, mid and late gestation were 195.10±49.43, 233.65±51.45 and 125.90±36.18 pg/ml respectively. The serum IL-1β concentration increases from early to mid-gestation and then declines in late gestation.

2.3. Oxidative stress markers

Oxidative stress occurs due to the production of reactive oxidative species and free radicals which causes decrease in anti-oxidant defence mechanism, in turn damages the DNA, enzymes and membranes. These changes decrease the efficiency of immune system of the body and causes health related disorders. Anti-oxidant defense mechanism includes essential enzymes; superoxide dismutase, catalase and glutathione peroxidase. During transition period, buffaloes are in negative energy balance which in turn increase production of free radicals and it exceeds the anti-oxidant capacity of the animal. This leads to impairment of immune system and increase in the susceptibility of the animal towards the health disorders.

Miller *et al*. (1993) did a study on 64 cows that were fed grass hay and an average of 5 kg/d of commercial dairy concentrate, which supplied about 160 IU/d of vitamin E. One-half of the cows were each given 1000 IU/d of additional vitamin E as 1 ex-tocopherol acetate by capsule. Serum ex-tocopherol decreased linearly during the last 6 weeks of gestation in cows that were not supplemented with vitamin E and was lowest at calving. Supplementation with 1000 IU/d of vitamin E was accompanied by increased ($p<0.01$) serum ex-tocopheroland plasma total antioxidants and by decreased erythrocyte substances reactive to thiobarbituric acid. Substances, reactive to thiobarbituric acid, an index of lipid peroxidation, were correlated ($p<0.01$) negatively with plasma total antioxidants ($r =-.57$) and ex-tocopherol ($r =-.27$), as expected when lipid peroxidation increases as antioxidant protection decreases.

Bernabucci*et al.* (2005) studied twenty-four dairy cows in their transition period. Body condition score (BCS) of the 24 animals at the initiation of the study (30.4±2 day before parturition) was between 2.0 and 3.6. Before calving, cows showed an increase of SOD, and GSHPx. After calving, cows showed a decrease of plasma and erythrocyte SH and SOD, and an increase of reactive oxygen species (thiobarbituric acid reactive susbstance). Cows with higher BCS at the beginning of the trial and greater loss of BCS after calving, had higher plasma reactive oxygen species, TBARS and lower SOD and erythrocyte SH in the early lactation. Cows with higher BHBA and NEFA showed higher reactive oxygen radicals and lower level of anti-oxidants in body.

Dimri*et al*. (2009) studied the erythrocyte lipid peroxides and superoxide dismutase activities in 28 pregnant water buffaloes supplemented with antioxidant nutrients, Vitamin E and selenium. Other 14 buffaloes did not receive any treatment during pregnancy and served as negative control. Lipid peroxidation level(oxidative stress) increases slowly and it increases marginally during 15 days of post-partum period, then sharply declined at 45 days of post-partum. Level of superoxide dismutase and catalase (anti-oxidant enzymes) shows gradual decrease in buffalo's erythrocytes as pregnancy progresses and level is maintained upto 45 days of post-partum period.

Pathan*et al*. (2010) did a study on trace minerals and oxidative stress markers in Mehsana buffaloes during periparturient period. Trace minerals, zinc and iron were significantly decreased ($p<0.01$) from 30 days before parturition to the day of parturition. There is higher oxidative stress and low anti-oxidant markers during advanced pregnancy.

Pedernera*et al*. (2010) conducted a study on 37 multiparous Australian Holstein–Friesian cows that were part of a feeding system study. Nineteen cows were fed on a typical Australian pasture-based system (pasture plus concentrates at milking) termed restricted production (Rp) and achieved a mean milk yield (±SE) of 6489±867 L of 4% fat-corrected milk (FCM)/cow/lactation. Eighteen animals were fed on a partial mixed ration to supplement pasture termed high production (Hp) and produced a yield of 8176 ± 1071 L of FCM/cow/lactation. Biomarkers of Oxidative stresswere measured in weekly blood samples taken immediately after morning milking from calving until 5 weeks post-partum. In both groups, the concentration of antioxidants was low and the concentration of ROMs high in the first 2 weeks of lactation. A higher OS level for cows with greater energy mobilisation (Hm) in the Rp than in the Hp group ($P < 0.05$) was observed. Also in both herds, cows with greater body reserve mobilisation tended to produce more milk than cows with medium or low levels of mobilisation ($P = 0.07$) (32.6, 31.2 and 28.7 L FCM yield/cow/day, respectively.

Sharma *et al*. (2011) conducted a study on twenty animals of HF and Sahiwal cross bred, that were categorised into two groups A and B, each contained cows. Group A cows were in last 4 weeks of pregnancy, while group B cows were in first 4 weeks of

lactation. The lipid peroxidation (plasma MDA production) was significantly ($p<0.001$) higher in Group B than in Group A. A positive relation was found in between catalase (anti-oxidants) and lipid peroxidation (oxidative stress) in cows during last 4 weeks of gestation, while non-significant negative correlation was found in group B cow. A significant positive correlation ($r = +0.831$, $p<0.01$) were found in MDA and catalase in group A cows, while SOD and GSH-Px level had negative correlation with MDA level of advanced pregnancy animals.

Aggarwal*et al.* (2012) did a study to determine the effect of α-tocopherol acetate on oxidative stress, antioxidants and heat shock protein 70 (Hsp70) during transition period in medium body score conditioned cows. Twenty crossbred Karan Fries cows with were selected 2 months before parturition. The cows were grouped into two groups: 10 cows were kept as control group and 10 were given with α-tocopherol acetate during dry period. Blood samples were collected at −30, −15, −5, 0, 5, 10, and 20 days of parturition. Superoxide dismutase and catalase activity in erythrocytes in control groups decreased towards parturition and further declined after parturition. In treatment animals, SOD and CAT activity also followed same patterns as in control, but their activity on all observation days was significantly ($P<0.05$) higher than corresponding values in control group.

Abuelo*et al*. (2013) sampled fortnightly 25 cows from 2 months before the expected date of parturition until 2.5 months after it. The markers of oxidative stress and antioxidants were measured in four different stages of production: (i) late lactation (22 to 21 months); (ii) prepartum (21 month until parturition); (iii) postpartum (delivery to 11 month) and (iv) peak of lactation (11 to 12.5 months). Anti-oxidant capacity is highest in PrP group followed by PsP>LL>PkL. Reactive oxygen species were found highest in PsP>PkL>PrP>LL groups.

Singh*et al*. (2015) did an experiment on 167 buffaloes that were sampled thrice during different stages of peri-parturient period viz. (i) Far off dry (FOD)->10 days following dry off and not < 30 days prior to calving, (ii) Close up dry (CUD)- Between 3 and 21 days prior to calving,(iii) Fresh- 3- 30 days in milk, from 12 organized dairy farms. During CUD stage, erythrocytic LPO showed negative correlation with the

glucose, TEC and Zn and positive correlation with NEFA and BHBA. SOD is a Cu-Zn dependent enzyme, thus during the late pregnancy period, when there is loss of Zn, there is resultant decrease in SOD along with an increase in LPO levels. During fresh stage, a negative correlation was observed between the SOD levels and the total proteins and albumin, while a positive correlation was observed between the LPO and NEFA and a negative correlation between LPO and glucose levels. GPx level was significantly decline from FOD and CUD to fresh stage.

Li *et al.* (2016) performed an experiment on thirty lactating Holstein multiparous cows of the same breed and age with similar milk production and body condition score. The cows were fed with total mixed ration (TMR) according to their daily nutrient requirements. Ten non-ketotic cows (NC, b-hydroxybutyric acid 3.75 mmol/l, and triglycerides >0.16 mmol/l), ten subclinical ketotic cows (SCK) and ten clinical ketotic cows (CK, b-hydroxybutyric acid >1.2 mmol/l, glucose) were taken. The plasma malonaldehyde level, were significantly higher ($p < 0.01$) in ketotic and subclinical ketotic cows than in non-ketotic cows. The plasma enzyme activities of superoxide dismutase and catalase were decreased ($p < 0.05$) in the ketotic cows than in the non-ketotic cows. A positive correlations between plasma BHBA concentrations and ALT, AST and LDH were seen and negative correlations between plasma BHBA concentration and TC, HDL, VLDL, vitamin E and inhibited hydroxyl radical capacity, suggesting that high plasma BHBA has association with compromising state of hepatic tissues and high levels of oxidative stress.

Colagoklu*et al.* (2017) did a study that was conducted at a commercial dairy farm in Western Thrace with Holstein cows housed in free stall resting barns. Holstein cows calving in January were assigned into winter group (n = 42), while cows calving in August were assigned into summer group (n = 42). All animals were fed a mixed *ad lib* ration containing grass, corn silage, and commercial concentrate twice a day. The blood were collected from the jugular vein into tubes with K2EDTA (for MDA analysis) and into tubes with heparin (for GSH-Px analysis) at 21, 14, and 7 days before calving (–21, –14, –7), at calving (0), and 7, 14, and 21 days after calving (7, 14, 21). In both groups of cows, GSH-Px activity decreased from 21 days before calving to day 0, and it gradually

continued to increase until 21 days after calving. GSH-Px activity was higher in winter group compared to summer group during the transition period ($p<0.05$). MDA levels in both groups increased over time starting from 21 days before calving to 0 day, but it gradually decreased thereafter. MDA levels were higher in summer group compared to winter group during the transition periods ($P < 0.05$).

Singh *et al*. (2017) conducted a study on hundred multiparous Murrah buffaloes in advanced pregnancy and grouped them according to transition stage *i.e.*, Far off dry, Close up dry and Fresh. The mean lipid peroxidation concentration in buffalo's significantly increased, whereas the mean SOD and GSH levels significantly decreased throughout periparturient period from far off dry to fresh stage.

Colakoglu *et al.* (2017) did a study on Holstein cows calving in January that were assigned into winter group (n = 42), while cows calving in August were assigned into summer group (n = 42). Blood samples were collected on 21, 14, and 7 days before parturition, at calving (0 day), and 7, 14, and 21 days after calving. GSH-Px activity decreased from 21 days before calving to day 0, and it gradually continued to increase until 21 days after calving. MDA levels in both groups increased over time starting from 21 days before calving to 0 day , but it gradually decreased thereafter. Negative correlation was recorded between GSH-Px and MDA during all examination days ($p<0.01$).

Fayed *et al.* (2018) conducted an experiment on seventy buffaloes during post parturient period, that were randomly selected from field cases in many localities in province of El-qalyubia during spring (season of berseem feeding) from 2016 to 2018, 30 clinically healthy buffaloes of similar description from the same areas were included for case control study. The level of serum inorganic phosphorus, copper, selenium levels and erythrocytic SOD, GPX, G6PD enzymes activity were significantly ($p\leq0.05$) lower when compared by control buffaloes, while MDA level in red blood cells was significantly ($p\leq0.05$) higher than in the healthy ones.

Sucupira*et al.*(2019) did a study to evaluate the effect of the parenteral administration of vitamins ADE blend during the transition period, 27 adult Santa Inês

sheep, which were healthy and in the last month of pregnancy, were distributed into two groups: a treated-group, which received 1 mL/50 kg BW of a vitamins ADE blend (270.000 UI vitamin A, 80.000 UI vitamin D and 80 UI vitamin E /mL) via intramuscular injection 30 days and 10 days before partum, and a control-group, which received saline solution at the same times and volumes. On days - 30, -10, 0, 7, 14 and 28 to parturition, the following parameters were taken; glucose, non-esterified fatty acids, beta-hydroxyburyrate (BHBA), serum activities of AST, GGT and CK enzymes and concentration of malondialdehyde (MDA), reduced glutathione (GSH) and erythrocyte superoxide dismutase (SOD). NEFA concentration reduced in the control and the ADE groups after lambing. Close to the lambing period, ROS production increased when stimulated by LPS and PI-Sa, and phagocytosis decreased in all sheep. The increase in ROS production in sheep treated with the ADE vitamin blend was milder than in the control group, either with stimulus of LPS or no stimulus. The use of two doses of ADE vitamin blend in sheep at 120 and 140 days of gestation improved in vitro the antioxidant capacity of PMN when animals were well fed and it presented a moderate, negative energy balance in the transition period.

Afshar *et al*. (2019) conducted an experiment to determine the effects of body condition score on periparturient period, parity on oxidative stress markers in 43 buffaloes during this period. The experiment comprises of four levels of BCS (2.5-2.99; 3-3.49; 3.5-3.99 and 4- 4.5) at two weeks before parturition and three groups of parity (1; 2- 5 and >5). Blood samples were collected from animals in three stages including: 14 days before parturition, parturition day and 14 days after parturition. The GPx activity and serum MDA content was at lowest level at BCS (2.5-2.99) significantly lower than that of other BCS groups. The higher serum catalase activity as well as TAC was observed in the BCS of 3.5-3.99. Based on the results, activity of GPx at 14 days of prepartum and calving day was higher than that of 14 days postpartum, and SOD and GGT activity at 14 days prepartum were significantly higher than that of other interval. The relationship between parity and oxidative stress factors in milking buffaloes is that GPx, catalase as well as TAC (total antioxidant capacity) in multiparous buffaloes were significantly more than that of primiparous buffaloes; while, SOD, ALT and MDA were not significantly affected by parity.

2.4. Haematological and biochemical parameters

Buffalo in transition period undergoes various physiological as well as pathological changes. Blood metabolic profile is used as one of the common indicator in the blood of animal, for assessing the nutritional status and animal health. During the different physiological state of the animal, these indicators shows great variation which can be used as diagnostic tool for the detection of metabolic diseases. Common metabolic indicators are NEFA, BHBA, TG, cholesterol, total protein, A:G ratio, ALT, AST, GGT, HDL, LDL, mineral profile etc. Haematological analysis include red blood cell (RBC), hematocrit, hemoglobin (Hb) and mean cell Hb concentration (MCHC). Sign of inflammation during periparturient period is evident by the significant increase of total leucocytes and monocytes.

Sarwar and Chaudhary(2001) conducted an experiment on ten hematological parameters that were studied in 160 apparently healthy productive milk Nili-Ravi buffaloes. Four groups were comprised of 40 lactating pregnant, 40 lactating not-pregnant, 40 dry pregnant and 40 dry not-pregnant buffaloes. Statistical analysis revealed that out of 10 hematological parameters studied: I) lactation altered seven parameters: raising ESR and neutrophil percentage in lactating but RBC, PCV, TLC, percentages of eosinophils and lymphocytes in dry buffaloes. ii) pregnancy affected a total of three parameters significantly: raising eosinophil percentage in pregnant but ESR and TLC in not-pregnant buffaloes. It is assumed that metabolic stress and hormonal changes may lead by and far changes in the blood composition in order to maintain the homeostasis.

Nazifi*eet al.* (2008) did an experiment on 50 postpartum healthy Holstein Frisian dairy cows that were selected from a large commercial dairy farm near Shiraz. The cows were evaluated on days 25 to 30 and days 50 to 60 post parturition. Significant difference in packed cell volume (PCV) between the 25– 30 days postpartum, 55–60 days postpartum, and the pregnant cows. The hematocrit in pregnant cows was significantly higher than in the prepartum cows ($P< 0.05$). The haemoglobin concentration in the pregnant cows was significantly higher than in the postpartum cows in 25–30 days after

parturition. The erythrocyte count and total leucocyte count in the pregnant cows was significantly higher than in the postpartum cows in 55–60 days after parturition.

Shahzad*et al.* (2008) did a study that was conducted to examine the influence of diets containing −110, +110, +220 and +330 mEq/kg dry matter (DM) of dietary cation anion difference (DCAD) on serum minerals, minerals balance (Na, K, Cl, S, Ca, Mg and P) and hypocalcemia in prepartumShahzad*et al.* (2008) did a study that was conducted to examine the influence of diets containing −110, +110, +220 and +330 mEq/kg dry matter (DM) of dietary cation anion difference (DCAD) on serum minerals, minerals balance (Na, K, Cl, S, Ca, Mg and P) and hypocalcemia in prepartumNili Ravi buffaloes. Twenty dry pregnant multiparous (3rd parity) Nili Ravi buffaloes (about 8 years old) were randomly allocated to four dietary treatments in a randomized complete block design, five buffaloes in each. The experiment lasted for 8 weeks; 6 weeks before and 2 weeks after parturition. The first two weeks of experimental period were adaptation period while the 3rd, 5th and 7th week served as collection period. Blood pH and serum HCO3 also increased linearly with increasing DCAD level. Serum Cl was high in buffaloes fed the −110 DCAD diet, while serum cation anion difference increased linearly with increasing the DCAD level. Serum Ca increased with decreasing the DCAD level while serum Mg, P and S remained unaffected. Urine pH increased with increasing DCAD level.

Solangi*eet al.* (2004) did an experiment on fifty samples that were collected from 3 groups (n=10) of buffaloes in surroundings of Tandojam. The animals were divided into groups as: A (Control group non-lactating and non-pregnant), B (Advanced Pregnant) and C (Hypocalcaemic). Group B was again categorized as B.1 (pre-parturient) and B.2 (Post Parturient). Similarly group C was sub classified as C.1 (Pre-treatment) and C.2 (Post treatment). The calcium, magnesium, phosphorus and glucose were determined by kit methods.The results revealed that serum calcium value decreased (5.07 mg/dl) in hypocalcaemic animals, which was statistically significant ($p<0.05$). The serum magnesium recorded 3.25 mg/dl significantly low in hypocalcaemic buffaloes. The serum phosphorus observed as decreased (3.66 mg/dl) in pre treatment buffaloes. While titre serum glucose levels was higher (73.70 mg/dl) in hypocalcaemic, which appeared significant in all other groups.

Sathya*et al.* (2010) did a study to evaluate polymorphonuclear leukocyte (PMN-L) functions in normally calved and dystocia affected buffaloes. Buffaloes (20) were divided into group 1 (6 Murrah buffaloes of the University dairy farm and calved normally) and group 2 included 14 Murrah buffaloes presented at Veterinary Clinics of the University for the treatment of dystocia. Blood was obtained from jugular venipuncture from normally calved buffaloes daily from day 3 pre-partum to day 3 postpartum and from dystocia affected buffaloes daily from the day of delivery by obstetrical means to day 3 post-partum using heparin as anticoagulant. In group 1, the TLC on day 3 prepartum gradually increased to reach a peak on day '0'. The counts declined non-significantly from day 0 to day 3 in both the groups. The TLC in group 2 was non-significantly lower than in group 1 on all the days of observation. The neutrophils were significantly lower ($P<0.01$) in group 2 than group 1 in all post-partum days.

Piccione*et al.* (2012) did a study on five clinically healthy cows, breed Holstein Friesian. Blood samples were taken two days before the expected date of calving, during the post-partum, in early lactation, 2nd, 5th and 15th weeks after calving, at the end of lactation period and at the dry period. Urea serum levels showed a significant increase ($P<0.01$) level in postpartum period compared to the late and end of lactation periods, urea showed a significant increase ($P<0.001$) compared to late gestation and beginning of lactation. There was significant decrease in the level of creatinine in 5^{th} week of lactation and at the end of lactation period compared to advanced pregnancy and early postpartum period. Total proteins level showed a significant increase during the 5th week after parturition and the end of lactation ($P<0.001$) compared to late gestation and the beginning of lactation. A significant increase in late lactation and decrease in the level of serum cholesterol during end of lactation, dry period were seen ($p<0.001$). NEFA level showed a slight increase compared with the late gestation and an important decrease during the end of lactation and the dry period ($P<0.001$). β-hydroxybutyrate serum levels, which increased just during the late lactation and the dry periods ($P<0.001$).

Abdulkarrem *et al.* (2013) conducted a study to evaluate some hematological and blood biochemical parameters of riverine buffaloes (*Bubalus bubalis*) in advanced

pregnancy and two months post-partum period. Blood samples were collected from each buffalo around calving and 15 days interval during two months. The Hb and PCV did not differ significantly at calving and PP period. Although of non-significance, AST and ALT were numerically higher at days 45 and 60 PP as compared with other periods. ALP activity was numerically lower (85.88±9.23 unit/ L) at day 60 PP. plasma glucose concentration was numerically lower (73.91±1.51 mg/dl) at day 15 PP in comparison with other periods. Plasma total protein and protein fractions did not differ significantly among periods studied.

Ashmawy*et al.* (2015) carried out an experiment on 24 buffalo (12 pregnant buffalo cows on the 60th day prior to parturition and 12 lactating buffalo cow from 10th day of lactation during winter feeding season). The buffalo cow was average 3 years old and healthy. There was a drop in calcium level during early stage of lactation (4.55±0.21) than pregnant period buffaloes (10.35±0.52 mg/dl).The concentrations of plasma phosphorous were lower during lactation period than in pregnant period. The concentrations of potassium, sodium were higher during pregnant period than in lactation period. Chloride concentrations varied non-significantly during pregnancy and lactation, the concentrations of plasma urea, cholesterol, glucose, triglyceride and total protein were lower during lactation period than in pregnant period. The concentration of plasma creatininewas higher during lactation period than in pregnant period. The blood enzymes (AST, ALT and AlP) activities were insignificantly higher during pregnant period than in lactation period.

Mohamed *et al.* (2015) did a study to evaluate the relation between lipid profile and total antioxidant capacity (TAC) and to investigate the changes in some hematological and biochemical parameters in buffalo heifers during pre and postpartum period. The study was conducted on 30 buffalo heifers from a buffalo's farm that belongs to Assiut City, Egypt.. The hematological indices including mean values of RBC, Hb, PCV, MCV, and MCH, TLC, lymphocyte % and neutrophil % revealed significant decrease in the early lactation period if compared with late pregnancy mean values. There were significant increase in total proteins, albumin and globulins in the early lactating group compared with late pregnancy group. The mean values of AST, revealed

significant increase in the early lactating group compared with late pregnancy group. A significant decrease in the mean values of triglycerides was observed in the early lactating period compared with late pregnancy results. The mean value of total cholesterol, HDL-C and total antioxidant capacity (TAC) revealed significant increase, in the early lactating group compared with late pregnancy group. There were a positive correlation between Total antioxidant capacity (TAC), albumin, total cholesterol and HDL-C.

Singh *et al.* (2015) did a study that was aimed to evaluate oxidative stress, hemato-biochemical and plasma mineral profile in transition buffaloes along with therapeutic efficacy of herbal Vitamin E-Selenium powder in reducing oxidative stress. Murrah buffaloes in advanced pregnancy were grouped according to transition stage i.e., Far off dry, Close up dry and Fresh. Total leukocyte count was significantly increased at F stage. Significant decrease was observed in levels of total plasma protein, albumin and glucose, whereas significant increase was observed in levels of plasma urea nitrogen, beta hydroxyl butyric acid and non-esterified fatty acids (NEFA) after parturition at F stage. Significantly low levels of potassium and copper were observed at F stage.

Pande*et al.* (2016) transition period of 12 high yielding buffaloes was monitored to assess the alterations in haemato-metabolic status and body condition. Twelve pregnant graded Murrah buffaloes in 2nd to 5th parity, having peak milk yield of 10 litres or greater in previous lactation. Blood samples (~6 ml) were collected aseptically on days -21, day 0 (Day of calving), and day +21 by jugular venipuncture for the assessment of haematological parameters and blood metabolites. The total leukocytes increased non-significantly on the day of parturition compared with before and after calving. An increase in monocytes and neutrophils accompanied by decrease in per cent lymphocytes and eosinophils at calving was evident. The eosinophils increased again at 21 DIM. Postpartum BUN and NEFA concentrations were higher as compared to the prepartum mean values, peaking at calving. The difference was highly significant ($p<0.01$) for NEFA. In the present study, plasma urea nitrogen and NEFA concentrations were highest at calving.

Fiore *et al.* (2018) studied the changes in level of Non-Esterified Fatty Acids, β-hydroxybutyrate, glucose, insulin, milk composition and yield in during advanced pregnancy and early lactation. From 50 buffaloes, blood samples were taken -7days before expected calving; +7; +30 and +50 days post-partum also, milk samples were collected at the same post-partum time. Lower BCS values were found at 50 days post-partum with respect to 7 days pre-partum; higher NEFA and BHB levels were found at 7 days post-partum respect to 50 days post-partum; lower glucose values were found at 7 days pre-partum respect to 7, 30 and 50 days post-partum, whereas insulin showed higher values at 50 days post-partum in comparison to 7 days pre-partum, 7days post-partum and 30 days post-partum. The values of BHB and NEFA were negatively correlated with milk yield. A positive correlation was found between insulin and milk yield.

Ruprechter*et al.* (2018) conducted a study on Holstein dairy cows (n=126 PP and n= 182 MP) from a 700-cow herd, with an approximate milk yield of 8000 kg per lactation. Cows were evaluated from 3 weeks before calving, until 4 weeks after calving. Cows were classified as healthy one, cows with one clinical occurrence, or cows with two clinical courses. All MP(multiparous) cows increased their NEFA concentrations from week -1 to week + 1, but sick MP cows presented higher NEFA concentrations than healthy MP cows at calving ($p<0.05$). In addition, healthy MP cows decreased their NEFA concentrations by week +3, while sick MP cows maintained higher NEFA concentrations ($P<0.05$). In PP cows, although NEFA concentrations increased from week - 1 to week +1, healthy PP cows presented the greatest NEFA concentrations at week +1 in comparison to sick PP ($P<0.05$). Healthy cows (MP and PP) presented higher cholesterol concentrations than sick cows (MP and PP) at week +2 and +3 ($p<0.05$). Healthy MP cows had higher albumin concentrations than sick MP cows. During close-up and fresh period ($p<0.05$ Subclinical hypocalcemia incidence was highest seen in multiparous cows than primiparous. Calcium concentrations declined sharply at calving in all MP cows, whereas it was not as evident in PP cows, reflecting the effect of parity at this time.

Golla*et al.* (2019) performed an experiment on nine Murrah buffaloes, including three heifers (control) and six early lactating animals in the 1st parity (experimental) were

considered in this study. The blood collection was done weekly from the Week 3 to the Week 12 of the postpartum. There was significant increase in the serum level of free fatty acids in lactating buffaloes ($p<0.05$) than heifers during 3rd and 4th weeks of postpartum period. Particularly, these levels were higher in HMY compared with LMY and H during the 3rd week. The average serum BHBA concentration appear to be higher in lactating buffaloes than heifers.

Afshar *et al.* (2019) did an experiment to evaluate the effect of body condition score, parity and biochemical parameters of 43 lactating buffaloes during transition period. Experiment comprises of four levels of BCS (2.5-3; 3-3.5; 3.5-4 and 4-4.5) at two weeks before calving. The effect of body condition score on ALT activity was significant, but there was not clear trend, that the higher and lower activity of the ALT was 3-3.49 and 2.5-3 BCS, respectively. Aspartate transaminase (AST) and ALP activity in the buffaloes with the higher BCS (4-4.5) was significantly higher than other BCS groups, while the gamma-glutamine transaminase activity was decreased with increasing BCS and activity of this enzyme in skinny buffaloes (2.5- 2.99 BCS) were significantly higher than other groups. The ALT activity significantly decreased in calving day than that of the 14 days pre- and post-calving. AST activity at 14 days of postpartum was significantly higher than calving and post-calving levels. The Alkaline phosphatase activity and TAC content was not affected during transition period of the buffalo.

Singh *et al*. (2019) conducted an experiment on twenty one buffaloes that were divided equally into three groups of seven animals each based on results of metabolic profile screening i.e., Group I: Healthy {Pre-partum Non esterified fatty acid (NEFA) levels at close up dry stage (CUD) ≤0.4mmol/L}; Group II (positive control): Pre-partum NEFA levels at CUD stage ≥0.4mmol/L and Group III: NEFA levels at CUD stage ≥0.4mmol/L and supplemented with oral propylene glycol @250 ml once a day for 5 days. Sampling was again done at fresh stage of transition period to evaluate the efficacy of PG as a prophylactic measure to prevent development of SCK in transition buffaloes. Results revealed significant increase in mean PCV percentage from 37.87±1.48% to 42.79±1.21% after propylene glycol administration in group III. Beta hydroxyl butyric acid (BHBA) levels increased significantly in group II from CUD (0.76±0.03 mmol/L) to

fresh stage (1.49±0.03 mmol/L) and were significantly higher than PG supplemented group (0.90±0.12 mmol/L) at fresh stage. Non esterified fatty acid (NEFA) recorded non significant increase and decrease in group II and PG supplemented group, respectively.

2.5 Nutritional interventions during transition period

Appropriate feeding during transition period ensures good milk yield and reproductive performance of the animal. There is dramatic change in the metabolic and physiological adaptation; as in advance pregnancy there is decrease in dry matter intake and increase in nutrient demand by the foetus. There are possibilities of imbalance between the nutrient requirement and utilization, therefore increase the chance of occurrence of metabolic and production diseases. So, it is necessary to maintain the balance between nutrient supplementation and utilization by the animal by providing balanced ration with adequate supplementation of minerals and vitamins to prevent the occurrence of metabolic disorders during peri-parturient period of buffaloes.

Bhimte*et al.* (2018) performed an experiment to investigate the effect of antioxidant (vitamin E) and trace minerals (selenium (Se), copper (Cu), zinc (Zn)), high energy diet supplementation and its effect on milk yield (MY) and biochemical parameters in crossbred cows, during the transition period. For this, advanced pregnant crossbred cows (n = 20) were selected. Treatment(TRT) (n=10) group was supplemented with Vit E, Se, Cu and Zn incorporated in wheat flour bolus from -4 to 8 week of calving and were provided with 20% additional concentrate from 2 to 8 week of calving. Control (CON) group was given only basal diet without any supplementation. Value of milk yield was significantly higher. Number of mastitis cases decrease in the TRT as compare to CON and milk production significantly increase. Total protein concentration was significantly ($p<0.05$) lower in CON group. The AST and ALT enzyme activities presented were significant increase ($p<0.05$) Ca level decreased in last gestation period ;1 week before calving, at calving and post-partum in CON cows compare to TRT.

Omur*et al.* (2016) did a study on 20 Brown Swiss dairy cows. The animals were divided into two groups: control (C) (n=10) and treatment (T) group (n=10). Vitamins (A, D, E) and trace elements (Cu, Mn, Se, Zn) were given to the cows of the treatment group.

In control group, there was significant changes ($p<0.001$) in the levels of non-esterified fatty acids, high density lipoprotein, low density lipoprotein, total protein (TP) ($p<0.05$) glucose, progesterone ($p<0.05$), total cholesterol (T.CHOL), triglycerides (TG), UREA, creatinine (CRSC) and total bilirubin (TBIL) ($p<0.001$) Whereas, significant changes in the levels of NEFA, total cholesterol, HDL, LDL($p<0.01$), TG, GLU, TAC($p<0.001$) were seen in treatment group. Khan *et al.* (2015) did an experiment that was designed to provide higher plane of nutrition, vitamin E and mineral supplementation for augmenting the improvement in reproductive performance. In the investigation, 10 Murrah buffaloes each in two groups were selected and randomly assigned to two experimental groups with 10 animals in each group; group 1 (C) was provided 20% higher nutrients than Kearl's Feeding Standard and group 2 (T) was provided 20% higher nutrients than Kearl's Feeding Standard along with vitamin E (2000 IU 8 weeks prepartum to 4 weeks postpartum and 1500 IU from 1 to 2 months of post-partum) and 50 gm of commercial mineral mixture. The blood samples were taken from 8 weeks pre-partum to 8 weeks post-partum. The supplemented group had higher concentrations of mineral (Ca, inorganic P, Zn, Cu and Mn), glucose, Vitamin E and lower level of biliary urinary nitrogen and non-esterfied fatty acids than the control group at all the stages. Plasma Ca decreased on days 30 and 15 pre-partum in control and day 30 only in supplemented group during the pre-partum period. Plasma inorganic P showed a specific trend. It increased in the supplemented group upto days 15 pre-partum and declined on calving day and showed an increasing trend thereafter, whereas in control group it declined upto day15 pre-partum and thereafter showed an increasing trend. Zn concentration dropped on day 30 pre-partum and day of calving and showed an increasing trend thereafter in supplemented buffaloes, whereas it showed an increasing trend at all stages except day of calving and day 15 postpartum in control group. Plasma glucose followed an increasing trend throughout the experiment period except on day 30 when it showed slight decrease in both the groups.

Parmar*et al.* (2015) did a study on the effect of vitamin E and selenium on oxidative stress during transition period in kankrej cows. Group I animals served as control and did not receive any treatment, while Group II cows were treated with vitamin-E (500 mg) and selenium (15 mg, Inj. E-CARE SeÒ , @ 10 ml i/m twice at 28th

& 14th day before expected date of parturition. In both groups the blood samples were collected on day -28, -21, -14, -7, 0 (day of parturition), 1, 2, 7, 14, 21 & 28 of postpartum. The blood biochemical markers of oxidative stress, viz., lipid peroxidation (in terms of malondialdehyde - MDA) & superoxide dismutase (SOD) were studied. The concentration of MDA was significantly ($P<0.05$) lower in Vit-E + Se supplemented group as compared to control group at stage-I, II and III (late pregnancy, recent parturition and early postpartum) during transition period in Kankrej cows.

Chapter-III

Materials And Methods

CHAPTER-3

MATERIALS AND METHODS

3.1 Study area

The study was conducted on organized and unorganized farms in and around Jammu district irrespective of their age, parity and body weight.

3.2 Animals

The present study was conducted on total 210 buffaloes that were in transition period (3 weeks pre-partum to 3 weeks post-partum).

3.3 Body condition score

Buffaloeswere scored on appearance and palpation of back and hind quarters according to Wildman *et al*.(1982). Factors considered were the thoracic and lumbar regions of the vertebral column (chine, loin, and rump), spinous processes (loin), anterior coccygeal vertebrae (tailhead), tuber sacrale (hooks), and tuber ischii (pin bones) (Fig. 1). Scorings were done on a scale of 1 to 5 by considering the factors mentioned above.

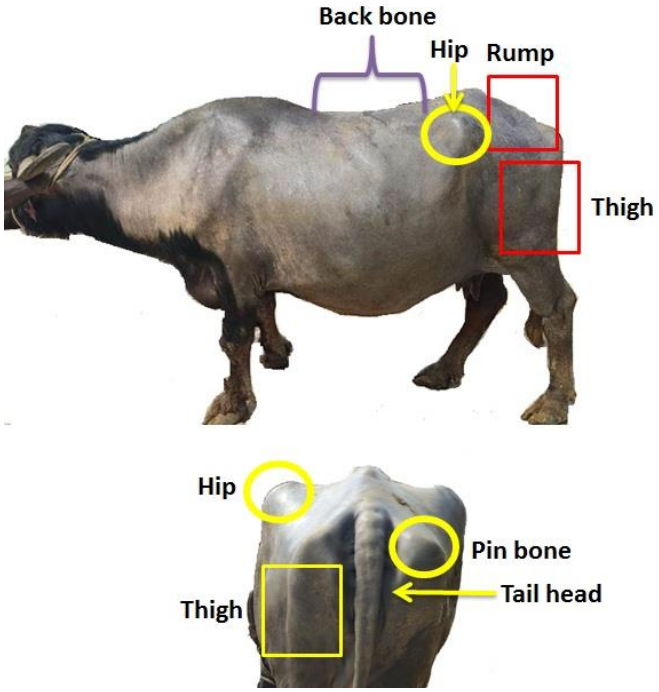


Fig. 3.1: Measurement of Body Score in buffaloes.

3.4 Samples

Blood, urine (during pre- and post-partum) and milk samples (during post-partum period) were collected for further studies. Blood samples were collected for hematological, biochemical parameters and oxidative markers. Urine samples were evaluated for Rothra's test, pH and different constituents present by using urine strips (Cat.U100E011A, Standard diagnostic, South Korea). Milk samples were evaluated for mastitis by Modified California Mastitis Test (MCMT), bacterial isolation and somatic cell count (SCC). Blood glucose level was estimated using Accu- check glucometer immediate after sampling.

3.4.1 Blood samples

Blood sample collected in EDTA (Ethylene diamine tetra acetic acid) containing vacutainersVACUETTE (Cat. 455036, Greiner bio-one, Austria) were used for hematology using MYTHIC 18 VET HemotologyAnalyser, (Compact diagnostics (India) Pvt Ltd.).

Blood samples collected in serum clot activator vacutainers VACUETTE (Cat. XLGA-C5, Greiner bio-one, Austria) were used for estimation of cytokines (INF-gamma and TNF-aplha) using their ELISA kits. Samples collected in heparin containingvacutainers VACUETTE (Cat. 455051, Greiner bio-one, Austria) were assayed for oxidative biomarkers; glutathione peroxidase (GPX), lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase.

3.4.2 Urine samples

3.4.2.1 Rothera's test

Urine samples were collected in the sterile Uricol (Cat. PW0176, Himedia, Mumbai). In a test tube, 5 ml of urine was taken and 1-2 gm of ammonium sulphate was added and mixed. After proper mixing, 1 ml of 5% solution of sodium nitroprusside was added. After that 1 ml of concentrated ammonium hydroxide was layered over the urine sample and without mixing further, waited for 2-3 minutes.

3.4.2.2 Urine analysis using SD Urocolor strips

Urine sample from the animals were taken and stored in sterile Uricol (Cat.PW016-1X100NO, Himedia) and taken to lab in an ice box within an hour. Then urine analysis strips (SD Urocolor, Standard diagnostics, INC, South Korea) were dipped in urine for seconds and removed immediately to avoid dissolving of reagents. These strips were compared with corresponding color chart on the bottle label and results were read immediately.

3.4.3 Milk samples

Milk samples from post-partum buffaloes were tested for mastitis on spot by using MCMT. Milk samples were collected in sterile tubes using aseptic precautions and transported to lab in an ice box for further processing. After proper disinfection of teat surface with 70% ethyl alcohol, few streams of milk from each quarter discarded. Milk collected in sterile polyethylene screw capped, wide mouth vials. The samples were kept in an ice box and carried to the laboratory for further processing.Milk samples were tested for somatic cell countsby using Cellcounter DCC, DeLaval (Sweden).MCMT positive (≥2 score) were subjected for cultural isolation of mastitis causing bacteria.

3.5 Oxidative stress markers

Various oxidative stress markers such as Superoxide dismutase (SOD), Glutathione peroxidase (GPx), Lipid peroxidase (LPO) and Catalase were evaluated from transition buffaloes and control group (mid lactation). For evaluation, 5ml of blood in heparin containing vacutainer was taken and centrifuged at 3000 rpm for 10 min. After that, plasma was stored and left over RBC lysate was used to determine the antioxidant status. Washing of RBC lysate was done with normal saline by diluting in a ratio 0f 1:1 and centrifuged for 10 min at 3000 rpm. Washing was done thrice. 1% lysate (100 µl of lysate and 990 µl of distilled water) and 33% lysate (330 µl of lysate and 640 µl of PBS with ph 7.4) was prepared

3.5.1 Lipid peroxidation

Lipid peroxidation in erythrocyte was determined by the evaluation of malondialdehyde production by the method of Rehman (1984). In brief, 1 ml of 33%

RBC haemolysate was taken and added with 1 ml of 10% w/v of trichloracetic acid. After thorough mixing, mixture was centrifuged at 3000 rpm for 10 min and supernatant was extracted. To 1 ml of the extracted supernatant added 1ml of 0.67% w/v of thiobarbituric acid and kept in water bath for ten minutes, cooled and diluted with 1 ml of distilled water. For control sample, same reagents were used except the haemolysate. Absorbance was noted at 535 nmin thespectrophotometer (Biospectrometer, eppendoff India Ltd.).

3.5.1.1. Calculation

Calculation was done by using the extinction co-efficient (EC, 13100 $M^{-1}cm^{-1}$) and results are be expressed in mM MDA per ml of blood, using the following formula.

$$\text{LPO (mM MDA/ml)} = \frac{\text{OD}}{\text{EC}} \text{X} \frac{\text{Total Volume of reaction mixture X1000 X DF}}{\text{Amount of sample taken}}$$

3.5.2 Catalase

The plasma catalase activity was determinedby the method described by the Aebi (1984). In brief, 2 ml of phosphate buffer along with 20 µl of 1% lysate were taken. This mixture then incubated with 1 ml of 30 mM of hydrogen peroxide at 37 ºC and decrease in the absorbance was observed at every 10 sec interval for one minute at 240 nm OD (optical density). The catalase activity was expressed as µmoles of H_2O_2 utilized/min/mg Hb using 36 as molar extinction coefficient of H_2O_2

3.5.3 Superoxide dismutase (SOD)

Theactivity of erythrocytic superoxide dismutase was determined by method of Marklund and Marklund (1974). In brief, 1.5 ml of 100 mMtrisHCl buffer, 20 µl of 1% haemolysate, 0.5 ml of 6 mM EDTA and 1 ml of pyrogallol was added. The rate of auto-oxidation of pyrogallol was taken from the increase in absorbance at 420 nm in a spectrophotometer, every minute after a lag of 30 sec up to 4 minutes. For the test, appropriate amount of enzyme was added to inhibit the auto-oxidation of pyrogallol to about 50%. A unit of enzyme activity is defined as the amount of enzyme causing 50% inhibition of the auto-oxidation of pyrogallol observed in blank. The activity of superoxide dismutase was expressed as SOD units/mg protein.

3.5.3.1 Calculation

$$\text{SOD activity (U/mg of Hb)} = \frac{(\Delta E0 - \Delta E)}{\Delta E0} X \frac{1}{2} X \frac{1}{\text{g of protein in 0.01 ml}}$$

ΔE_0 = change of absorbance of pyrogallol.

ΔE = change of absorbance of sample.

3.5.4 Glutathione peroxidase (GPx)

Theactivity of glutathione peroxidase in erythrocytes was assayed by Hafeman *et al*. (1974). In brief, 0.1 ml of erythrocyte was taken. Addition of 1 ml of glutathion peroxidase, 1ml of 0.4 M sodium phosphate containing EDTA, 0.5 ml of 0.01 M of NaN_3 and distilled water to make a volume of 5 ml was made. The solution was incubated for 5 minutes. After the incubation, 1 ml of 125 mM hydrogen peroxidase added and kept for incubation of about 3 minutes. 1 ml liquated from the incubation mixture and 4 ml of meta-phosphoric acid was added to it. Then, 2 ml of this solution was added with 2 ml of 0.4 M $NaHPO_4$ and 1 ml of DTNB reagent. For blank solution, water was used instead of hydrogen peroxidase and rest of the procedure was same. OD was taken at 412 nm against blank solution

$$\text{Activity of GPx} = 10 \log \frac{Co}{C}$$

3.6 Extraction of total RNA from blood

Total RNA was extracted from EDTA preserved blood by trizol method (Invitrogen Life Technologies, Carlsbad, CA). Trizol reagent (200 ml, Cat. 15596018; Thermo Fisher Scientific) contains various components viz guanidine isothiocyanate, phenol and isoamyl which facilitate the extraction of RNA with high yield. In a sterile centrifuge tube, 5 ml of blood was diluted with 1x PBS at a ratio of 1:1. In another falcon, 5 ml of HiSep LSM-1077 (Cat. LS001, HiMedia, Mumbai, India) was taken, over this 5 ml of diluted blood was layered without mixing. Centrifugation at 2400 rpm for 24 minutes at 4 ºC was done in a refrigerated centrifuge (Centrifuge 5430R, eppendoff India Ltd.). A layer of buffy coat was seen, separated and diluted with 1x PBS. Centrifugation at 1780 rpm for 10 minutes was done. This step was repeated

three times until a clear pellet was obtained at the bottom of the falcon tube. The supernatant was removed and 1 ml of TRIzol(Cat. 15596026; Thermo Fisher Scientific) was added and mixed properly with pellet. Mixture was incubated for 30-40 minutes at -20 ºC. After thawing, 300 µl of chloroform(Cat. MB109, Himedia, Mumbai, India) was added and mixed properly in 2 ml of microcentrifuge tube. Centrifugation at 12,000 rpm at 10 minutes was done. Total 500 µl of isopropanolol was added and mixed, then subjected to centrifugation at 12000 rpm for 10 minutes. Ethanol washing was given to the pellet thrice at 75000 rpm for 5 minutes. Air drying of the pellet for one hour and addition of 20 µl of diethyl pyrocarbonate (DEPC) treated water (Cat. R0601; Thermo Fisher Scientific)was done. Optical density (OD) of the final pellet was taken spectrophotometrically for their concentration and purity in spectrophotometer (Biospectrometer, eppendoff India Ltd.). 100 µl of nuclease free water was taken as blank in cuvette and OD at 260 nm and ratio of 260/280nm was recorded. For sample OD, 1 µl of sample was added in 99 µl of blank water

$$\text{Calculation of RNA concentration (ug/ml): } A260 \times \text{Dilution factor} \times 100$$

3.6.1 Gel electrophoresis of total extracted Ribonucleic acid(RNA)

Gelelectrophoresis was done to check the purity and concentration of RNA bands (28s and 18s) when compared with ladder. A 1% agarose gel of molecular grade was made with 1x TAE buffer (40x molecular grade; Cat. V4281, Promega, USA) treated in DEPC water (Cat. MB076,Himedia, Mumbai, India) in 250 ml conical flask. Flask was heated for few minutes for proper mixing of agarose in TAE buffer.The flask was cooled down and 4 µl of EtBr (Cat. H5041, Promega, USA) in gel was added. The gel was poured in horizontal gel plate along with combs and allowed to cool down at room temperature for 20-30 min. After solidification of gel, it was placed in gel tray and dipped in 1x TAE buffer. After the removal of comb, samples were mixed with loading dye (6x loading dye; Cat.G190A, Promega, USA).Then, 100bp ladder (GeneRuler 100bp ladder, Cat. SM0328, Thermoscientific) was charged in first well and samples in the consecutive wells. The voltage 1-5 V/cm was applied across the gel until the bromophenol blue migrated to appropriate distance. Resultant band in the gel was seen with gel documentation system (Vilber, eppendoff India Ltd.), under the illumination of UV light. 28s band and 18s band was seen and

confirmed the presence of RNA. After the confirmation, RNA sampleswere further processed for cDNA synthesis.

3.6.2 cDNA synthesis from isolatedtotal RNA

Total isolated RNA was subjected to cDNA synthesis using Hi-cDNA synthesis kit (Cat. MBT076-100R, Himedia, Mumbai, India). A reaction mixture of 1 µg of RNA template, 2 µl of oligo-dt primer (10 pmol) and 7 µl of nuclease free water in sterile PCR tubes was made**.** This mixture was subjected to incubation of 5 minutes at 65 ºC in the thermal cycler (BioRad T100 Thermal cycler). Other components from the kit; 4 µl of RT buffer, 2 µl of 10x solution, 10 mMdNTP solution, 0.5 µl of ribo-nuclease inhibitor and 1 µl of reverse transcriptase enzyme were added in template RNA and primer mixture to make a volume of 20 µl. This mixture was subjected to PCR conditions; one cycle of 42 ºC for 60 minutes, 70 ºC for 5 minutes and hold at 4 ºC in a thermal cycler. A negative control reaction mixture was made, containing all the kit components except RNA template. cDNA reaction was validated with GAPDH primer (housekeeping primer)

3.6.3 Validation of cDNA with GAPDH primer

GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used as a house keeping gene for validation of cDNA as the expression of these genes remain constant in cells or tissues under investigation. A reaction mixture of 20 µl containing 10 µl of PCR pre-mix (Thermo scientific), 2 µl of cDNA, 0.3-0.5 µl of GAPDH forward and reverse primer (10 pm each) and nuclease free water to make a final volume in sterile PCR tubes were made. These reactionswere subjected to PCR cyclic conditions (Table 3.1).

Table 3.1: PCR cyclic conditions for GAPDH primer for validation.

Step	Temperature	Time	Cycle
Initial Denaturation	94	3 min	1
Denaturation	94	30 sec	35
Annealing	58.9	30 sec	35
Extension	72	45 sec	35

After the PCR cycles, reaction mixture was run in gel for electrophoresis and the visualization of cDNA band along with negative controlin gel documentation system (Vilber, Eppendoff India Ltd.) was done. Positive reaction containing cDNA was observed at 456 bp (size of GAPDH primer)and negative control showed no band with ladder**.** This step verified the synthesis of cDNA from the total extracted RNA.

3.6.4 Quantitative Real time PCR (qRT-PCR) for quantification of expression of gene

Real time PCR helps to determine the extent of expression of a particular gene. It detects the amplification of the targeted molecule a real time. cDNA made from all the samples were subjected to real time PCR for determination of the quantification of gene of interest. qRT-PCR was conducted by Quantitative Real Time PCR cycler-Mygo mini (MyGo 002). Software was installed, named as MyGo Mini PCR software which was connected to MyGo Mini PCR for the observation of Cq value of the gene of interest in the form of graph. A reaction mixture was prepared containing 10 µl Himedia Hi-SYBr master mix (Cat. MBT074-100R, Himedia, Mumbai, India), 0.5 µl GAPDH forward and reverse primer and nuclease free water upto 20 µl. RT-PCR cyclic conditions are as given below in Table 3.2.

Table 3.2: Quantitative Real-time PCR cyclic condition.

Steps	Temperature	Cycles	Time
Pre incubation	95 ºC	1	5min
Amplification (3 steps)	95 ºC	40	20 sec
	Annealing temperature of the gene of interest	40	15 sec
	72 ºC	40	15 sec
Pre melt	95 ºC	1	5 sec
Melting	70 ºC	1	1 min
Melting	95 ºC	1	40 sec
Cooling	40 ºC	1	30 sec

3.6.4.1 *In-silico* analysis of molecular markersfor milk fever

3.6.4.1.1 Datasets and their normalization for analysis of gene expression in milk fever

In order to identify the molecular markers , gene expression datasets of 4 samples of normal cowsand 4 samples of milk fever cows were retrieved (GEO ID: GSE46901;Agilent-018964 NIAS Designed Custom Agilent Bovine 15K Oligo DNA Microarray) and analyzed by a variety of bioinformatics tools. All samples from healthy non-diseased cows were considered as controls. Milk fever samples were considered as Experimental or test group. The expression file was subjected to quantilealgorithm to make the samples comparable. Expression values were computed based on corresponding probe set annotations. AltAnalyze python scripts were used to call the Affymetrix Power Tools, distributed with the GPU license(Lockstone, 2011).

3.6.4.1.2 Gene expression analysis

The analyses of gene expression levels and gene ontology (GO) annotation were carried outwith GO-Elite software, using default options (Zambon*et al*., 2012). Microarray expression values were reported as log2 values. The log-fold was calculated by geometric subtraction of the experimental group from the control groups for each pair-wise comparison. The max log-fold was calculated as the log2 fold value between the lowest group mean and the highest group expression mean for all conditions in the dataset. These statistics were intended for further data filtering and prioritization in order to assess putative transcription differences between the genes. Differentially expressed genes were identified using a combination of a >2-fold change in expression with a statistical significance of $p<0.05$ (Moderated t-test) using Benjamini-Hochberg correction method (Emig*et al*., 2010).

3.6.4.1.3Clustering Analysis

Differentially expressed genes were subjected to hierarchical clustering to identify gene clusters within the control and test groups (Fendri*et al*., 2013). Gene-set enrichment analysis and comparison was executed using the GO-Elite in AltAnalyze, where only terms with an FDR adjusted enrichment$p<0.05$ was considered for further evaluation.

3.6.4.1.4Identification of milk fever-specific Markers

To identify tissue/cell markers in both healthy and milk fever samples, we first filtered the genes with expression level of >90 percentile of all samples, and then we compared the abundantly expressed genes in both ketosis and non ketosis samples to identify both common and uniquely expressed genes. A gene was defined as enriched in tissue 'X' if the average expression of the gene in tissue 'X' was at least 3 times greater than its average expression in all other 66 tissues. We defined a tissue-specific gene marker as the gene not only enriched in tissue 'X', but also expressed highest in tissue 'X' and when expression in tissue 'X' was at least 1.5 times higher than its expression in any other tissues. Further, Marker Finder algorithm in AltAnalyze was performed within each independent dataset to derive putative cell-population-specific markers for gene-set enrichment (Hulin*et al*., 2019).

3.6.4.2*In-silico* analysis of molecular markersfor ketosis

3.6.4.2.1Datasets and their normalization for analysis of gene expression in ketosis

In order to identify the molecular markers , gene expression datasets of 14 samples of normal liver of healthy cows of early postpartumand 14 samples of ketosis cows of early postpartum ketosis cows were retrieved (GEO ID: GSE4304;UIUC Bostaurus 13.2K 70-mer oligoarray) and analyzed by a variety of bioinformatics tools. All samples from healthy non-diseased cows were considered as controls (Non-ketosis) ketosis samples were considered as Experimental or test group. The .expression file was subjected to RMA algorithm to make the samples comparable. Expression values were computed based on corresponding probe set annotations. Alt Analyze python scripts were used to call the Affymetrix Power Tools, distributed with the GPU license (Lockstone, 2011).

3.6.4.2.2Gene Expression Analysis

The analyses of gene expression levels and gene ontology (GO) annotation were carried out with GO-Elite software, using default options(Zambon*et al*., 2012). Microarray expression values were reported as log2 values. The log-fold was calculated by geometric subtraction of the experimental group from the control groups for each pair-

wise comparison. The max log-fold was calculated as the log2 fold value between the lowest group mean and the highest group expression mean for all conditions in the dataset. These statistics were intended for further data filtering and prioritization in order to assess putative transcription differences between the genes. Differentially expressed genes were identified using a combination of a >2-fold change in expression with a statistical significance of $p<0.05$ (Moderated t-test) using Benjamini-Hochberg correction method (Emig*et al*., 2010).

3.6.4.2.3Clustering Analysis

Differentially expressed genes were subjected to hierarchical clustering to identify gene clusters within the control and test groups (Fendri*et al*., 2013). Gene-set enrichment analysis and comparison was executed using the GO-Elite in AltAnalyze, where only terms with an FDR adjusted enrichment $p<0.05$ was considered for further evaluation.

3.6.4.2.4Identification of Ketosis-specific Markers

To identify tissue/cell markers in both ketosis and healthy samples, we first filtered the genes with expression level of >90 percentile of all samples, and then we compared the abundantly expressed genes in both ketosis and non ketosis samples to identify both common and uniquely expressed genes. A gene was defined as enriched in tissue 'X' if the average expression of the gene in tissue 'X' was at least 3 times greater than its average expression in all other 66 tissues. We defined a tissue-specific gene marker as the gene not only enriched in tissue 'X', but also expressed highest in tissue 'X' and when expression in tissue 'X' was at least 1.5 times higher than its expression in any other tissues. Further, Marker Finder algorithm in Alt Analyze was performed within each independent dataset to derive putative cell-population-specific markers for gene-set enrichment (Hulin*et al*., 2019).

3.6.4.3*In-silico* analysis of molecular markersfor Mastitis

3.6.4.3.1 Datasets and their normalization for analysis of gene expression in mastitic mammary gland

In order to identify the pathways and markers in mastitis, gene expression datasets of 10 samples of *E.coli* infected mammary gland and 20 healthy samples were retrieved

(GEO ID:GSE15025; Affymetrix Bovine Genome Array platform) and analyzed by a variety of bioinformatics tools. All samples from healthy mammary gland were considered as controls (Healthy) whereas E.coli infected mammary gland were considered as Experimental or test (Mastitis) group. All the .CEL files in raw format were retrieved and the data were subjected using RMA algorithm to make the samples comparable. Expression values were computed based on corresponding probe set annotations in the downloaded CDF file. In Affymetrix array analyses, AltAnalyze python scripts call the software Affymetrix Power Tools, distributed with the GPU license (Lockstone, 2011).

3.6.4.3.2Gene Expression Analysis

Microarray Expression values were reported as log2 values. The log-fold was calculated by geometric subtraction of the experimental group from the control groups for each pair-wise comparison. The max log-fold was calculated as the log2 fold value between the lowest group mean and the highest group expression mean for all conditions in the dataset. These statistics were intended for further data filtering and prioritization in order to assess putative transcription differences between genes. In our study Gene expression levels and GO annotation with GO-Elite software were carried out, using default options. Genes differentially expressed were identified using a combination of a >2-fold change in expression with a significance of $P< 0.05$ (Moderated t-test) and Benjamini-Hochberg correction method (Emig*et al*., 2010).

3.6.4.3.3Clustering Analysis

Differentially expressed genes (DEGs) were subjected to hierarchical clustering to identify gene clusters within the control and test groups (Fendri*et al*., 2013). Gene-set enrichment analysis and comparison was executed using the GO-Elite in AltAnalyze, where only terms with an FDR adjusted enrichment $p <0.05$ was considered for further evaluation.

3.6.4.3.4Pathway analysis

The identified DEGs were submitted to David Functional annotation tool for the identification of pathways responsible for mastitis.

3.6.4.3.5Lineage Analysis

Cell type / tissue prediction approaches were applied in this study for the independent confirmation. In order to identify the time specific variations in the tissue and cell markers LineageProfiler gene marker database was used which was derived from the hundreds of distinct cell and tissue markers in the GO-Elite software. For these tissue and cell prediction analyses, GO-Elite Fischer-Exact enrichment test p <0.05 was required for downstream analyses. To identify tissue/cell markers in both mastitis and healthy samples we first filtered genes with expression level >90 percentile of all samples, and then we compared the abundantly expressed genes in both mastitis and non mastitis samples to identify both common and uniquely expressed genes. We defined a gene as enriched in tissue A if the average expression of the gene in tissue A was 3 times greater than its average expression in all other 66 tissues. We defined a tissue specific gene marker as the gene not only enriched in tissue A, but also expressed highest in tissue A and its expression in tissue A was at least 1.5 times higher than its expression in any other tissues.

3.6.4.3.6Condition Specific Markers

MarkerFinder algorithm in AltAnalyze was performed within each independent dataset to derive putative cell-population-specific markers for gene-set enrichment using GO-Elite MarkerFinder.

3.6.5. Validation and expression analysis of up- and down regulated genes of milk fever, ketosis and mastitis

After In-silico analysis identified up- and down-regulated genes expression was validated by the qRT-PCR. Up- and down-regulated genes one each for all three production diseases viz. milk fever, ketosis and mastitis were selected for validation (Table 3.3)

Table 3.3: Primer sequences and annealing temperatures of genes of interest.

Diseases	Genes	Annealing temperature	Size	Sequence
Milk fever	NUAKI (Upregulated)	58.3 º	202 bp	F: TGAAAATGCCAAGTGCTCTC R: AGGAATGATACCATGCCAGT
Milk fever	NESP55 (Downregulated	62.3 º	116bp	F: TTTTTAATGCTGCACAACACGAT R: GCCACAAATGTTCCTTCTCTCTTT
Ketosis	CPT1A (Upregulated)	58.7 º	292bp	F: TTATGTGAGCGACTGGTGGG R; GTGCTGGATGGTGTCTGTCT
Ketosis	IGF1 (Downregulated	59.6 º	140bp	F:CATCCTCCTCGCATCTCTTC R: GAAATAAAAGCCCCTGTCTCC
Mastitis	TNF-alpha (Upregulated)	58.9 º	272bp	F: TAACAAGCCGGTAGCCCACG R:TCTTGATGGCAGACAGGATG
Mastitis	INF-gamma (Upregulated)	58.7 ºC	98bp	F:TGGAGGACTTCAAAAAGCTGATT R:TTTATGGCTTTGCGCTGGAT

3.6.6 Calculation and analysis of Cq/Ct level from qRT-PCR

Calculation of the Cq value was done by double delta analysis by taking the averageCq value of housekeeping gene and gene being tested for the experiment. Four values were obtained; gene of interest being tested for experiment (TE), gene being tested control (TC), housekeeping gene experimental (HE) and housekeeping gene control (HC). The ΔCTE and ΔCTC were obtained by calculation the difference between TE-TC and HE-HC respectively. To arrive at double delta analysis, difference between ΔCTE and ΔCTE werecalculated. Further calculation by a formula 2^-ΔΔCt was done to get the expression fold change of gene of interest with the control one.

3.7 Biochemical parameters estimation

All the biochemical parameters in the present study were estimated by UV spectrophotometer using Erba diagnostic kits (Transasia Biomedical Ltd. Mumbai, India) except for blood electrolytes; sodium, potassium using Liquizyme kit (Beacon

Diagnostics Pvt. Ltd. Navsari, India) and magnesium, inorganic phosphorus using Agappe diagnostic kits (Ernakulam, Kerala). Total serum protein, Total serum albumin were estimated in g/dl. Total serum globulin was determined by taking a difference between total protein and total albumin and A:G ratio was calculated subsequently.

Serum calcium, blood urea nitrogen (BUN), cholesterol, triglyceride, high-density lipoproteins (HDL) and creatinine were derived and expressed in mg/dl whereas serums serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetate transaminase (SGOT) and Gamma glutamyl transaminase (GGT) were estimated in IU/L. Serum electrolytes were expressed in mmol/lt or mEq/lt.

3.8 Assaying of early diagnostic markers

3.8.1 Beta-hydroxy butyric acid (BHBA) estimation

Beta-hydroxy butyric acid (BHBA) was estimated using ELISA kits (Immunotag; GBiosciences, USA) following their standard protocols supplied with kits as described below.

3.8.1.2 Reagent preparation

1. All the reagents were brought to room temperature(18-25ºC) before use.
2. The standard was reconstituted with 120 µl of standard diluents to generate 2400 nmol/ml stock solution.
3. Then serial dilution of standard was done in a ratio of 1:2 to produce 1200 nmol/ml, 600 nmol/m, upto 150 nmol/ml; with 120 µl of diluents.
4. Wash buffer (25x) was diluted with distilled water to yield 1x solution.
5. Mixed the chemicals gently until crystals have completely dissolved.

3.8.1.3 Assay procedure

1. Prepared all the reagents, standard solutions and samples as instructed. The assay was performed at room temperature.

2. In assigned standard wells, 50 µl of standard was added.

3. In remaining wells, 40 µl of samples were loaded and 10µl of anti-BHBA antibody was added to sample wells.

4. In sample and standard wells, 50 µl of streptavidin-HRP was added.

5. Covered the plate with sealer and incubated it for 60 minutes at 37ºC.

6. The sealer was removed and washing was done 5 times with wash buffer.

7. Wells were soaked with 0.35 ml of wash buffer for 30 sec to 1 minutes for each wash.

8. 50 µl of Substrate A was added to each well then, 50 µl of substrate B solution was added. Plate was incubated for 10 minutes at 37º in the dark.

9. Stop solution was added in each well, the blue colour was changed into yellow .

10. Optical density of each well read at 450nm within 10 minutes of adding stop solution.

3.8.2 Estimation of tumor necrosis factor-α(TNF-α)

Blood serum tumor necrosis factor- α (TNF-α) was estimated by using ELISA kit (Cat. ELB-TNFa, RayBio, Georgia, USA) following standard protocol supplied with kits as given below.

3.8.2.1 Reagent preparation

1. All the reagents were brought to room temperature (18-25º) before use.

2. Assay diluents B was diluted 5-fold with de-ionised water before use.

3. The standard vial (Item C) was diluted with 400 µl of assay diluent A to prepare 200 ng/ml standard solution.

4. Further dilution were done as per instructions given in the manual and a blank solution with diluent only was made.

5. Diluted the 20 ml of wash buffer with 400ml of distilled water to obtain a final concentration of 1x.

6. For preparation of antibody solution, it was diluted about 80 fold with 1x assay diluents.

7. HRP-streptavidin was diluted 600 fold with 1x assay diluents, about 20 µl of HRP-streptavidin with 12 ml of diluents was mixed.

3.8.2.2 Assay procedure

1. All the reagents were brought to room temperature.

2. In 96 well plate, 100 µl of standard, sample and blank were loaded in the marked wells and incubated for 2.5 hours at room temperature with gentle shaking.

3. After incubation, solutions were discarded and washing was done with wash buffer four times with the help of multi channel pipette.

4. Complete removal of wash buffer after final washing was essential. The plate was inverted for blotting with tissue.

5. 100 µl of 1x prepared antibody was added to each well and incubate for 1 hour at room temperature with gentle shaking.

6. Discarded the solution and washing was done 4 times.

7. After that, 100 µl of streptavidin solution was added to each well and incubated for 45 minutes at room temperature with gentle shaking.

8. Solution was discarded and washing was repeated four times.

9. TMB one step substrate reagent was added to each well (100 µl) and incubated for 30 minutes at room temperature in the dark with gentle shaking.

10. Stop solution was added to each well, around 50 µl and absorbance was taken at 450 nm immediately.

3.8.3 Estimation of Bovine interferon gamma (IFN-γ) using ELISA kit (Cat. ELB-IFNg, RayBio, Georgia, USA)

Blood serum Bovine interferon gamma (IFN-γ) was estimated by using ELISA kit (Cat. ELB-IFNg, RayBio, Georgia, USA) following standard protocol supplied with kits as given below.

3.8.3.1 Reagent preparation

1. All the reagents were brought in room temperature before use (18-25 degree).
2. Assay diluent (E) was diluted 5 fold with distilled water before use.
3. Preparation of standard: vial C was briefly centrifuged and 400 µl of assay diluents too prepare 100 ng/ml and further serial dilution was done as per given instruction in their manual. Blank was prepared with diluent alone.
4. Wash buffer was diluted with distilled water to get 1x concentration.
5. For detection antibody concentrate , 100 µl of assay diluents was added and further diluted 80 fold with 1x diluents.
6. HRP-streptavidin concentrate was diluted 600 fold with 1x assay diluents i.e 20 µl of HRP concentrate in 12ml of 1X assay diluents.

3.8.3.2 Assay procedure

1. All the reagents were brought in room temperature 18-25 º before use.
2. Standard and sample were added in their respective wells and incubated for 2.5 hours at room temperature with gentle shaking.
3. Solution was discarded after incubation and washed with 1x wash buffer four times.Proper decanting and aspiration of wash buffer was a necessity
4. Then, 100 µl of 1x prepared biotinylated antibody was added to each well and incubated for 1 hour at room temperature with gentle shaking.

5. Washing was repeated.

6. Prepared streptavidin solution was added (100 µl) in each well and incubated for 45 minutes at room temperature with gentle shaking.

7. Solution was discarded and washing was repeated.

8. Added 100 µl of TMB one substrate reagent in each well and incubated for 30 minutes at room temperature in the dark with gentle shaking.

9. Stop solution of about 50 µl was added in each well and absorbance at 450 nm was taken immediately.

3.9 Formulation of transition feed for buffaloes

3.9.1 Preparation of feed

A transition feed was prepared using various locally available feed ingredients and supplemented daily on the basis of animal requirement from day -30 to +30 of calving. Balanced concentrate mixture provided to the buffaloes containing essential components (crushed maize, oats, rice bran, and mineral mixture, vitamins (A,D and E).

3.9.2 Proximate principle analysis of feed

3.9.2.1 Moisture (%) and dry matter (DM) of feed

Moisture determination or dry matter was calculated by putting a known quantity (2-5 g) of sample in hot air oven and air dried at 100º for 8-12 hrs**.** The loss of weight was expressed as moisture percentage.

Calculation: Difference in weight of sample in a moisture cup before drying and sample in moisture cup after drying

$$\text{Dry matter (\%)} = 100 - \text{moisture (\%)}$$

3.9.2.2 Total ash

Feed sample of about 2-5 g into clean and dry pre- weighed silica basin. Sample was de-carbonised in silica basin on heater till no clear smoke was emitted. Silica basin

with de-carbonised sample was put in muffle furnace and ignited at 550º for 2-4 hours until no black particles were left. Ash in silica basin was cooled down in desicator and weighed quickly

Calculation: $\text{Total ash (\%)} = \text{weight of ash} \div \text{weight of sample} \times 100$

3.9.2.3 Crude protein (CP)

The crude protein of Weende analysis estimated the nitrogen present in sample and predicts the protein content based on nitrogen concentration in the protein. This analytical procedure is known as kjeldahl method. The method of estimation includes digestion, distillation and titration. In kjeldahlfalsk, 1 gm of sample and 50 ml of commercial sulfuric acid was added along with 5 gm of digestion mixture($CuSo_4$:KOH/NaOH). The content in flask was boiled for 5-8 hours till the solution was clear without having any black undigested material in it. Adhered material inside flask was washed 2-3 times after cooling. Digested material was transferred into volumetric flask after cooling and made upto final volume of 250 ml. For distillation process, a conical flask was placed with 10 ml of tashiro's indicator at the end of condenser of micro-kjeldahl apparatus. Digested sample from volumetric flask was taken (5-10 ml) into distillation flask. Sodium hydroxide (40%) was added to make content alkaline and washing with distilled water was done. Receiving end was closed immediately with cock/cotton. Funnel was sealed with little amount of distilled water to avoid escape of ammonia. Content in distillation unit was steam distilled by boiling the water in round bottom flask connected to distillation unit. About 30-50 ml of distillate was collected to ensure that all the nitrogen in the form of ammonia was distilled. Red/dark pink colour changed to green. Conical flask with distillate after washing the tip of the condenser was removed. After that, titration of the distillate in conical flask against standard N/7 H_2SO_4 solution in burette was done till faint color appeared. Volume of N/7 H_2SO_4 consumed at end point was noted.

Calculation:$\text{CP (\%)} = \text{A} \times (\text{B} - \text{B1}) \times 0.002 \times 6.25 \times 100 \div \text{C} \times \text{W}$

A = Volume (ml) of made out of digested material

B = Volume (ml) of N/7 H_2SO_4 consumed for titration of sample

B1 = Volume (ml) of N/7 H_2SO_4 consumed for titration of blank sample

C = Volume (ml) of aliquot taken for distillation

W = Weight (gm) of sample taken for digestion

6.25 = Factor of converting nitrogen into protein of feed stuff

3.9.2.4 Ether extract (Crude fat)

Ether extract was determined by extracting a moisture free sample with a fat solvent like petroleum ether in Soxhlet ether extract apparatus. This method in addition to glycerides, fatty acids contains free fatty acid, cholesterol, sterols etc thus also known as crude fat. A small amount of moisture free sample was weighed and put in a thimble made of whatman no. 1 filter paper. Thimble was plugged with cotton and placed in extractor having small cotton plug at the bottom to avoid spillage of particles. Extractor was fixed to a clean dry pre-weighed oil flask. Required amount of solvent was poured from the top of condenser with the help of funnel about 1.5 times capacity of extractor and cotton was plugged at the top of condenser to avoid evaporation of solvent. Extraction time was between 8-12 hours with minimum of 250 condensations. The flask was oven dried to a constant weight and sample free of fat and moisture was used for estimation of crude fat.

$$\textit{Calculation}: \text{Ether Extract } (\%) = (\mathrm{Y} - \mathrm{X}) \times 100 \div \mathrm{W}$$

Y = Weight of oil flask after extraction

X = Weight of flask before extraction

W = Weight of oven dried sample

3.9.2.5 Crude fiber

Crude fiber analysis partitions the carbohydrates into soluble and insoluble fibrous substances. The fibrous part is estimated as crude fiber fraction. The moisture and fat free sample was refluxed with weak sulfuric acid and alkali, each followed by filtration and repeated hot water washing .the remaining residue was air dried and ashed. The loss of weight was expressed as crude fiber. 1 gm moisture and fat free sample was taken in a spoutless beaker and 25 ml of 2.04 N H_2SO_4 was added. Distilled water was

added to make up the volume upto 200 ml. The content in the beaker was boiled for 30 minutes, after placing the round bottom flask with cold water as condenser on the top of beaker. The content in the beaker was filtered after cooling and washed with hot water and filtrate on muslin cloth was collected. Then, the residue/filtrate was treated in a same way with 2.50 N NaOH (alkali). After final washing with hot water, filtrate was collected in silica basin and oven dried at 100º to constant weight. Weight of dried residue was noted down. Decarbonisation of the residue was done on heated flame and then was put in muffle furnace at 550º for 1-2 hours. The left over ash in silica basin was weighed after cooling in dessicator.

$$\textit{Calculation}: \text{Crude fibre } (\%) = (a - b) \div w \times 100$$

a = Weight of silica basin oven dried residue after acid and alkali treatment

b = Weight of silica basin plus ash

c = Weight of oven dried sample

3.9.3 Transition feed trial

3.9.3.1 Animals and supplementation of transition feed

Management of production diseases during transition period in buffaloes was done by using a formulated transition feed. For nutritional interventions, a total of 20 buffaloes (1-3 parity) divided into 2 groups viz. Group-I (Control, no transition feed) and Group-II (transition feed supplemented group), each comprising 10 animals.In Gp.-II, transition feed was supplemented @ 1 kg/animal/day during transition period (from -30 day to +30 day of parturition) in addition to basal diet.

3.9.3.2 Evaluation of effect of transition feed supplementation

Transition feed supplementation effect was evaluated by various parameters at different intervals viz. -30 day, -15 day, 0 days (day of calving) and +30 days post calving.Sampling was done from each group at -15, 0 and +30 day postpartum. Blood samples collected for analysis including hematology, oxidative stress markers, cytokine and biochemical parameters in a same manner as mentioned above (3.4, 3.5. 3.7 and 3.8). Milk samples after calving were subjected for MCMT, SCC.

Total RNA isolation was done from the EDTA contained blood samples of feed and control group at pre-defined time (-15, 0, +15 and +30 days). Synthesis of cDNA from the extracted RNA and quantification of expression of selected genes was done with the aid of thermal cycler PCR and qRT-PCR respectively (procedure mentioned above).

The expression level of selected genes for hypocalcemic condition, negative energy balance and inflammatory condition/mastitis (Table 3.3) were seen in both the groups at pre- defined time interval.

Chapter-IV

Results

CHAPTER-4

RESULTS

4.1 Prevalence and associated risk factors of the production diseases in buffaloes during transition period

A total of 210 buffaloes were examined in the transition period (-30 to +30 days) during the period from August 2019 to November 2020. Animals in transition period were divided into three groups (n=70) viz. 3 weeks pre-partum (Group I), calving day (Group II) and 3 weeks post-partum (Group III). For prevalence of hypocalcaemia and ketosis, parameters like calcium, phosphorus, Ca:P ratio, Rothera's test for urine and blood glucose level were estimated. Group III (3 weeks postpartum) was analyzed for Modified California Mastitis Test (MCMT) and somatic cell count (SCC). The screened buffaloes were in the age between 4-10 years, with 3-6th parity and milk yield between 1800-2058 kg per lactation. The previous milk yield per day in buffaloes was 6.8 to 7.9kg.

4.1.1 Prevalence of production diseases on the basis of biochemical/ field tests.

4.1.1.1 Prevalence of milk fever/ hypocalcemia in buffaloes during transition period.

In our present study, it was seen that there was no evidence of clinical cases of milk fever/typical hypocalcemia in buffaloes during transition period when subjected for calcium estimation. However, mean values of calcium (mg/dl) showed significant decrease ($p<0.05$) from group I to group III (8.40±0.03, 8.1±0.02 and 7.98±0.02). Therefore, on the basis of blood serum calcium level, prevalence of milk fever in buffaloes was zero. Further, these samples were subjected for calcium metabolism related genes expression study as molecular diagnostic tools.

4.1.1.2 Prevalence of ketosis in buffaloes during transition period.

Out of 210 buffaloes in different time interval of transition period, there was no positive case found for ketosis by Rothera's test and urine strips used for detection of ketone bodies. The beta-hydroxy butyric acid (BHBA) values were also under the normal range (<1200 nmol/ml) in all the three groups. However, blood serum BHBA increased

significantly ($p<0.05$) from Gp-I to Gp-III (313.96±2.81 nmol/ml, 397.36±1.69nmol/ml and 483.69±1.98 nmol/ml, respectively). Blood glucose levels in all three groups were also under normal range. Further, these animals were subjected for gene expression studies. Urine analysis of the selected buffaloes showed negative result for Rothera's test (Fig. 4.1) and urine strips (Fig. 4.2) for ketone bodies when compared with positive control.

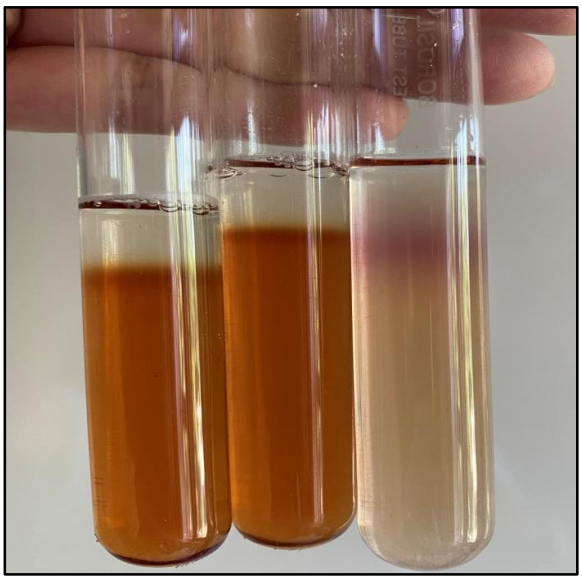


Fig. 4.1: Figure depicting negative results of Rothera's test with positive control (purple ring)

Fig 4.2: Negative result of ketone bodies detection in urine strips with positive control

4.1.1.3: Prevalence of mastitis in buffaloes during transition period (Post-partum group).

For evaluation of prevalence of mastitis among buffaloes, animals after calving (Gp-III) were examined for MCMT and SCC, 62.85% were MCMT negative and had an average of 168.41±0.8 ($\times 10^3$/ml) somatic cell count whereas 37% were CMT (T/+) with SCC of 278±0.67 ($\times 10^3$/ml).

4.1.2 Prevalence of production diseases on the basis of gene expression/ molecular tests.

4.1.2.1 Identification of production disease specific markers by *in-silico* analysis.

4.1.2.1.1 *In-silico* analysis for milk fever markers

The distribution of the probe intensities are shown in Fig.4.3 and it varied from 100 to 1600 observations between both normal and milk fever samples.

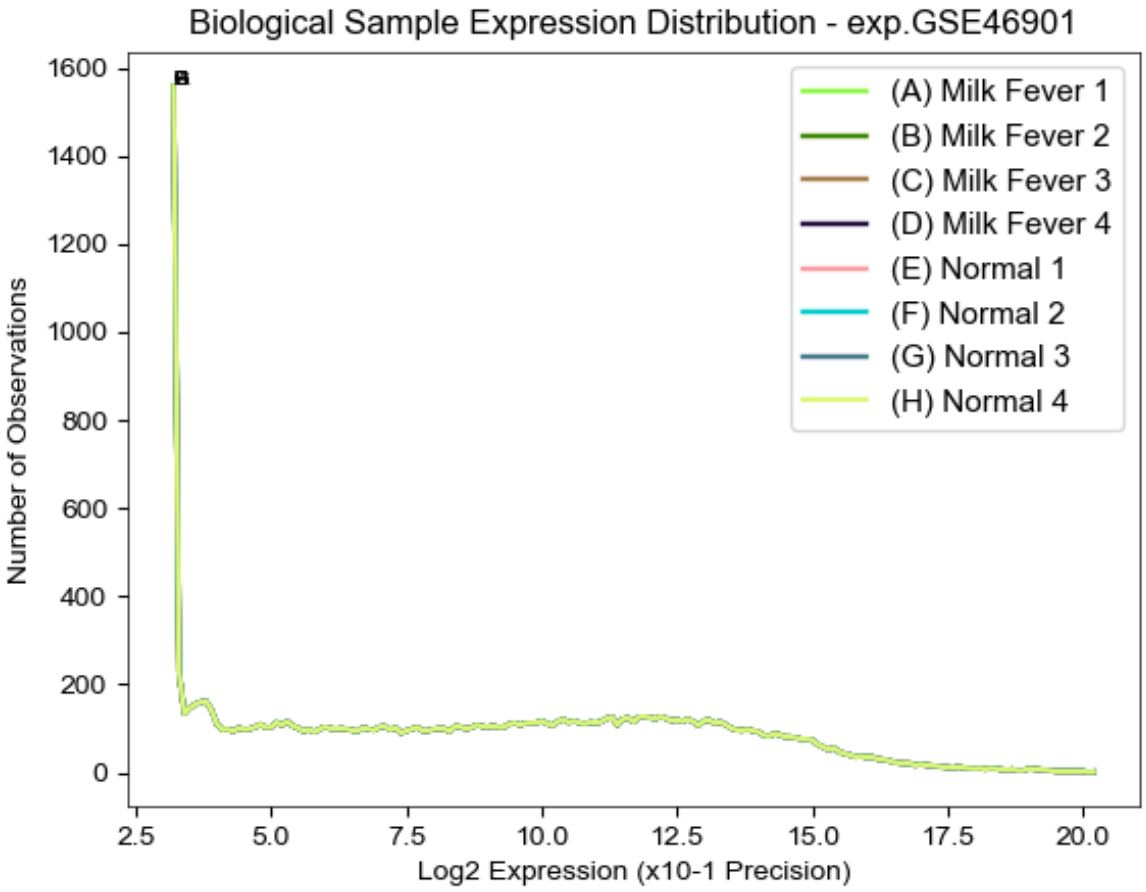


Fig. 4.3: Distribution of microarray probe intensities in milk fever and normal samples

4.1.2.1.1.1 Differentially expression analysis, clustering and principal component analysis

Compared with normal and milk fever samples 1,761 were found to be differentially expressed in the milk fever samples. Among these 918 found to be upregulated and 843 genes were found to be downregulated. In order to study the relationship between the gene expressions patterns in different samples, clustering of the differentially expressed genes was used (Fig.4.4). Both milk fever and normal groups were clustered separately by Hierarchical clustering. Principal component analysis (Fig.4.5) of the two groups revealed that all milk fever samples were sharing one component and all normal samples were sharing other principal components. The top 10 upregulated and downregulated genes are shown in Table 4.1.

Table 4.1: Top 10 up- and down-regulated genes in milk fever cows.

GeneID	log fold	p_value
	Upregulated Genes	
ABR398	1.071196	0.002996446
ABR396	1.097558	0.029088045
A_73_106705	1.061531	0.003337784
ABR7885	2.713719	0.001812921
ABR7884	1.155886	0.027572363
ABR5953	2.232789	0.022460489
A_73_120623	1.499876	0.007967889
ABR942	2.952955	0.003940428
ABR2688	1.738063	0.012512236
ABR1141	1.032224	0.017780196
Downregulated Genes		
ABR2473	-1.13247	0.026255498
ABR1652	-1.46121	0.003761416
ABR1655	-1.11553	0.039544881
ABR1656	-1.01527	0.007367563
ABR2872	-1.12142	0.000183830
A_73_107929	-1.47057	0.013874517
A_73_121348	-1.08098	0.011505187
ABR6020	-1.89149	0.030677436
ABR3650	-1.30661	0.000814204
A_73_113950	-1.32781	0.003349288

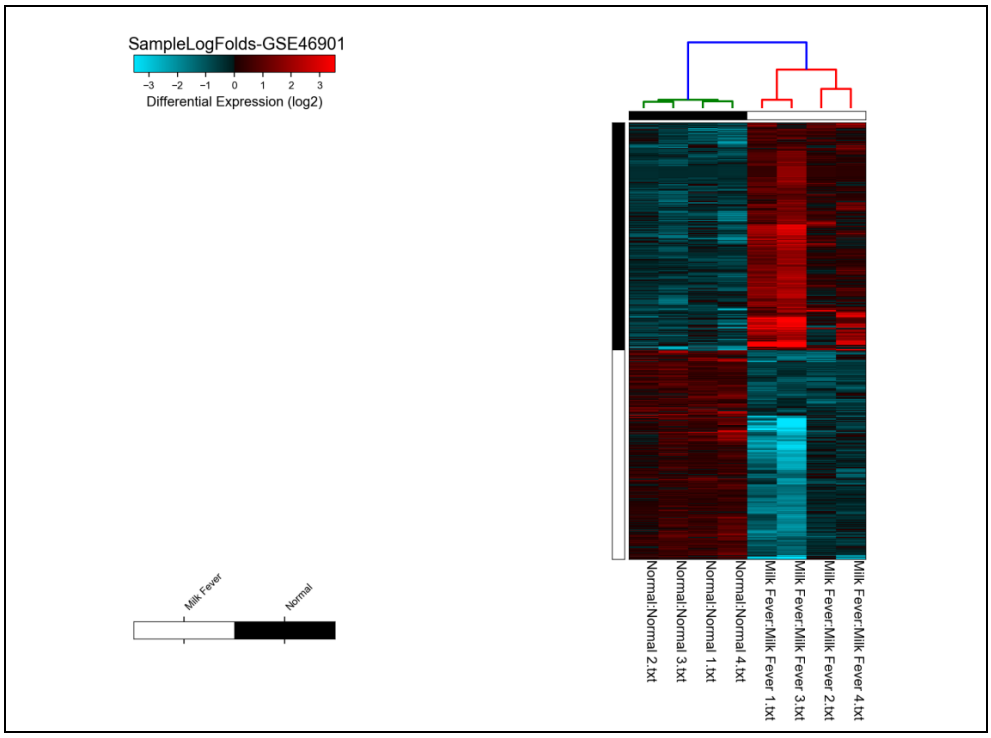


Fig. 4.4: Heatmap showing the log-fold changes of genes and hierarchal clustering of various milk fever and normal samples.

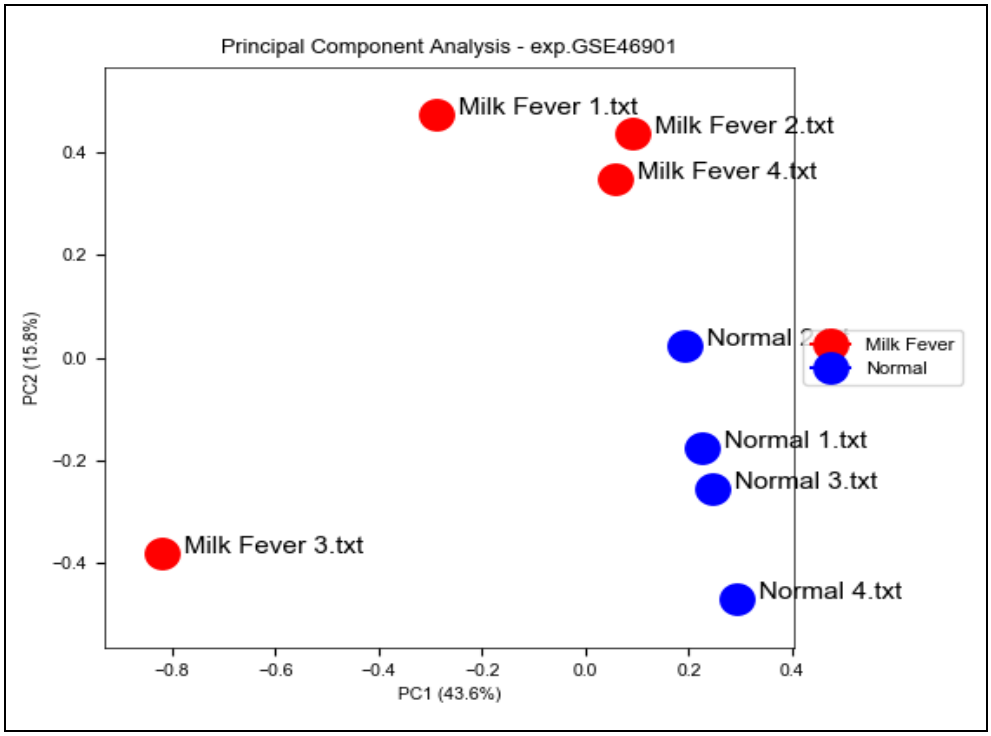


Fig. 4.5: Principal component analysis (PCA) of milk fever and normal samples sharing different principal components. Red spheres represent milk fever samples whereas blue spheres represent normal samples.

4.1.2.1.1.2 Marker genes identification

Total 60 milk fever specific genes were determined by Pearson correlation coefficient. The top 10 correlated genes determined with at least a 0.3 Pearson correlation coefficient were selected as the gene signature for each cell clusters. The top 10 genes identified as markers in milk fever include top 10 genes identified as marker genes in normal samples shown in Fig. 4.6. The list is shown in Table 4.2.

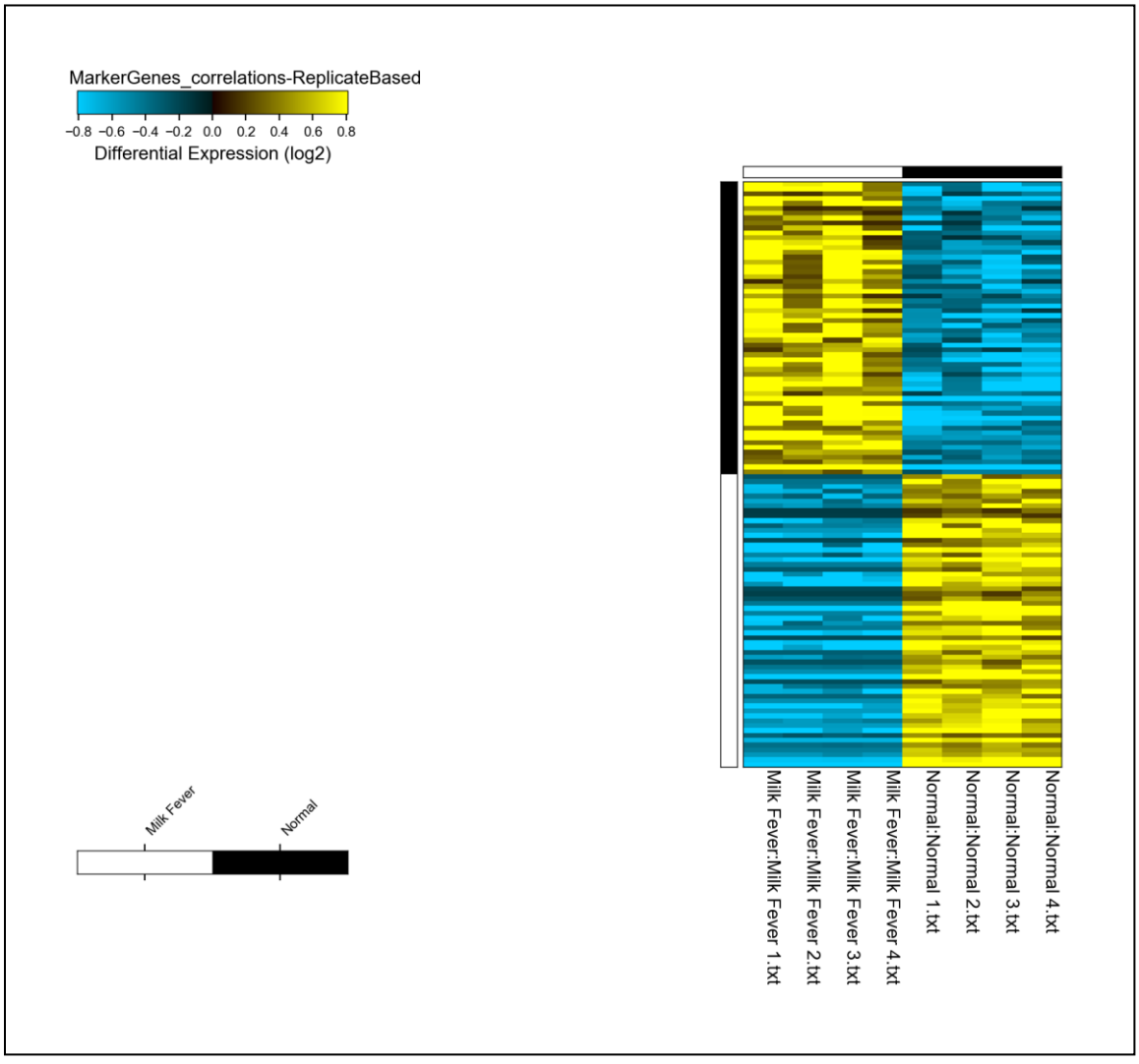


Fig. 4.6: ICGS analysis of transcriptomes for finding markers of milk fever and normal samples.

Table 4.2: Potential marker genes for detection of milk fever in cows

Symbol	Pearson rho	Pearson p-value	Cell State
ABR8148	0.949382599	0.000312036	Normal
ABR5353	0.999181884	1.37E-09	Normal
ABR4997	0.997859354	2.45E-08	Normal
ABR5665	0.994908004	3.29E-07	Normal
ABR2328	0.994252266	4.73E-07	Normal
ABR5114	0.989924544	2.54E-06	Normal
ABR4354	0.986367468	6.27E-06	Normal
ABR7171	0.985147870	8.10E-06	Normal
ABR6474	0.984616208	9.00E-06	Normal
ABR203	0.982293107	1.37E-05	Normal
ABR8862	0.973230117	4.70E-05	Milk Fever
ABR168	0.971139743	5.88E-05	Milk Fever
ABR178	0.969982231	6.61E-05	Milk Fever
ABR6614	0.969759295	6.76E-05	Milk Fever
A_73_107895	0.964875740	1.05E-09	Milk Fever
ABR1852	0.959190340	1.64E-09	Milk Fever
A_73_112653	0.957624656	1.84E-09	Milk Fever
A_73_121091	0.954477217	2.27E-09	Milk Fever
A_73_101935	0.953866179	2.37E-09	Milk Fever
ABR8148	0.949382599	3.12E-09	Milk Fever

4.1.2.1.2 *In-silico* analysis for ketosis markers

The distribution of the probe intensities are shown in Fig. 4.7 and it varied from 0 to 8000 for ketosis and 0 to 12000 observations for healthy samples.

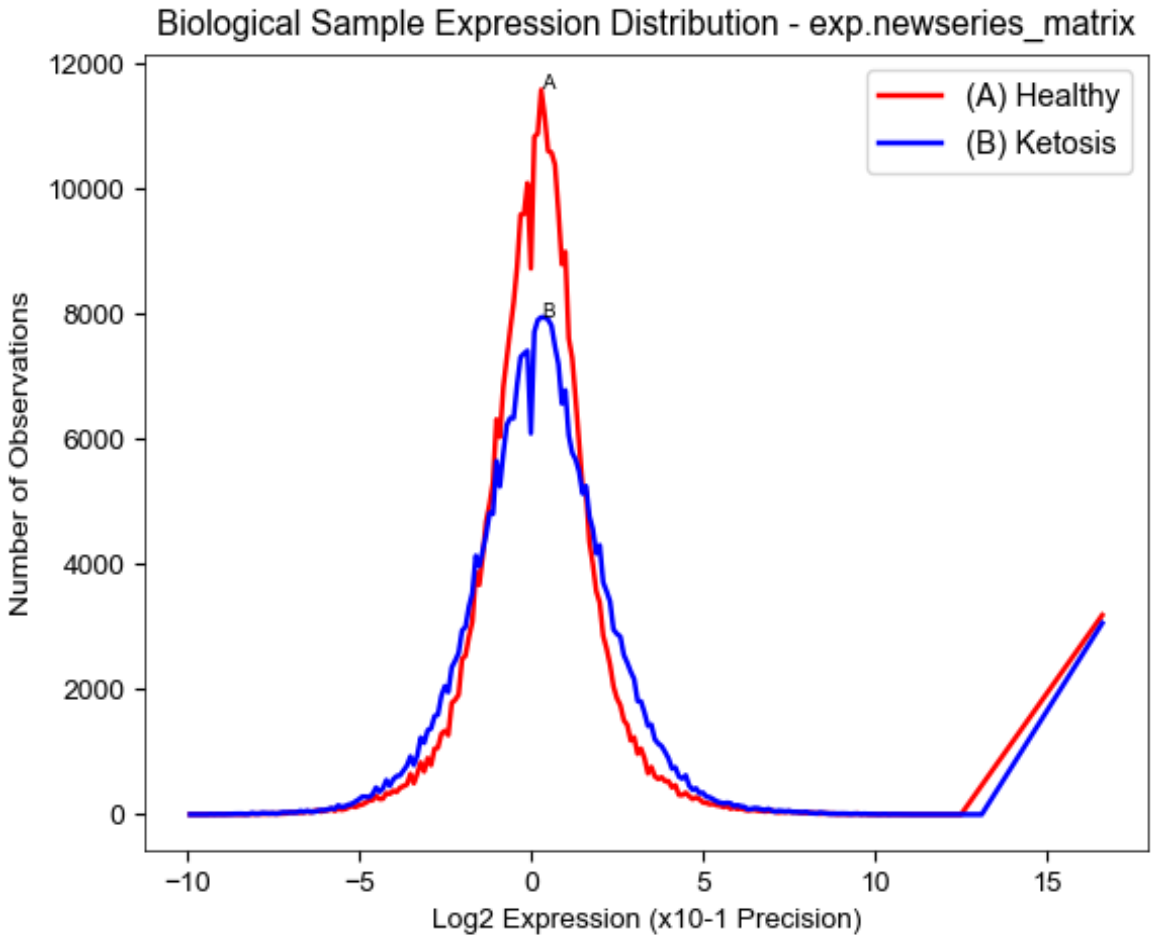


Fig. 4.7: Distribution of microarray probe intensities in ketosis and healthy samples.

4.1.2.1.2.1 Differentially expression analysis, clustering and principal component analysis

Compared with normal non-ketosis samples, 2,121 were found to be differentially expressed in the ketosis samples. Among these 1,053 found to be upregulated and 1,068 genes were found to be downregulated. In order to study the relationship between the gene expression patterns in different samples, clustering of the differentially expressed genes was used (Fig. 4.8). Both ketosis and healthy groups were clustered separately by Hierarchical clustering. Principal component analysis (Fig. 4.9) of the two groups revealed that all ketosis samples were sharing one component and all healthy samples were sharing other principal components.

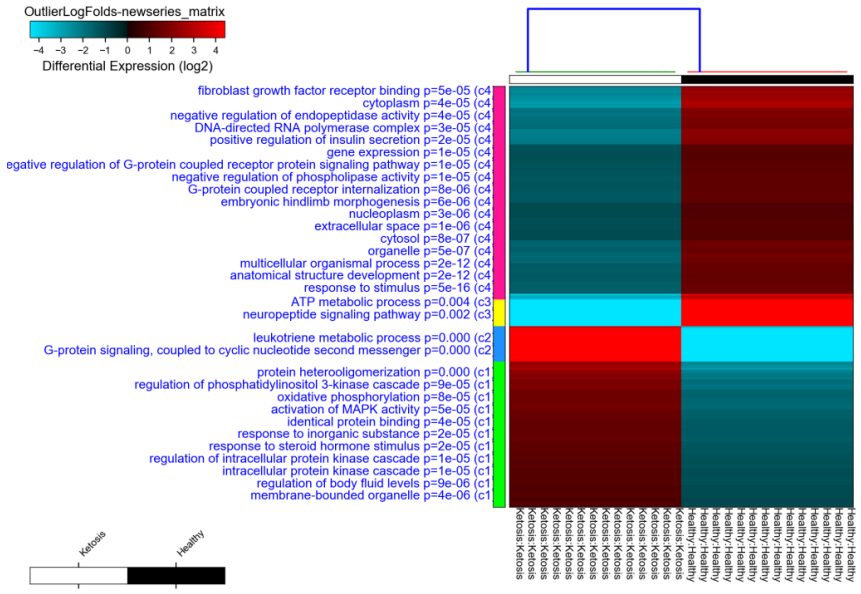


Fig. 4.8: Heatmap showing the log-fold changes of genes and hierarchal clustering of various Ketosis and Healthy samples.

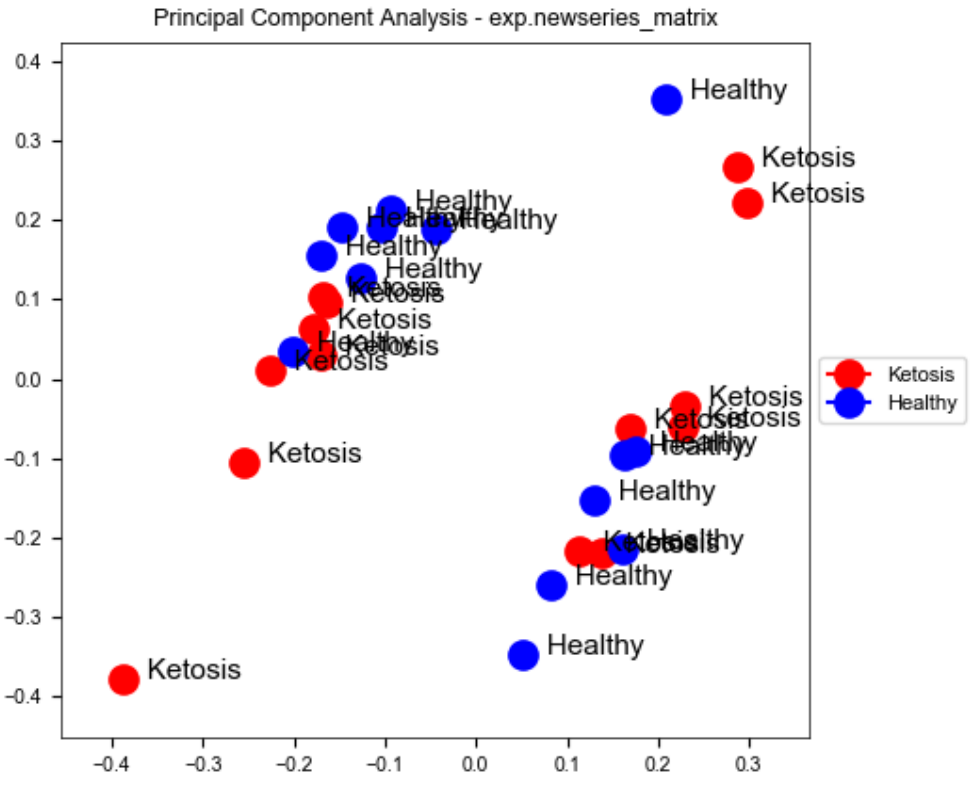


Fig. 4.9: Principal component analysis (PCA) of ketosis and healthy samples sharing different principal components. Red spheres represent ketosis samples whereas blue spheres represent healthy samples.

4.1.2.1.2.2 Gene ontology and pathway analysis

Gene ontology analysis revealed that some of the genes were directly related to the immune response, inflammatory response, and chemotaxis and leukocyte cell-cell adhesion. Pathway analysis of the up-regulated genes (Table 4.3; Fig. 4.10) was related to the immune response, with the majority of genes involved in the induction and regulation of the local inflammatory response. Upregulated genes include IL6, IL3, CCR1, IFNB1, IL12RB1 and LEP. Pathways associated with these genes were pathways involved in Cytokine-cytokine receptor interaction. The group of down-regulated genes (Table 4.3) in the ketosis found to involve in Glycosphingolipid biosynthesis (Fig. 4.11) and fat digestion and absorption pathways (Fig. 4.12). It is well known that cows with ketosis experience decreased milk production. However, the affected molecular mechanisms related to fat synthesis in those cows has been undefined. The results presented may suggest that ketosis reduces the expression of genes involved in fat metabolism. However, further research is needed to verify whether these genes actually influence the total amount of fat secretion in the milk or instead influence the fat percentage in the milk.

Table 4.3: Top 10 up- and down-regulated genes in bovine ketosis.

Gene Symbol/ ID	Log fold value	p value
Upregulated genes		
LOC539627	2.06	0.004198
LOC514162	2.29	0.003647
MYOD1	2.03	0.009818
3453	2.02	0.000611
18184	2.34	0.003018
20440	2.99	0.001995
10354	8.08	0.006570
24762	10.20	0.001088
16680	2.03	0.014555
12089	6.60	0.011377
Downregulated genes		
UMOD	-2.14	0.002491
25009	-2.12	0.000604
12815	-2.03	0.001730
ZP4	-2.70	0.000699
23213	-6.10	0.007947
LOC767868	-5.26	0.008864
BOLA-NC1	-6.72	0.010887
SAMM50	-5.78	0.010735
14012	-2.17	0.004844
20902	-6.59	0.013437

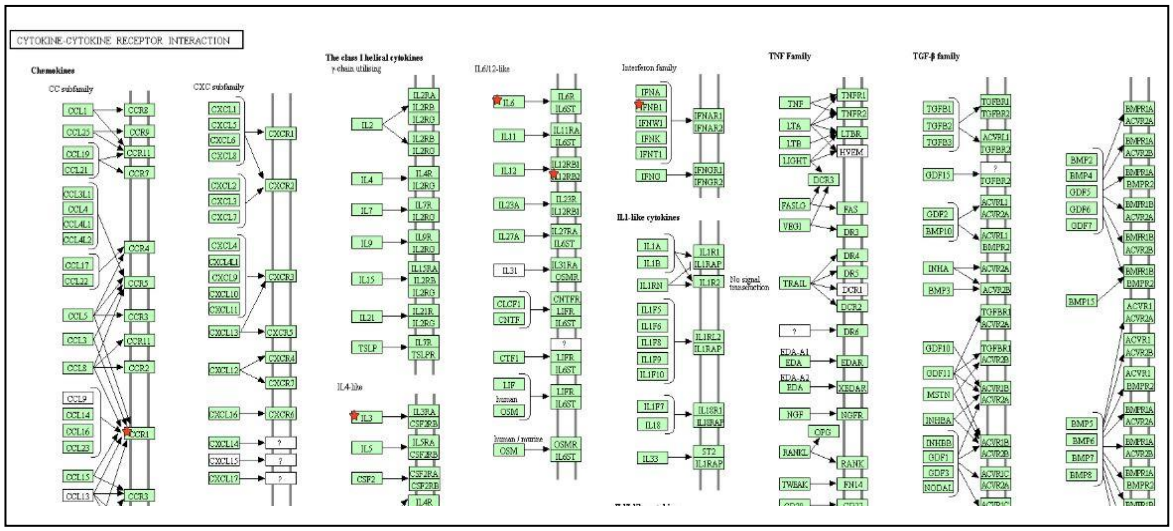


Fig. 4.10: Participation of up-regulated genes in cytokine receptor signaling pathways. Genes in star marked were found to be up-regulated in ketosis samples.

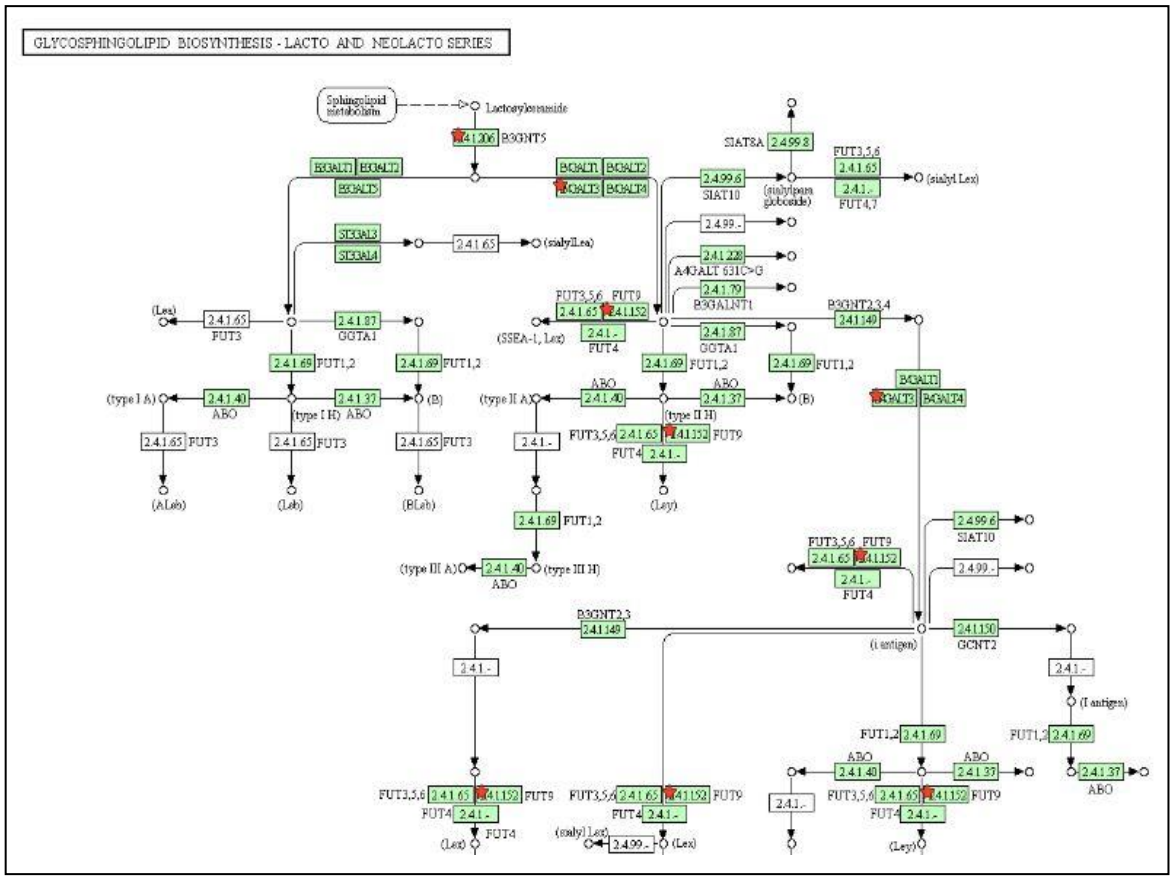


Fig. 4.11: Participation of down-regulated genes in Glycosphingolipid biosynthesis. Genes in star marked were found to be down-regulated in ketosis samples.

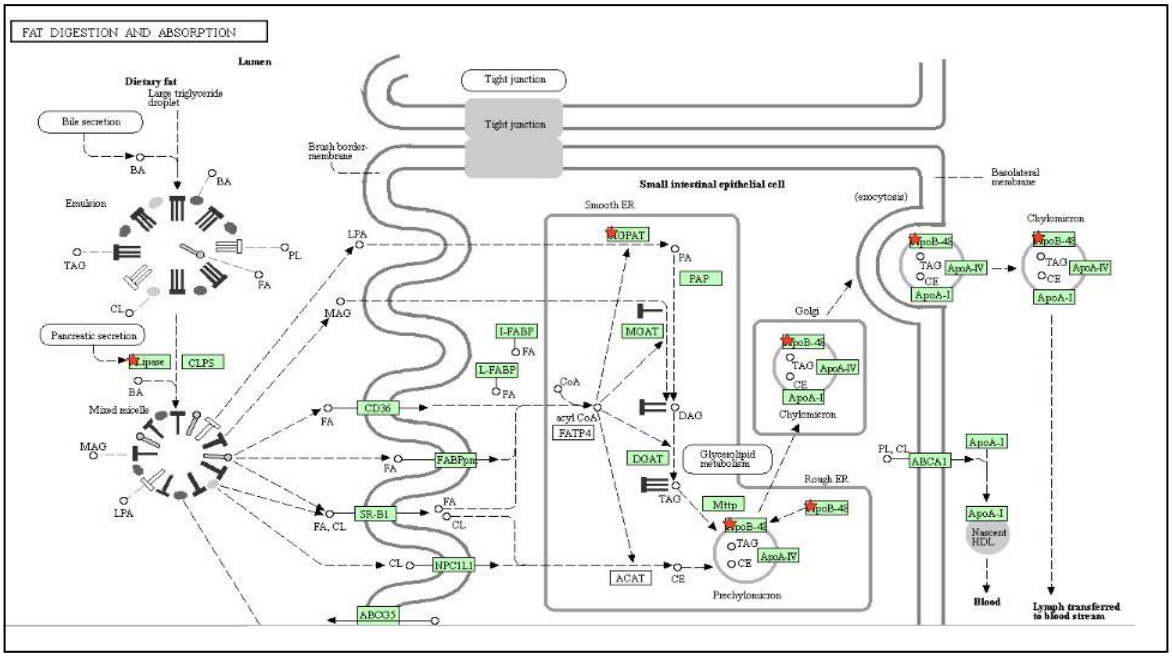


Fig. 4.12: Participation of down-regulated genes in fat digestion and absorption. Genes in star marked were found to be down-regulated in ketosis samples.

4.1.2.1.2.3 Marker genes identification for ketosis

A total 60 ketosis- specific genes were determined by Pearson correlation coefficient. The top 10 correlated genes determined with at least a 0.3 Pearson correlation coefficient were selected as the gene signature for each cell clusters (Fig. 4.13). The top 20 genes identified as markers in Ketosis include the IL6, IL3, CCR1, IFNB1, IL12RB1 and LEP. On the other end, top 10 genes identified as marker genes in healthy samples are B3GNT5, B4GALT3 and FUT9. The complete list is shown in Table 4.4.

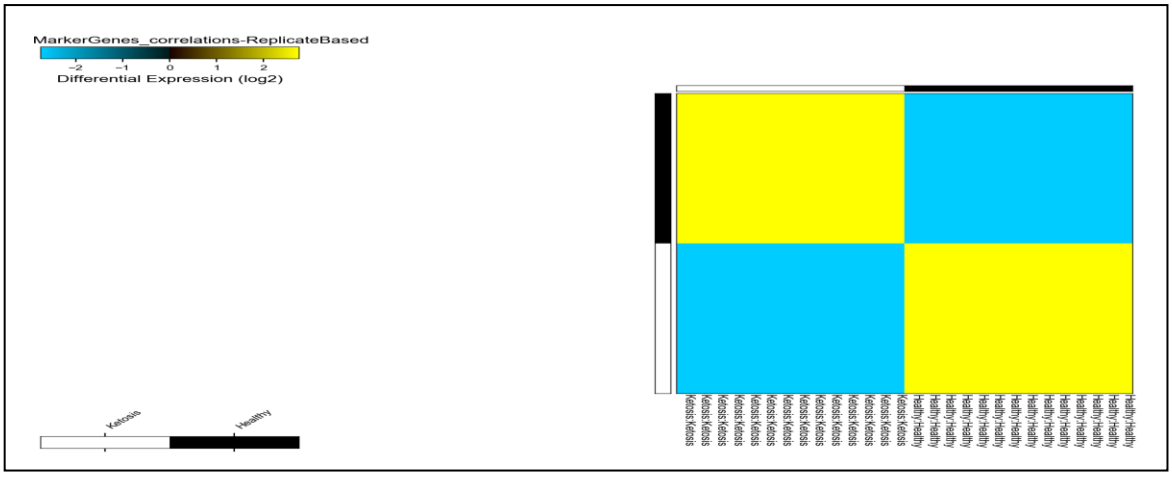


Fig. 4.13: ICGS analysis of transcriptomes for finding markers of ketosis and healthy.

Table 4.4: Potential marker genes for detection of bovine ketosis.

Gene Symbol/ ID	Pearson rho	Pearson p-value	Cell State
LOC510604	-4.55E-05	0.9998167	Healthy
GUCA1B	-1.30E-04	0.9994769	Healthy
MGC151578	-1.59E-04	0.9993597	Healthy
4031	-1.65E-04	0.9993331	Healthy
LOC784882	-1.88E-04	0.9992407	Healthy
18181	-2.06E-04	0.9991703	Healthy
7889	-2.19E-04	0.9991167	Healthy
GRTP1	-3.19E-04	0.9987156	Healthy
18929	-3.23E-04	0.9986973	Healthy
728	-3.80E-04	0.9984709	Healthy
14241	-8.45E-06	0.9999659	Ketosis
ASMTL	-5.31E-05	0.9997861	Ketosis
21265	-6.18E-05	0.9997509	Ketosis
20145	-6.53E-05	0.9997369	Ketosis
SNCA	-7.23E-05	0.9997085	Ketosis
AEBP1	-8.36E-05	0.9996631	Ketosis
MGC155209	-1.24E-04	0.9994985	Ketosis
LOC508367	-1.40E-04	0.9994373	Ketosis
CLPP	-1.53E-04	0.9993812	Ketosis
MGC160122	-1.59E-04	0.9993588	Ketosis

4.1.2.1.3 *In-silico* analysis for mastitis markers

4.1.2.1.3.1 Multiple basic quality control

Multiple basic quality control (QC) plots were produced to evaluate the sample quality and overall technical similarity to other samples in the dataset. Different QC

metrics were applied. Three output QC files were generated: 1) distribution of normalized log_2probeset intensity values, 2) mean raw signal intensities of each array and 3) mean absolute deviation (MAD) of the RMA residuals for each array. The source data for all of these three QC metrics were derived from the Affymetrix Power Tools. The distribution of the probe intensities are shown in Fig.4.14 and it varied from 0 to 550 observations between both mastitis and Healthy samples. The signal intensity shown in the Fig.4.15 revealed that the signal intensity value varies from 400 to 800 of both healthy and mastitis samples.

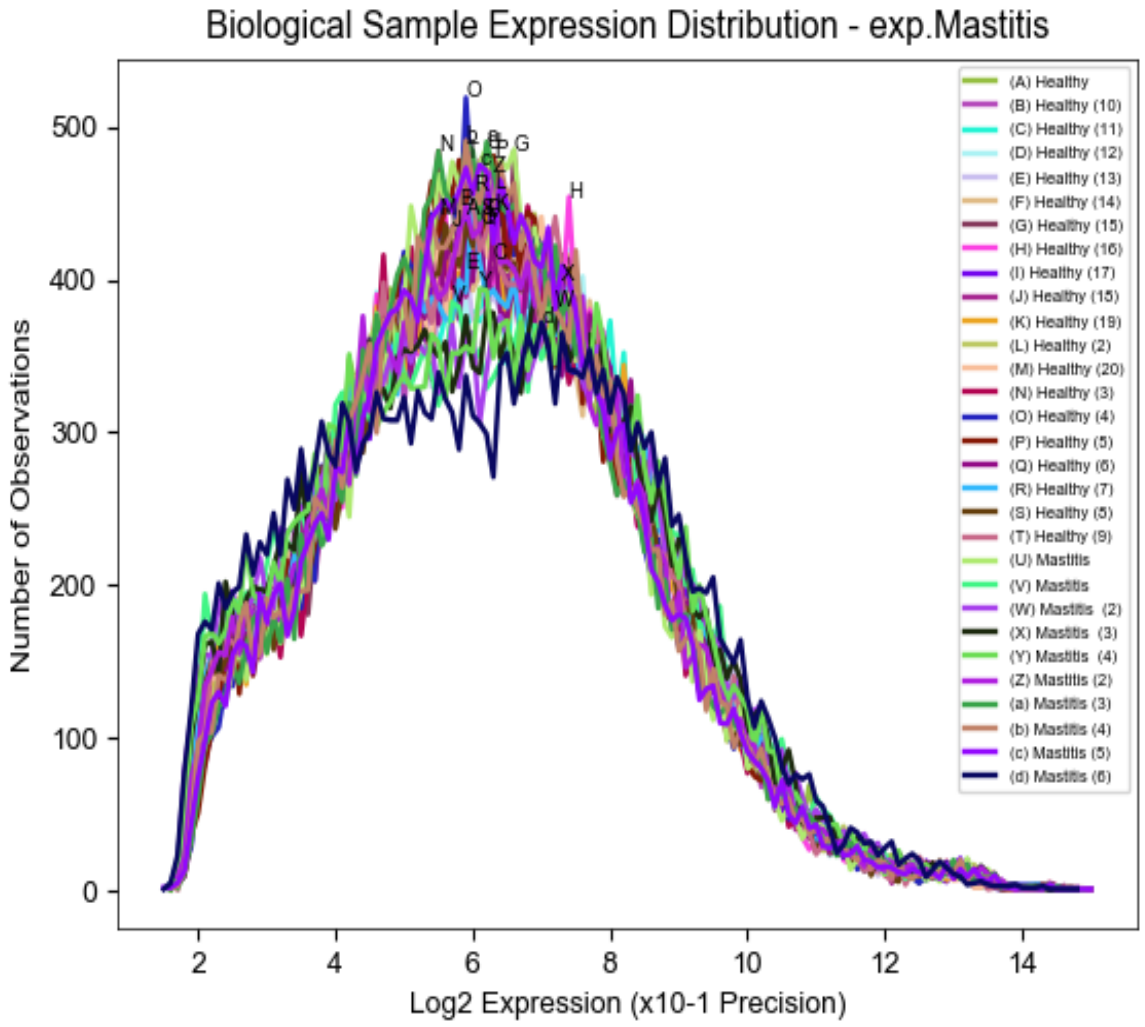


Fig. 4.14: Distribution of microarray probe intensities in mastitis and healthy samples.

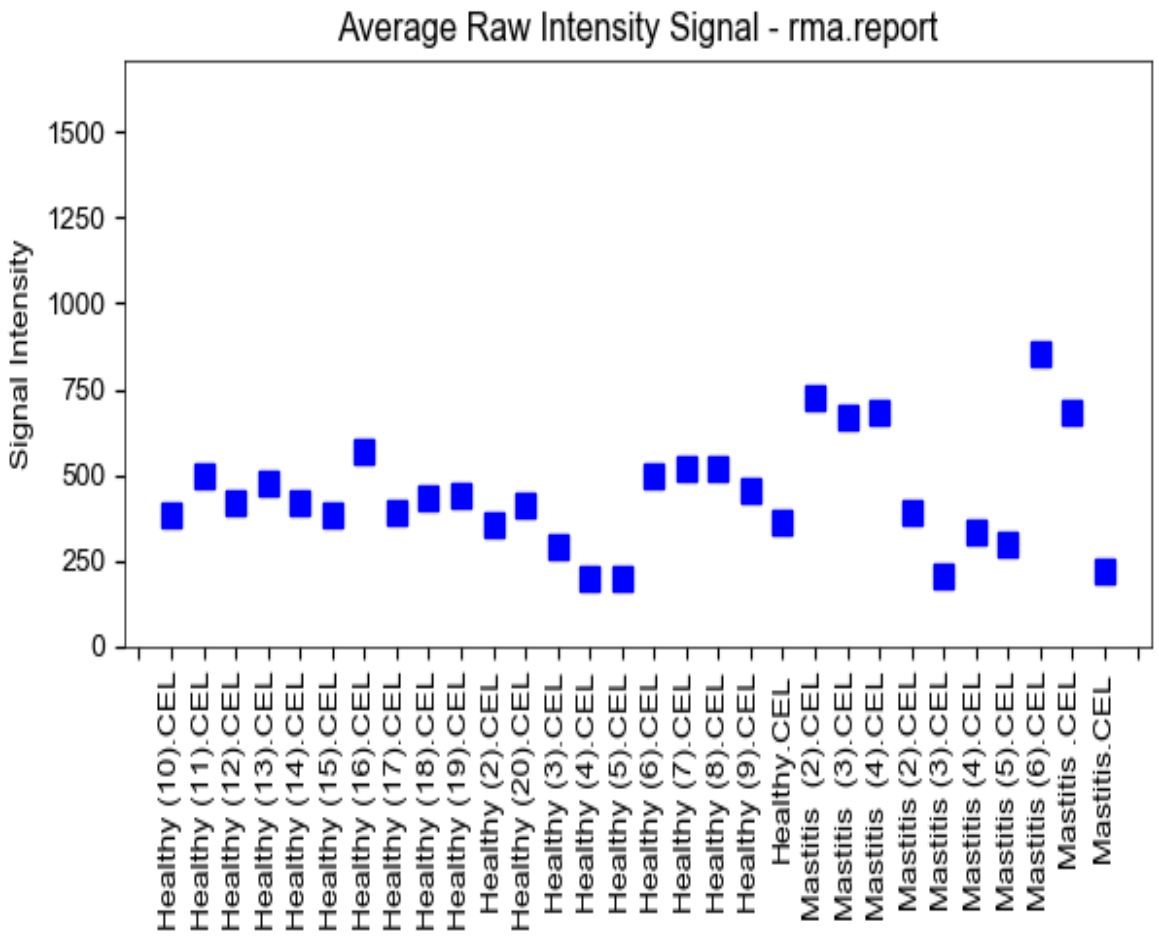


Fig. 4.15: Signal intensity robust multi-array analysis algorithm analysis in mastitis and healthy samples for normalizing the distribution

4.1.2.1.3.2 Differentially expression analysis, clustering and principal component analysis

Compared with Healthy samples 409 were found to be differentially expressed in the mastitis samples. Among these 306found to be upregulated and 103 genes were found to be downregulated (Table 4.5). In order to study the relationship between the gene expressions patterns in different samples, clustering of the differentially expressed genes was used (Fig. 4.16). Both mastitis and healthy groups were clustered separately by Hierarchical clustering. Principal component analysis (Fig. 4.17) of the two groups revealed that all mastitis samples were sharing one component and all healthy samples were sharing other principal components.

Table 4.5: Top 10 up- and downregulated genes in mastitis of mammary gland.

Gene Symbol/ ID	Log fold value	p-value	Biological Process
Upregulated genes			
HP	3.7181205	0.0002224	defense response
S100A9	3.477803	0.000836	response to wounding
PTX3	3.397747	1.47E-09	leukocyte aggregation
CXCL2	3.2492945	0.0001117	inflammatory response
S100A8	3.193347	0.0003501	immune system process
CXCL8	3.1167785	5.78E-05	defense response to bacterium
CXCR1	3.082216	5.92E-05	immune response
S100A12	3.048458	0.0009306	cellular response to zinc ion
BOLA	3.0204685	0.0016988	neutrophil aggregation
SAA3	2.9137555	2.49E-05	RNA destabilization
Downregulated genes			
LOC516849	-2.378388	0.0004804	positive regulation of antigen
ALOX15	-2.3277265	0.0012386	regulation of antigen processing
MSTN	-2.2944785	0.0019767	positive regulation of antigen processing
CAPN6	-2.2861555	0.0008425	regulation of antigen processing
TRAF3IP3	-2.2834185	0.0002498	positive regulation of antigen processing
TMEM120B	-2.2023025	0.0024439	lymphocyte chemotaxis
NFASC	-2.166867	0.0015806	hepatic immune response
SHCBP1	-2.1486475	0.0008298	myeloid dendritic cell activation
FASN	-2.1284355	0.0018092	leukocyte chemotaxis
LOC16	-2.1226	0.0166853	innate immune response pathway

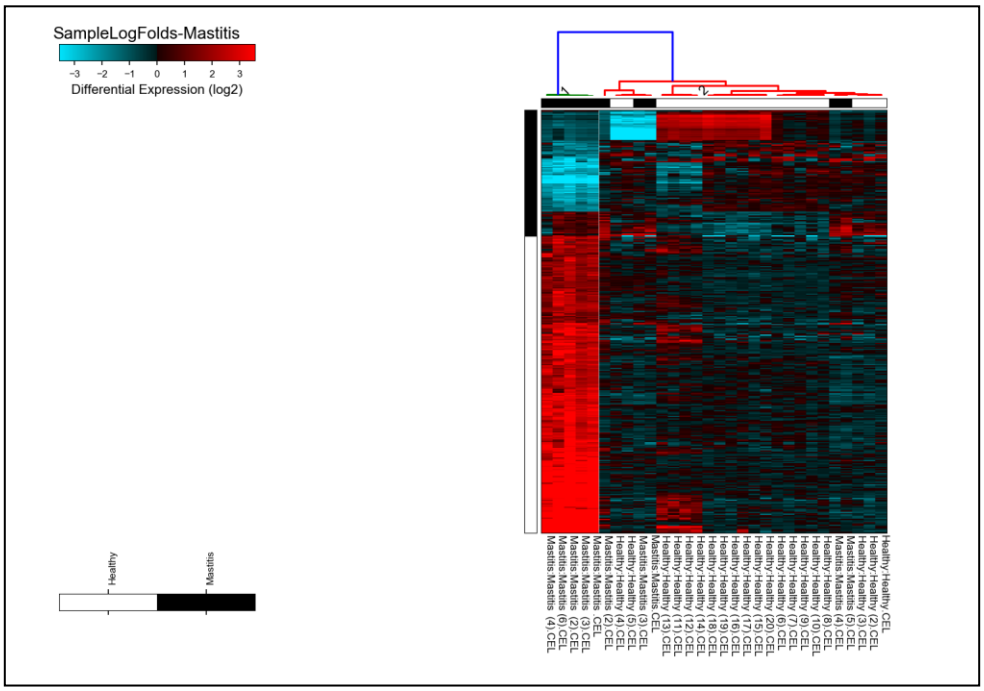


Fig. 4.16: Heatmap showing the log-fold changes of genes, and hierarchal clustering of various mastitis and healthy samples.

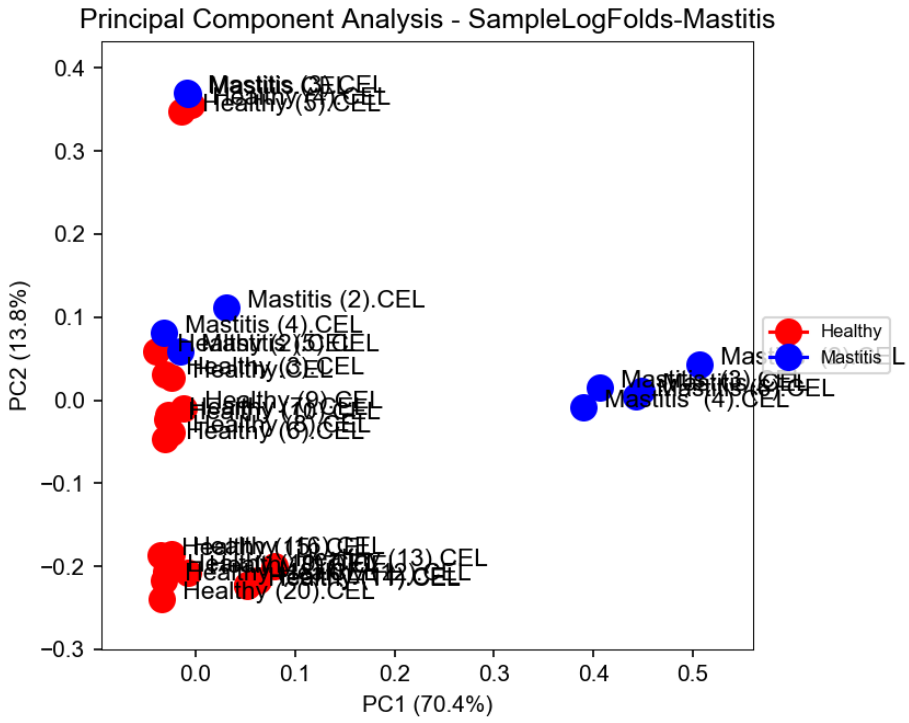


Fig. 4.17: Principal component analysis of mastitis and healthy samples sharing different principal components. Red spheres represent mastitis samples, whereas blue spheres represent healthy samples.

4.1.2.1.3.3 Gene ontology and pathway analysis

Gene ontology analysis revealed that some Biological process between the genes was directly related to the immune response, immune system process, inflammatory response and leukocyte aggregation. Pathway analysis of the upregulated genes were related to the immune response, with the majority of genes involved in the induction and regulation of the local inflammatory response. Pathways associated with these genes were Toll-like receptor (TLR) signaling (Fig.4.18), TNF signaling pathway (Fig.4.19) and chemokine signaling pathway (Fig.4.20). These finding indicates the importance of recruitment and activation of macrophages and neutrophils to sites of infection during mastitis. It is well known that cows with mastitis experience decreased milk production. However, the affected molecular mechanisms related to fat synthesis in those cows has been undefined. The results presented may suggest that Mastitis reduces the expression of genes involved in fat metabolism. However, further research is needed to verify whether these genes actually influence the total amount of fat secretion in the milk or instead influence the fat percentage in the milk.

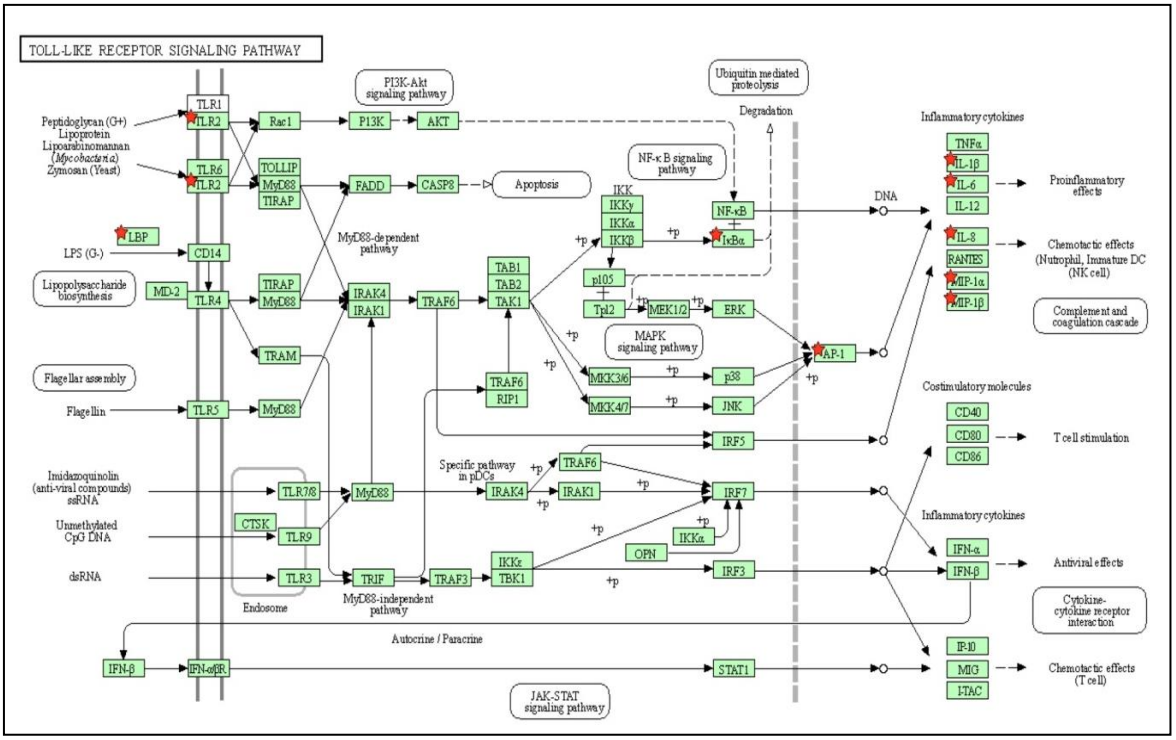


Fig. 4.18: Participation of upregulated genes in bovine toll like receptor signalling pathway. Genes highlighted in star symbol were found to be upregulated in mastitis samples.

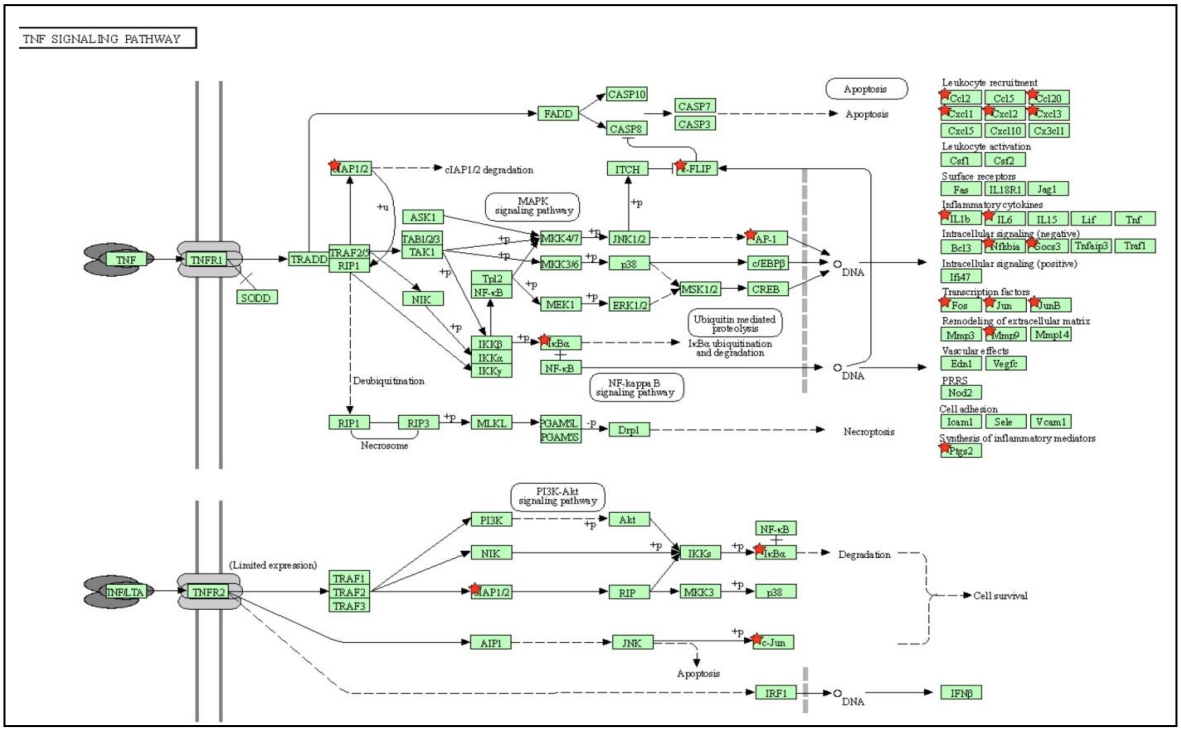


Fig. 4.19: Participation of upregulated genes in bovine TNF signalling pathways. Genes highlighted in star symbol were found to be upregulated in mastitis samples.

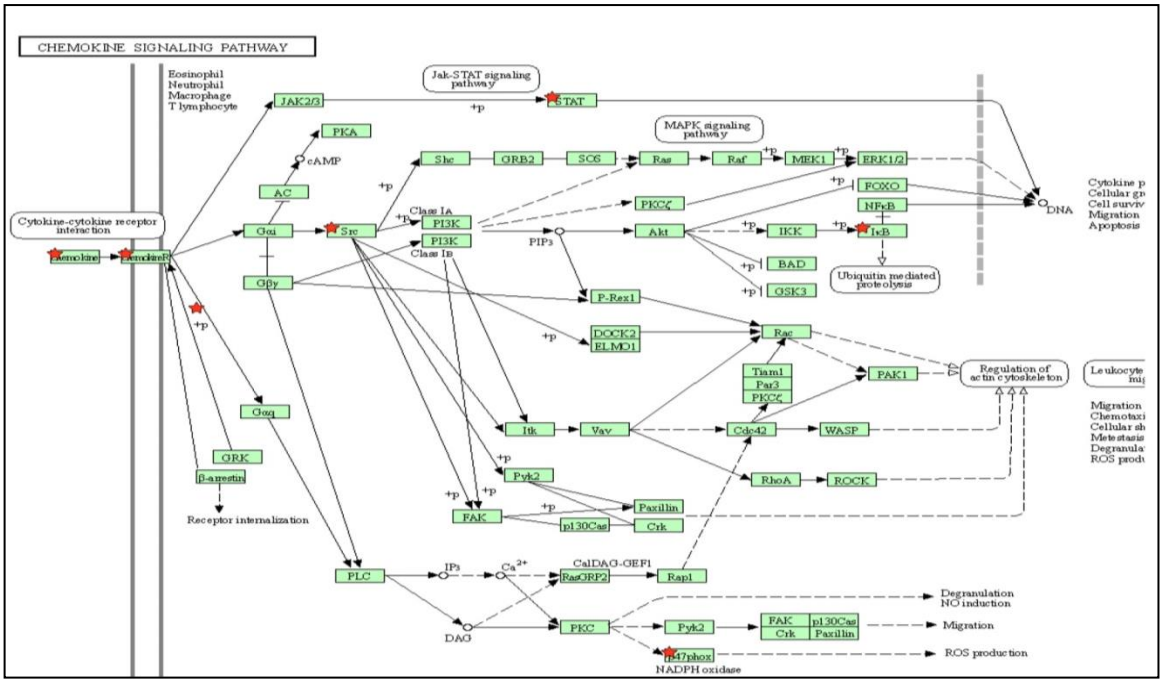


Fig. 4.20: Participation of upregulated genes in bovine Chemokine signalling pathway. Genes highlighted in star symbol were found to be upregulated in mastitis samples

4.1.2.1.3.4 Lineage analysis

Result of lineage analysis is shown as lineage correlation heat map (Fig.4.21). The lineage correlation heat map revealed that both mastitis and healthy Z score sharing was high in both lymph nodes and macrophages. The z score of mammary gland was high in healthy samples when compared to mastitis samples. In bone marrow and spleen, the Z score is more in mastitis when compared to healthy samples indicating that these genes are highly expressed in the tissues.

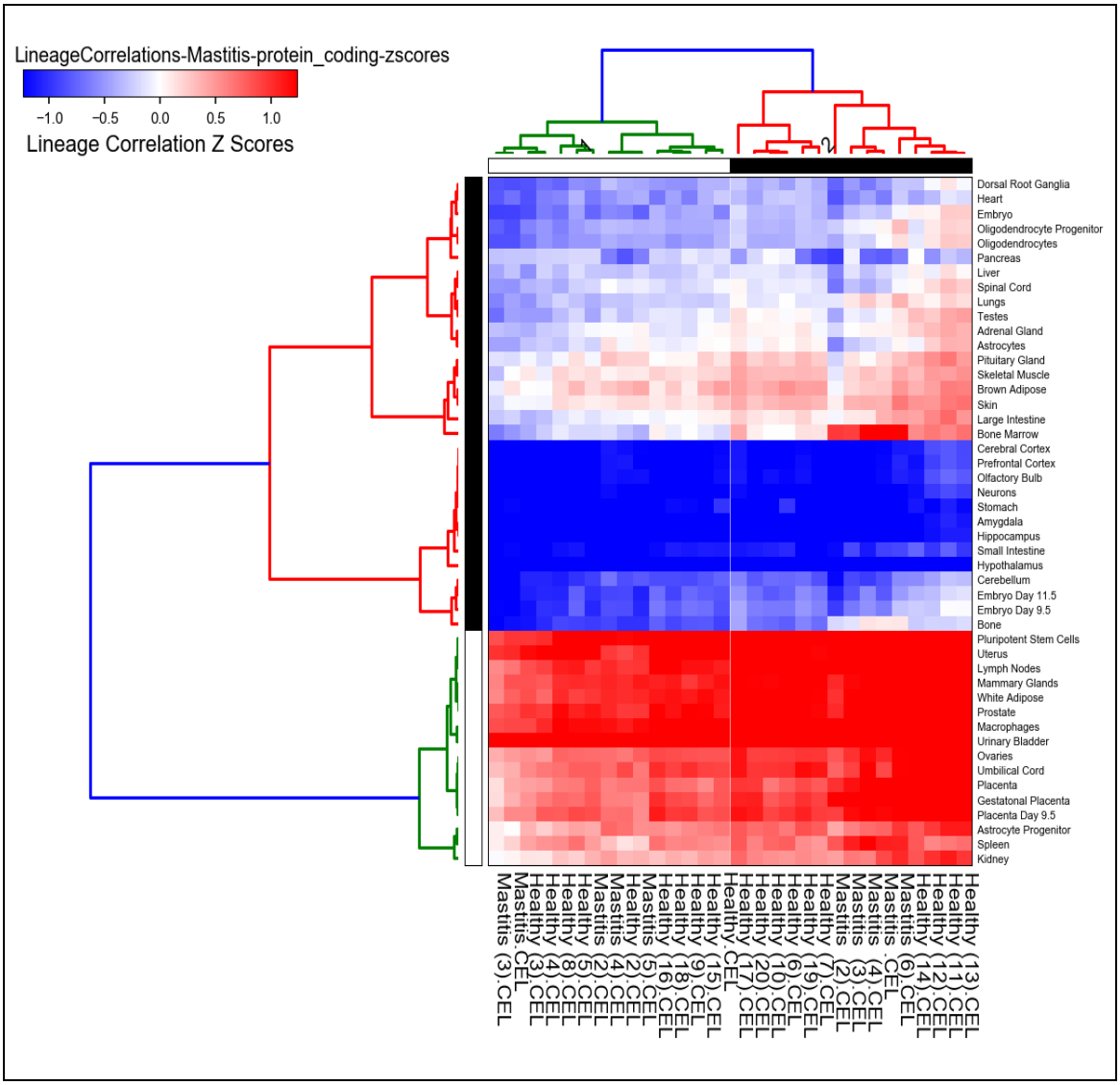


Fig. 4.21: Lineage correlation analysis of genes, expressed in mastitis and healthy samples, with other cell and tissue types.

4.1.2.1.3.5 Marker genes identification for mastitis

60 mastitis- specific genes were determined by Pearson correlation coefficient (Fig.4.22). The top 10 (Table 4.6) correlated genes determined with at least a 0.3 Pearson correlation coefficient were selected as the gene signature for each cell clusters. The top 10 genes identified as markers in Mastitis include JUN, RP2, AGPAT3 etc. On the other end, top 10 genes identified as marker genes in healthy samples are CPEB4, ARV1, ADCY6 etc.

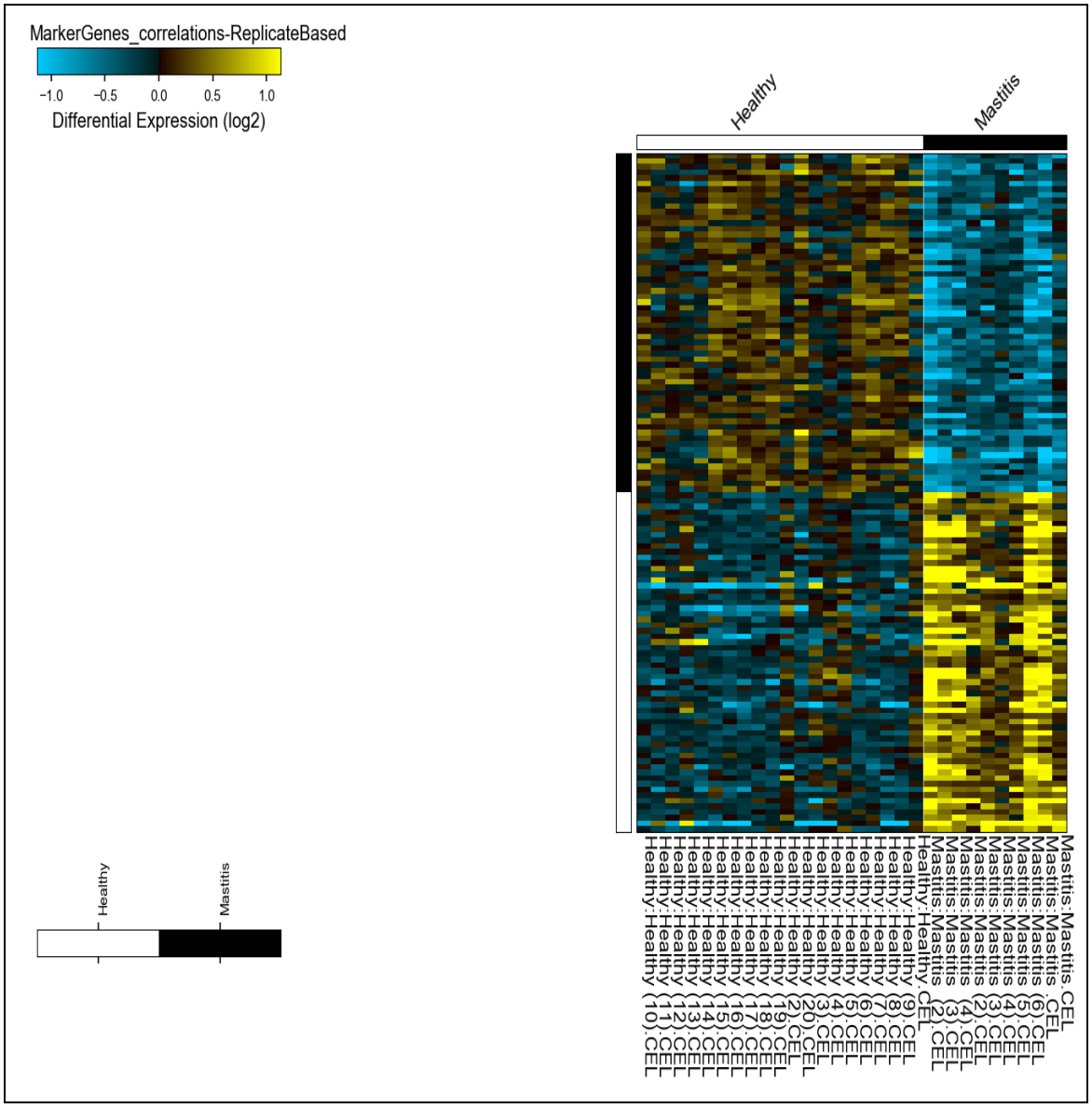


Fig. 4.22: ICGS analysis of transcriptomes for finding markers of mastitis and healthy samples.

Table 4.6: Potential marker genes for detection of mastitis in mammary gland.

Gene Symbol	Pearson r-value	Pearson P-value	Cell State
CPEB4	0.8362	8.73E-09	Healthy
Bt.14209.1.A1_at	0.8302	1.38E-08	Healthy
ARV1	0.8269	1.77E-08	Healthy
Bt.7593.1.S1_at	0.8138	4.51E-08	Healthy
ADCY6	0.8040	8.72E-08	Healthy
Bt.21930.2.S1_at	0.8024	9.63E-08	Healthy
LOC512408	0.7937	1.67E-07	Healthy
Bt.10734.2.S1_at	0.7878	2.38E-07	Healthy
RAB11FIP5	0.7860	2.63E-07	Healthy
LOC785659	0.7842	2.93E-07	Healthy
Bt.16818.1.A1_at	0.8353	9.36E-09	Mastitis
PTX3	0.8299	1.42E-08	Mastitis
Bt.19227.1.S1_at	0.8107	5.57E-08	Mastitis
TRA2B	0.8044	8.45E-08	Mastitis
DMBT1	0.7986	1.23E-07	Mastitis
FOS	0.7892	2.18E-07	Mastitis
AGPAT3	0.7853	2.76E-07	Mastitis
JUN	0.7818	3.37E-07	Mastitis
POLR2A	0.7753	4.87E-07	Mastitis
RP2	0.7717	5.93E-07	Mastitis

4.1.2.2 Prevalence of milk fever/hypocalcemia on the basis of gene expression

Out of 210 buffaloes, 17.14% animals in Group-I (n=70), 38% animals in Group-II (n=70) and 45% animals in Group-III (n=70) (Fig. 4.22) were found to have hypocalcemic condition when subjected to molecular level diagnosis via quantification of selected genes (NUAKI; upregulated and NESP55; downregulated) in spite of their

clinical healthy condition. They showed a significant difference (P<0.05) in the upregulation of selected genes for hypocalcemia/ milk fever condition. It was seen that fold change in the expression level of selected genes; NUAKI gene (upregulated) upregulation in hypocalcemic animals in all three groups increased significantly (p<0.05) from -30 to 0 days; whereas, NESP55 gene (downregulated) expression was significantly downregulated (p<0.05) from Group-II to Group-III (Table 4.7). The animals with low calcium range were in 3-7th parity with average lactation yield of 2058 kg of milk/ lactation.

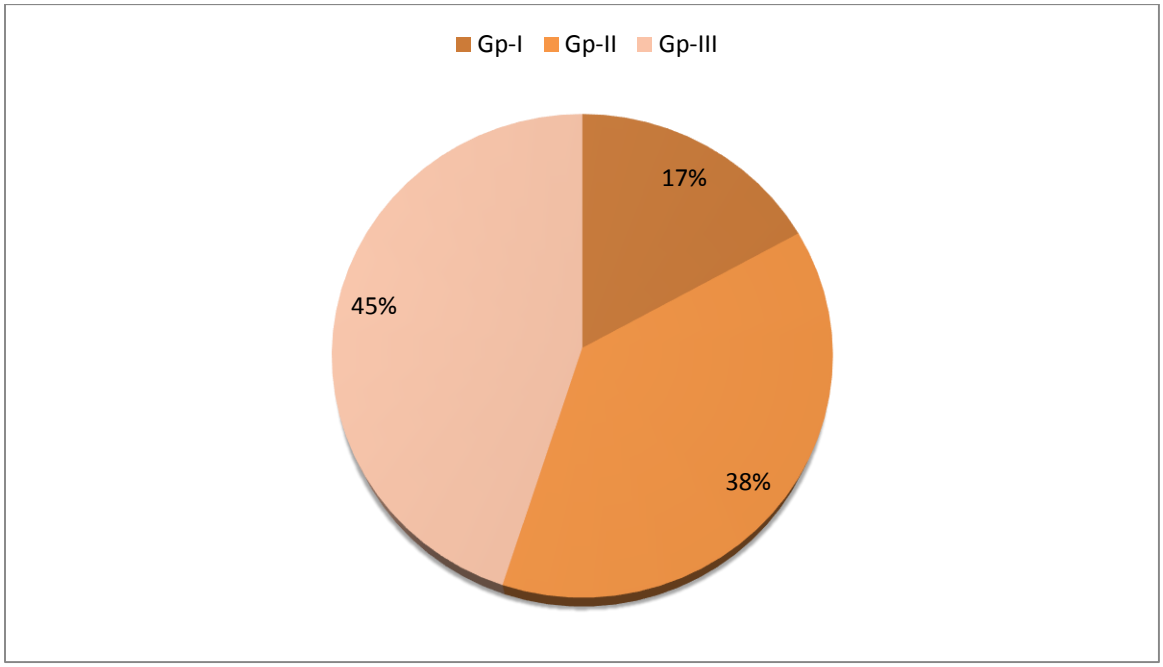


Fig. 4.23: Percentage of buffaloes with hypocalcemic condition in three groups of study

Table 4.7: Microarray data of Hypocalcemia/milk fever genes expression from PBMC of buffaloes during the transition period.

Genes	Fold change		
	-30 days	0 days	+30 days
NUAKI (Upregulated)	0.901^{a}±0.04	1.084^{b} ± 0.05	1.032^{b}± 0.02
NESP55(Downregulated)	0.402^{b}±0.01	0.383^{b}±0.01	0.332^{a} ±0.01

^{a,b,c}means with different superscripts between rows differs significantly (p< 0.05)

4.1.2.3 Prevalence of ketosis on the basis of gene expression

For the estimation of prevalence of ketosis/negative energy balance, cDNA samples from all the buffaloes of three groups were subjected for quantification of selected genes (Up- and down-regulated) for negative energy balance and ketotic condition. The genes for negative energy balance/ketotic condition viz CPT1A (Upregulated) in buffaloes showed a significant ($p<0.05$) increased expression from day -30 to +30 days and IGF-1 (Downregulated) showed significant ($p<0.05$) increase in downregulation from -30 to 0 days and non-significant difference was observed between 0 day and +30 days (Table 4.8). The percentage of animals that showed positive result for negative energy balance via fold change in the expression of CPT1A and IGF-1 were 24%, 47% and 62% in group I, II and III.

Table 4.8: Microarray data of genes expression for ketotic or negative balance energy from PBMC of buffaloes during the transition period.

Genes	Fold change		
	-30 days	**0 days**	**+30 days**
CPT1A (Upregulated)	$0.96^{a}\pm0.06$	$1.336^{b}\pm0.02$	$1.372^{c}\pm0.02$
IGF-1 (Downregulated)	$0.839^{a}\pm0.05$	$1.063^{b}\pm0.06$	$1.123^{b}\pm0.06$

^{a,b,c}means with different superscripts between rows differs significantly ($p< 0.05$)

4.1.2.4 Prevalence of mastitis on the basis of gene expression

For evaluation of prevalence of mastitis among buffaloes, animals from Gp-III were examined for MCMT and SCC, 62.85% were MCMT negative and had an average of 168.41 ± 0.8 ($\times10^3$/ml) somatic cell count whereas 37% were CMT (T/+) with SCC of 278 ± 0.67 ($\times10^3$/ml).

Blood samples from buffaloes (n=25) with CMT (T/+) and SCC of 278 ± 0.67 ($\times10^3$/ml) were subjected to qRT-PCR for genes (TNF-α and interferon gamma) regulated for inflammation condition. They showed upregulation of both the genes (3.372^{a} ± 0.20 and 3.09^{a} ± 0.3891 respectively). Whereas, buffaloes (n=45) with MCMT

negative showed significantly less upregulation of TNF-alpha and interferon gamma ($2.96^{b} \pm 0.20$ and $2.94^{b} \pm 0.24$) when compared to positive results

4.2 Risk factors associated with occurrence of production disease in transition period.

4.2.1 Changes in body condition score (BCS) during transition period.

Profile of BCS was studied at different time intervals -30 days (Gp.-I), 0 day (Gp.-II) and +30 days (Gp.-III) of calving. Mean±SE of BCS among three groups revealed significant decrease ($p<0.05$)3.14±0.05, 3.04±0.05 and 2.88±0.05 in group I, group-II and group III, respectively, suggestive of reduced BCS from pre-partum to post-partum period. Range of BCS considered in the study was 2-2.5, 3-3.5, 3.5-4 and 4-4.5. Highest percentage of buffaloes with BCS 2.5, 3-3.5, 3.5 and above was found in Gp-III, Gp-II and Gp-I, respectively. Maximum number (37.20%) of animals had lowest BCS (2-2.5) during post-partum period (Gp.-III) followed by Gp.-I and Gp.-III (Table 4.9).

Table 4.9: Range BCS during transition period in buffaloes.

BCS range	Group I (n=70)	Group II(n=70)	Group III(n=70
2-2.5	14 (20%)	13(18.75%)	**26 (37.20%)**
3-3.5	30 (42.80%)	**36 (51.40%)**	27 (38.50%)
3.5-4	**20(28.50%)**	17 (27.14%)	15 (21.80%)
4-4.5	**6 (8.50%)**	4 (5.70%)	2 (2.80%)

4.2.2 Association between BCS, anti-oxidative markers and production of BHBA during transition period

In our study, 210 buffaloes during transition period were monitored for various parameters. Based on conjugation of BCS profile, metabolic indices and oxidative markers, we found a pattern those animals with greater loss in BCS during postpartum group (Gp-III) had a higher level of BHBA (483.69±1.98) than other two groups (Table 4.13)

However, animal (GP-III) with low BCS score had lowest Mean±SE values of SOD (37.97±0.36) and catalase (119.30±0.27) when compared to other groups (4.14)

4.2.3 Effect of dry period length on milk yield

From the collected data of 70 buffaloes (Gp-III) it was found that buffaloes with shortened dry period (42.21±0.59) had low milk yield (6.81±0.10 kg/day) compared to others with longer dry period (53.83±0.48)having milk yield 7.98±0.87 kg/day.

4.3 Assaying of cytokines during the transition period in buffaloes

In our study, serum cytokines viz. Tumor necrosis factor-α (TNF-α) and interferon gamma (IFNγ) levels in Group -I, -II and -II were 1.36±0.03, 2.15±0.02, 1.97±0.04 and 0.79±0.01, 1.11±0.02, 1.08±0.01, respectively. Results showed significant increase ($p<0.05$) in cytokines from Group-I to -III, with highest values in Group-II.

There was a strong association between the serum concentration of cytokines and fold change in the expression level by qRT-PCR of TNF-α and IFNγduring the transition period. There was significant increase ($p<0.05$) in both serum values and real time expression of the selected genes from -30 to 0 day (Gp-I to Gp-II) and thereafter decreased upto +30 days (Table 4.10 and 4.11)

Table 4.10: Estimated Mean±SE values of TNF-alpha and interferon gamma via ELISA kit during the transition period in buffaloes.

Attributes	Group I(n=70)	Group II(n=70)	Group III(n=70)
TNF-α	1.36^{a}±0.03	2.15^{c}±0.02	1.97^{b}±0.04
Interferon- γ	0.79^{a}±0.01	1.11^{c}±0.02	1.08^{b}±0.01

a,b,c means with different superscripts between rows differs significantly($p< 0.05$)

Table 4.11: Data of fold change in the expression level of selected genes quantitatively for inflammatory conditions/mastitis in buffaloes during transition period.

Genes	Fold change		
	-30 days (Group-I)	0 day (Group-II)	+30 days (Group-III)
TNF-α (upregulated)	3.082^{a}±0.05	3.243^{b}±0.12	3.227^{c}±0.12
INF- γ (upregulated)	3.007^{a} ± 0.16	3.131^{b} ± 0.10	3.010^{c}±0.09

^{a,b,c}means with different superscripts between rows differs significantly ($p< 0.05$)

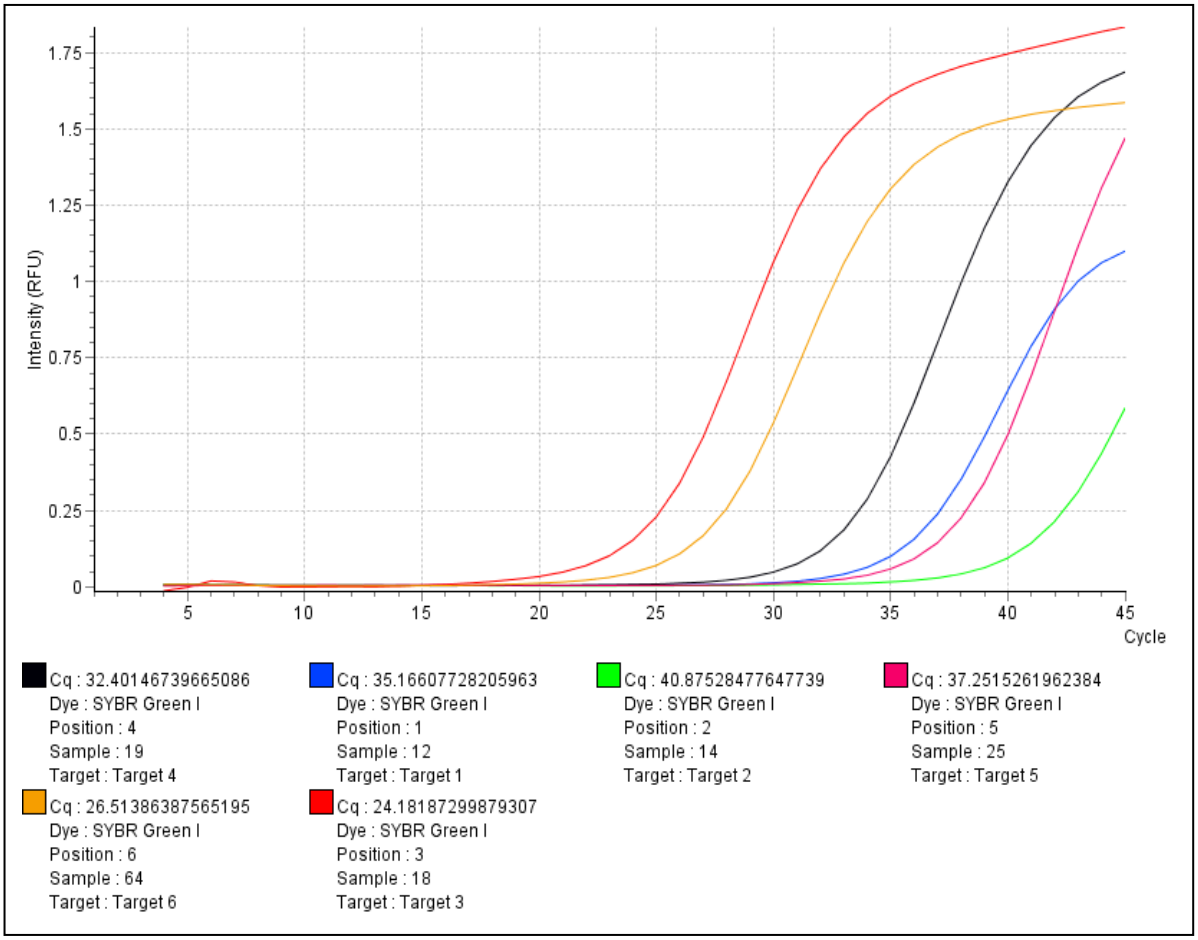


Fig. 4.24: Figure showing representation of threshold curve in qRT-PCR for TNF-α and IFN- γ. Sample with lowest Cq value is having highest curve in the graph (red line) and those with high Cq value are having lowest threshold curve (green and blue).

Excl.	Sample	SYBR Green I	Type	Cq	+	Quantity
	Sample 25(-15)	Target 1	U	29.295	✓	
	Sample 28(30	Target 2	U	23.272	✓	
	Sample 29(-7)	Target 3	U	22.816	✓	
	Sample 29(0)	Target 4	U	18.271	✓	
	Sample 29(15)	Target 5	U	24.244	✓	
	Sample 29(30)	Target 6	U	21.112	✓	
	Sample 41(-15)	Target 7	U	22.184	✓	
	Sample 41(0)	Target 8	U	22.194	✓	
	Sample (41-!5	Target 9	U	23.566	✓	
	Sample 36(0)	Target 10	U			
	Sample 36(30)	Target 11	U	23.99	✓	
	Sample 138(-15)	Target 12	U	20.828	✓	
	Sample 38(15	Target 13	U	22.713	✓	
	Sample 38(30	Target 14	U	19.595	✓	

Fig. 4.25: Representation of Cq values of selected genes in qRT-PCR software (Mygo mini)after the completion of cyclic conditions. The samples were selected against the target SYBR green dye which binds with the template and increases its florescence during cyclic condition until it reaches to its threshold cure (Cq value). SYBR green in qPCR tracks linearity.

4.4. Hematological changes during transition period

In our study, whole blood sample of the buffaloes were used for analysis of hematological parameters. Mean±SE values of hematological parameters in all the three groups (Group-I, II and III) are depicted in Table 4.12.

Mean±SE values of WBC(x10^3/µl) in all the groups increased significantly ($p<0.05$) with highest values in Group-III (7.57±0.09). The Mean±SE values of lymphocytes (45.78±0.19, 48.12±0.19 and 46.17±0.19) and granulocytes (43.66±0.33, 46.65±0.67 and 43.62±0.16, respectively) differ significantly ($p<0.05$) in all three groups, with highest value in Group-II particularly.

Mean±SE values of monocytes decreases significantly ($p<0.05$) from Goup-I to Group-II, thereafter significant increase ($p<0.05$) in the values upto +30 days were found (4.51±0.04, 4.09±0.02 and 4.41±0.02, respectively). The mean±SE values of RBC's (10^6/µl) in all the groups were 5.19±0.06, 4.98±0.03 and 5.21±0.04, respectively. There was significant ($P<0.05$) decrease in Group-II from other groups. The mean±SE values of hemoglobin (Hb, g/dl) decreased significantly ($p<0.05$) along the progression of

transition period from –30 days to +30 days (10.65±0.13, 9.67±0.11and 9.25±0.08, respectively). Hematocrit values (HCT) decreased significantly (P<0.05) in Group-II (0 day) compared to other groups. The values are depicted in Table 4.12. Mean±SE values of mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) decreased significantly (p<0.05) from Group-I to Group-II (Table 4.12). The mean±SE values of mean corpuscular hemoglobin concentration (MCHC) showed non- significant difference among all the groups. There is significant increase (p<0.05) in the platelet count from Group-I to Group-III (338.81±6.10, 380.40±5.67 and 397.97±5.90 respectively).

Table 4.12: Mean±SE values of different hematological indices in different stages of transition buffaloes.

Parameters	**-30 days (Gp-I) (n=70)**	**0 day (Gp-II) (n=70)**	**+30 days (Gp-III) (n=70)**
WBC (x10³/µL)	6.45^{a}±0.05	7.27^{b}±0.05	7.57^{c}±0.09
Lymphocytes (%)	45.78^{a}±0.19	48.12^{b}±0.19	46.17^{a}±0.19
Monocytes (%)	4.51^{c}±0.04	4.09^{a}±0.02	4.41^{b}±0.02
Granulocyte (%)	43.66^{a}±0.33	46.65^{b}±0.67	43.62^{a}±0.1
RBC (x10^{6}/µL)	5.19^{b}±0.06	4.98^{a}±0.03	5.11^{b}±0.04
Hb (g/dl)	10.65^{c}±0.13	9.67^{b}±0.11	9.25^{a}±0.08
HCT (%)	33.34^{b}±0.31	30.89^{a}±0.45	31.22^{a}±0.23
MCV(fl)	57.20^{a}±0.33	56.80^{b}±0.14	56.64^{b}±0.09
MCH (pg)	17.90^{b}±0.10	16.07^{a}±0.20	17.83^{b}±0.09
MCHC (pg)	32.41^{a}±0.18	31.86^{a}±0.59	32.67^{a}±0.11
PLT ((x10³/µL)	338.81^{a}±6.10	380.40^{b}±5.67	397.97^{c}±5.90

a,b,c means with different superscripts between rows differs significantly(p<0.05)

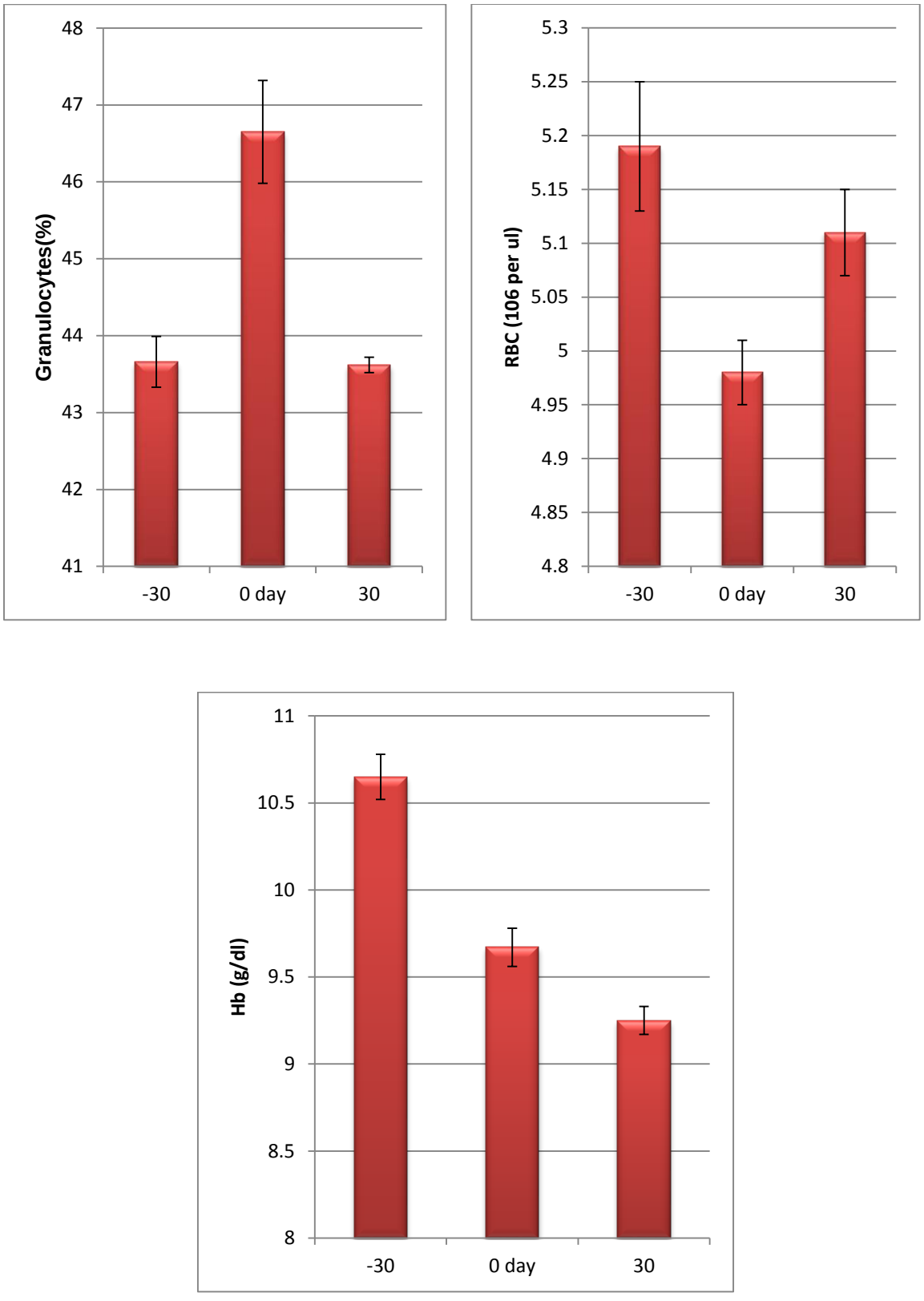


Fig 4.26: Graphical representation of hematological parameters changes occur during transition period

4.25 Biochemical changes during transition period in buffaloes

In present study, serum sample collected from the buffaloes were subjected to biochemical parameters analysis and all the mean±SE values of the biochemical parameters are given in Table 4.13 depicting significant and non-significant difference among the three groups (Group-I, -II and -III).

Mean±SE values of albumin, total protein and A:G ratio decreased significantly ($p<0.05$) from Group-I to Group-III and values are given in Table 4.13. Whereas, non-significant ($p<0.05$) difference in the mean±SE values of globulin among the groups was observed.

There is significant increase ($p<0.05$) in the mean±SE values of serum glutamic-oxaloacetic transaminase (SGOT) from Group-I to Group-III, ranging from 98.39±0.5, 102.27±0.28 and 109.69±0.54, respectively. Mean±SE values of Gamma-glutamyl transferase (GGT) (U/L) amongst three groups showed significant ($p<0.05$) difference with highest value in group II (24.33±0.14) as compared to group I (15.38±0.18) and group III (21.29±0.20).Mean±SE values of cholesterol showed significant increase ($p<0.05$) in Group-III (81.62±0.47) than Group-I (79.16±0.67) and Group-II (70.76±0.54). Similar pattern was also noted in Serum glutamic pyruvic transaminase (SGPT) (U/L) and high-density lipoproteins (HDL) (mg/dl) in which there was significant increase ($p<0.05$) in Group-III compared to other groups (Table 4.13).

Mean±SE values of BHBA (nmol/ml) of Group-I, -II and -III were (313.96±2.81, 397.36±1.69and 483.69±1.98, respectively (Table 4.13). The data showed a significant increase ($p<0.05$) in the values from Group-I to Group-III.

Mean±SE values of blood urea nitrogen (BUN) (mg/dl) showed non-significant difference ($p<0.05$) between Group-I and -II. Whereas there was a significant increase ($p<0.05$) in group III compared to other two (Table 4.13). The mean±SE values of creatinine in all the groups showed significant increase ($p<0.05$) in group II (1.24±0.01) as compared to the group I, III (1.22±0.01 and 1.20±0.01, respectively).

The calcium level (mg/dl) showed significant decrease ($p<0.05$) from Group-I to Group-III (8.40±0.03, 8.1±0.02 and 7.98±0.02). For phosphorus, there was a non-significant ($p<0.05$) decrease in the Group-I and Group-II. But there was a significant decrease ($p<0.05$) in the mean±SE values of Group-III from other two's. Mean±SE values of Ca:P ratio showed significant decrease ($p<0.05$) from Group-I to -III, with lowest value seen in Group-II (Table 4.13).

Sodium and potassium (mEq/L) showed non-significant ($p<0.05$) decrease from Group-I to Group-II. Whereas, there was a significant decrease ($p<0.05$) in the Group-III in comparison to other groups (138.05±0.36, 137.73±0.3, 135.70±0.26 and 3.88±0.03, 3.86±0.02 and 3.72±0.01, respectively).Serum magnesium level (mg/dl) showed a significant decrease ($P<0.05$) from Group-I to -II, but there was non-significant ($P<0.05$) decrease amongst Group-II and -III (Table 4.13).

Table 4.13: Biochemical changes during transition period in buffaloes.

Parameters	**-30 days (Gp-I) (n=70)**	**0 day (Gp-II) (n=70)**	**+30 days (Gp-III) (n=70)**
Albumin (g/dl)	3.12^{c}±0.02	2.93^{b}±0.02	2.81^{a}±0.02
Total protein (g/dl)	7.13^{c}±0.03	6.96^{b}±0.02	6.83^{a}±0.02
Globulin (g/dl)	4.01±0.01	4.03±0.01	4.02±0.01
A:G Ratio	0.78^{c}±0.01	0.73^{b}±0.01	0.70^{a}±0.01
SGOT (U/L)	98.39^{a}±0.51	102.27^{b}±0.28	109.69^{c}±0.54
SGPT (U/L)	24.60^{b}±0.26	23.76^{a}±0.23	25.79^{c}±0.21
GGT (U/L)	15.38^{a}±0.18	24.33^{c}±0.14	21.29^{b}±0.20
BHBA (nmol/ml)	313.96^{a}±2.81	397.36^{b}±1.69	483.69^{c}±1.98
Cholesterol (mg/dl)	79.16^{b}±0.67	70.76^{a}±0.54	81.62^{c}±0.47
BUN (mg/dl)	11.05^{a}±0.14	11.61^{a}±0.19	12.56^{b}±0.29
Creatinine (mg/dl)	1.22^{a}±0.01	1.24^{b}±0.01	1.20^{a}±0.01
Triglyceride(mg/dl)	24.24^{c}±0.20	21.49^{b}±0.32	19.04^{a}±0.23
HDL (mg/dl)	40.39^{b}±0.39	39.47^{a}±0.28	45.04^{c}±0.22
Calcium (mg/dl)	8.80^{c}±0.03	8.5^{b}±0.02	8.35^{b}±0.02
Sodium (mEq/L)	138.05^{b}±0.36	137.73^{b}±0.30	135.70^{a}±0.26
Potassium (mEq/L)	3.88^{b}±0.03	3.86^{b}±0.02	3.72^{a}±0.01
Magnesium(mg/dl)	1.18^{b}±0.01	1.16^{a}±0.01	1.16^{a}±0.01
Phosphorus (mg/dl)	4.90^{b}±0.01	4.88^{b}±0.01	4.74^{a}±0.01
Ca:P ratio	1.71^{c}±0.01	1.66^{a}±0.01	1.69^{b}±0.00

a,b,c means with different superscripts between rows differs significantly($p<0.05$)

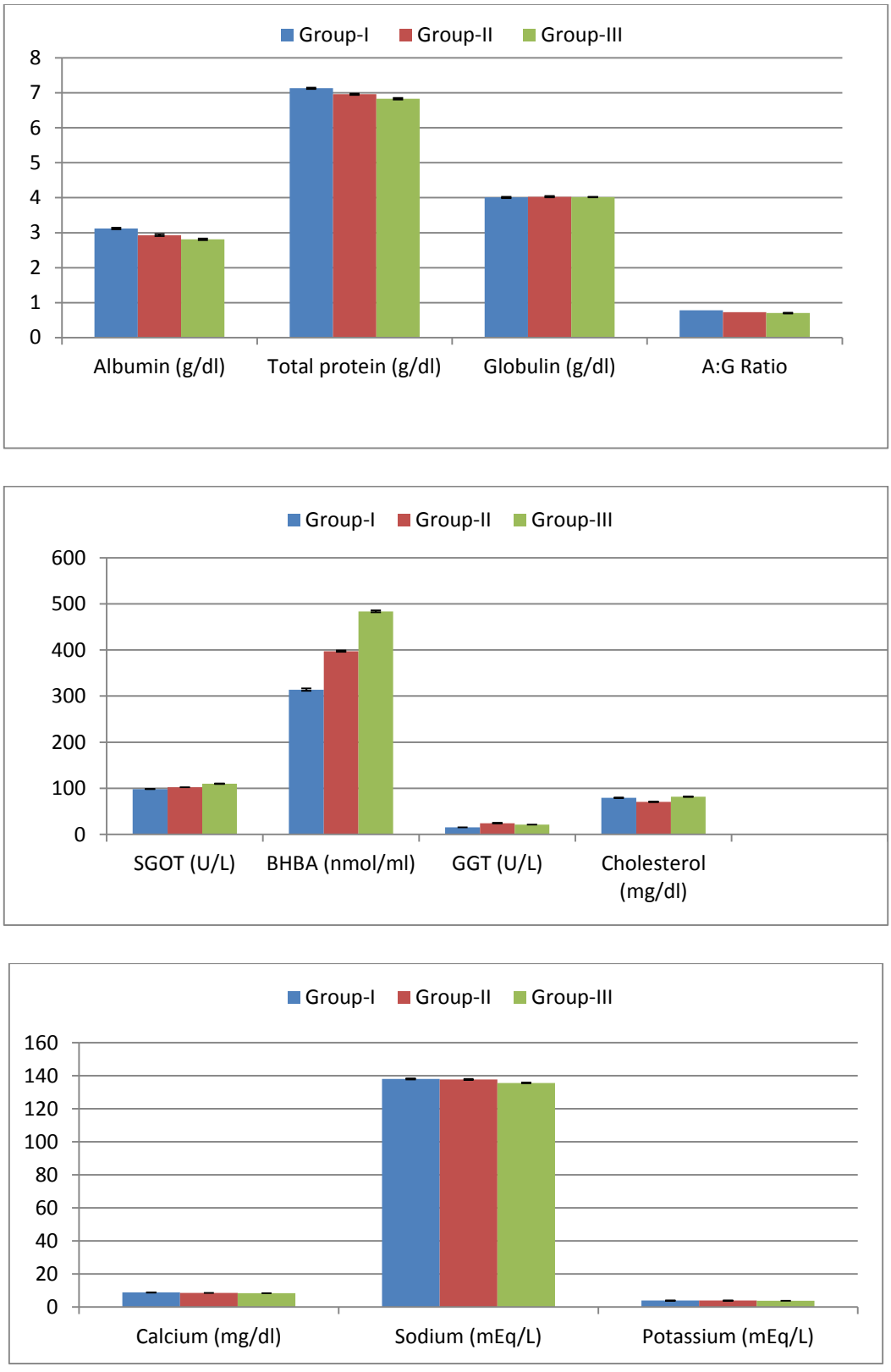


Fig 4.27: Graphical representation of changes in biochemical parameters in all three different groups during different time intervals of transition period.

4.6 Changes in oxidative stress markers during transition period in buffaloes.

The results of imbalance between oxidative marker and anti-oxidant markers level revealed oxidative stress. On analysis of these anti-oxidant and oxidative markers, our results showed a significant difference ($p<0.05$) amongst these groups providing sufficient evidences about oxidative biomarkers and anti-oxidants production during transition period in buffaloes.

Present study showed significant ($p<0.05$) increase in the mean±SE values of malondialdehyde (MDA) (nmoles of MDA produced/ g of Hb /hr) in Group-II (7.01±0.08) in comparison to Group-I and -III (6.09±0.10 and 6.31±0.13). Moreover, there was non-significant difference ($p<0.05$) in values of Group-I and -III (Table 4.14).

Blood serum catalase (CAT) level in all three Groups (I, II and III) were 125.89±0.16, 123.05±0.14 and 119.30±0.27, respectively. The data showed a significant decrease ($p<0.05$) in the values from Group-I to Group-II (Table 4.14).

Mean±SE values of superoxide dismutase (SOD, U/mg of Hb) in Group-I, -II and -III were 43.0±0.39, 39.10±0.45 and 37.97±0.36, respectively. The study showed a significant decrease ($p<0.05$) in the values from Group-I to Group-III, with highest level in Group-I (Table 4.14). Blood serum glutathione peroxidase (GPx, mg/Hb) level was highest in Group-I (3.16±0.03) followed by Group-II and –III (2.07±0.02 and 2.21±0.03), respectively. There was significant decrease ($p<0.05$) in the values from Group-I to -III.

Table 4.14: Oxidative biomarker and anti-oxidants level during transition period in buffaloes

Parameters	**-30 days (Gp-I) (n=70)**	**0 day (Gp-II) (n=70)**	**+30 days (Gp-III) (n=70)**
MDA (nmoles of MDA produced/ g of Hb /hr)	6.09^{a}±0.10	7.01^{b}±0.08	6.31^{a}±0.13
CAT (µmoles of H_2O_2 utilised min^{-1} mg^{Hb-1}	125.89^{c}±0.16	123.05^{b}±0.14	119.30^{a}±0.27
SOD (Units /mg of Hb	43.0^{c}±0.39	39.10^{b}±0.45	37.97^{a}±0.36
GPx (mg/Hb)	3.16^{c}±0.03	2.07^{a}±0.02	2.21^{b}±0.03

a,b,c means with different superscripts between rows differs significantly($p< 0.05$)

4.7 Transition feeding trial for management of production diseases in buffaloes.

Total 20 buffaloes in transition period (-30 days to +30 days) irrespective of age and parity were selected for transition feeding trial for the management of production diseases. These animals were divided into two groups viz. Group-I (Transition feed) and Group-II (Control, no transition feed), each comprising 10 animals. Transition feed was formulated from locally available feed ingredients and offered to the Group-I at rate of 1 kg per day in addition to their basal diet.

4.7.1 Proximate principle analysis of transition feed.

The formulated feed for the transition period was evaluated for the proximate principle analysis (Weende analysis) including moisture percentage, total ash, crude protein, ether extract and crude fiber. The dry matter of feed was found to be 91.50% with total ash (8.50%), crude protein (17.50%), ether extract (1.75%) and crude fiber (17.00%).

4.7.1 Analysis of the effect of the transition feed during transition period in buffaloes.

Effect of transition feed on buffaloes was evaluated on the basis of various parameters such as body condition score, hemato-biochemical changes, oxidative markers and molecular markers for production diseases. Blood samples were collected at different time intervals viz. -30 days, -15 days, 0 day, +15 days and +30 days of calving for analysis various blood parameters and gene expression studies to find out the impact of transition feed.

4.7.1.1 Effect of transition feed on body condition score.

In our present study, the BCS was evaluated among transition feed group and the control group in pre-defined time interval on a scale of 1-5 with 0.5 increment on scale. Mean±SE values of BCS showed non-significant difference ($p<0.05$) among transition feed trial group and control group. Whereas, there was a significant ($p<0.05$) decrease in the BCS from -30 to 0 day of study in feed group where as in control group there was significant decrease ($p<0.05$) from 0 to +30 days.

Table 4.15: Profile of body condition score during transition period at different time interval in buffaloes.

BCS	Periods			GM
	-30	0	+30	
Group-I	3.25^{a}±0.11	3.00^{b}±0.07	2.90^{b}±0.08	**2.98^{A}±0.07**
Group-II	3.10^{a}±0.07	3.00^{a}±0.02	2.55^{b}±0.05	**2.88^{A}±0.05**
PM	**3.18^{a}±0.07**	**3.00^{b}±0.04**	**2.83^{c}±0.05**	

$^{A,B\&a,b,c}$means with different superscripts between columns and rows differs significantly ($p< 0.05$)

PM and GM denotes period mean and group mean respectively

4.7.1.2 Effect of transition feed on hematology.

To study the effect of transition feed during the different time interval in transition buffaloes, the animals were divided into two groups, each carrying equal number of animals(n=10). Mean±SE values of hematological and biochemical parameters in transition feed group (Group-I) and control group (Group-II) are depicted in Table 4.16.

Group mean±SE values of WBC's (7.13±0.09 and 6.91±0.08), lymphocytes (46.69±0.26 and 45.54±0.27), granulocytes (44.65±0.34 and 43.48±0.24), RBC (5.20±0.05 and 5.07±0.03), Hb (10.05±0.15 and 9.71±0.08), HCT (32.71±0.25 and 31.87±0.24) and glucose (56.58±0.36 and 55.68±0.24) showed significant difference ($p<0.05$) between transition feed trial group (Gp-I) and control group (Gp-II), respectively (Table 4.16). Whereas, there was non-significant difference ($p<0.05$) in the Mean±SE values of monocytes, MCH, MCHC and platelets among feed trial group (Gp-I) and control group (Gp-II) (Table 4.16).

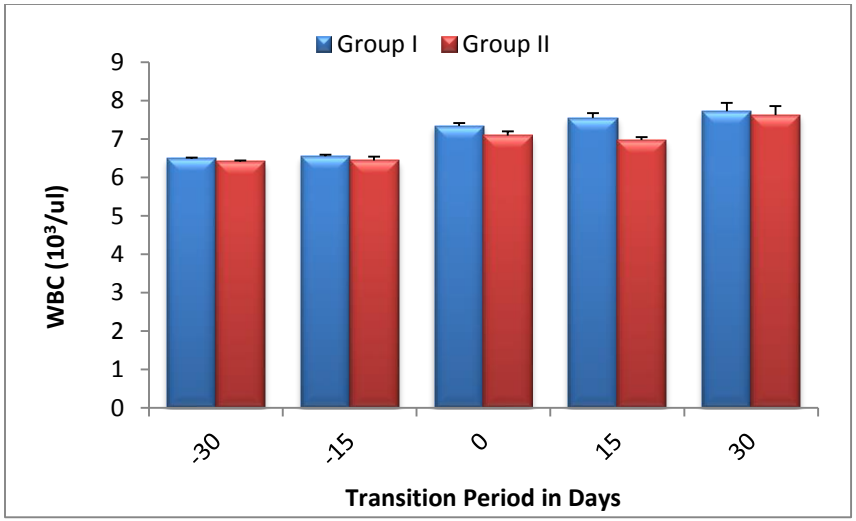
The difference in mean±SE values of hematological parameters along the different time interval (-30, -15, 0, +15, +30 days of calving) in transition feed trial group (Gp-I) and control group (Gp-II) are shown in Table 4.16. Mean±SE values of WBC's depicted significant increase ($p<0.05$) from -15 days to +15 days (6.50±0.05, 7.21±0.07, 7.27±0.10 and 7.68±0.15 respectively).

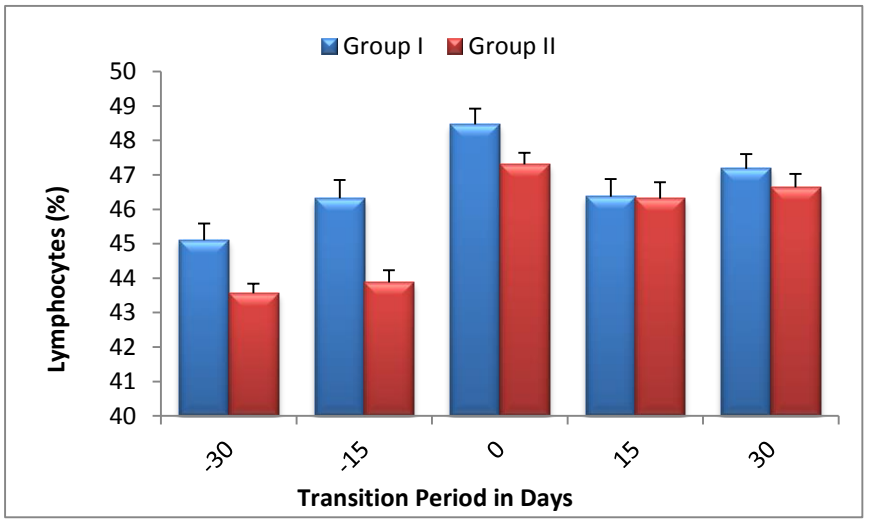
Table 4.16: Mean±SE values of hematological parameters within time period (-30, -15, 0, 15, 30 days) and between the groups (Transition feed group and control group)

Groups	Transition Period (Days)					GM
	-30	-15	0	+15	+30	
WBC						
Group I	6.49^{a}±0.03	6.55^{a}±0.04	7.34^{b}±0.08	7.55^{c}±0.13	7.73^{d}±0.21	7.13^{A}±0.09
Group II	6.42^{a}±0.02	6.44^{a}±0.10	7.09^{b}±0.11	6.98^{ba}±0.07	7.63^{c}±0.23	6.91^{B}±0.08
PM	6.46^{a}±0.02	6.50^{a}±0.05	7.21^{b}±0.07	7.27^{ca}±0.10	7.68^{c}±0.15	
Lymphocytes						
Group I	45.10^{a}±0.49	46.32^{b}±0.53	48.47^{c}±0.45	46.37^{d}±0.51	47.18^{da}±0.42	46.6A^{A}±0.26
Group II	43.56^{a}±0.28	43.88^{a}±0.35	47.31^{b}±0.33	46.31^{c}±0.48	46.64^{ca}±0.39	45.54^{B}±0.27
PM	44.33^{a}±0.33	45.10^{a}±0.42	47.89^{b}±0.30	46.34^{c}±0.34	46.91^{ca}±0.29	
Monocytes						
Group I	4.60^{a}±0.08	4.74^{b}±0.09	4.02^{c}±0.06	4.44^{d}±0.04	4.43^{d}±0.04	4.45^{A}±0.04
Group II	4.60^{a}±0.08	4.55^{a}±0.10	4.16^{b}±0.05	4.37^{c}±0.06	4.40^{ca}±0.05	4.42^{A}±0.04
PM	4.60^{a}±0.06	4.64^{a}±0.07	4.09^{b}±0.04	4.41^{c}±0.04	4.42^{ca}±0.03	
Granulocytes						
Group I	42.68^{a}±0.39	44.01^{b}±0.49	47.75^{c}±0.91	44.10^{d}±0.37	44.73^{e}±0.36	44.65^{A}±0.34
Group II	42.09^{a}±0.24	43.10^{b}±0.48	45.84^{c}±0.33	42.50^{d}±0.34	43.86^{e}±0.27	43.48^{B}±0.24
PM	42.39^{a}±0.23	43.56^{b}±0.35	46.79^{c}±0.52	43.30^{d}±0.30	44.30^{e}±0.24	
RBC (x10^{6}/µL)						
Group I	5.14^{a}±0.11	5.25^{b}±0.12	5.10^{c}±0.08	5.25^{d}±0.11	5.26^{d}±0.11	5.20^{A}±0.05
Group II	5.00±0.05	5.01±0.12	5.08±0.07	5.07^{a}±0.06	5.18^{ba}±0.07	5.07^{B}±0.03
PM	5.07^{a}±0.06	5.13^{b}±0.09	5.09^{b}±0.05	5.16^{ba}±0.07	5.22^{c}±0.07	
Hb						
Group I	10.70^{a}±0.35	10.83^{a}±0.41	9.83^{b}±0.18	9.46^{ca}±0.14	9.42^{ca}±0.20	10.05^{A}±0.15
Group II	10.04^{a}±0.20	10.11^{a}±0.14	9.84^{b}±0.11	9.30^{c}±0.17	9.28^{d}±0.16	9.71^{B}±0.08
PM	10.37^{a}±0.21	10.47^{b}±0.23	9.83^{c}±0.10	9.38^{d}±0.11	9.35^{d}±0.12	

HCT						
Group I	33.29^{a}±0.48	34.53^{b}±0.45	32.18^{c}±0.35	32.08^{c}±0.25	31.49^{d}±0.66	32.71^{A}±0.25
Group II	32.86^{a}±0.56	32.39^{a}±0.36	31.87^{a}±0.53	31.02^{b}±0.46	31.20^{ba}±0.58	31.87^{B}±0.24
PM	33.07^{a}±0.36	33.46^{a}±0.37	32.02^{b}±0.31	31.55^{ba}±0.28	31.34^{b}±0.43	
MCV						
Group I	53.87^{a}±0.80	55.54^{b}±0.70	57.72^{c}±0.30	57.43^{c}±0.31	57.44^{c}±0.24	56.40^{A}±0.31
Group II	51.87^{a}±0.80	53.99^{a}±0.49	57.54^{b}±0.33	57.50^{ba}±0.27	56.61^{c}±0.21	55.90^{A}±0.31
PM	53.87^{a}±0.55	54.76^{b}±0.45	57.63^{ca}±0.22	57.46^{c}±0.20	57.02^{c}±0.18	
MCH						
Group I	15.51^{a}±0.64	16.33^{b}±0.62	18.06^{c}±0.30	17.92^{c}±0.26	17.87^{c}±0.17	17.14^{A}±0.24
Group II	15.19^{a}±0.51	15.30^{a}±0.49	17.99^{ba}±0.29	17.69^{b}±0.27	17.41^{b}±0.11	16.72^{A}±0.23
PM	15.35^{a}±0.40	15.82^{a}±0.40	18.03^{ba}±0.20	17.81^{b}±0.18	17.64^{b}±0.11	
MCHC						
Group I	32.70^{a}±0.23	33.56^{a}±0.22	32.70^{a}±0.21	33.24^{a}±0.29	32.98^{a}±0.29	33.04^{A}±0.12
Group II	31.48^{a}±0.23	32.46^{a}±0.53	30.26^{b}±2.99	32.12^{c}±0.18	32.34^{c}±0.28	31.98^{B}±0.60
PM	32.70^{a}±0.16	33.01^{a}±0.31	31.48^{b}±1.49	32.68^{c}±0.21	32.66^{c}±0.21	
RDW						
Group I	17.49^{a}±0.41	16.84^{b}±0.39	14.59^{c}±0.23	15.81^{d}±0.29	16.56^{e}±0.22	16.26^{A}±0.20
Group II	18.67^{a}±0.41	17.35^{a}±0.56	15.86^{b}±0.23	16.47^{c}±0.29	16.65^{ca}±0.25	16.76^{B}±0.18
PM	17.49^{a}±0.28	17.09^{b}±0.34	15.23^{c}±0.21	16.14^{d}±0.21	16.61^{d}±0.16	
PLT						
Group I	353.80^{a}±12.74	356.30^{a}±12.99	374^{b}.00±12.86	390.50^{ca}±9.27	380.00^{c}±15.78	370.92^{A}±5.88
Group II	345.80^{a}±11.92	348.40^{a}±16.41	373.60^{b}±10.41	379.90^{ba}±10.93	378.00^{b}±15.94	365.14^{A}±6.11
PM	349.80^{a}±8.54	352.35^{a}±10.23	373.80^{b}±8.05	385.20^{ba}±7.08	379.00^{b}±10.92	
Glucose						
Group I	59.40^{a}±0.43	58.30^{b}±0.37	57.00^{c}±0.39	55.00^{d}±0.37	53.20^{e}±0.33	56.58^{A}±0.36
Group II	58.00^{a}±0.26	56.50^{b}±0.31	55.80^{c}±0.25	54.60^{d}±0.22	53.50^{e}±0.17	55.68^{B}±0.24
PM	58.70^{a}±0.29	57.40^{b}±0.31	56.40^{c}±0.27	54.80^{d}±0.21	53.35^{e}±0.18	

$^{A,B\&a,b,c,d,e}$means with different superscripts between columns and rows differs significantly ($p < 0.05$)





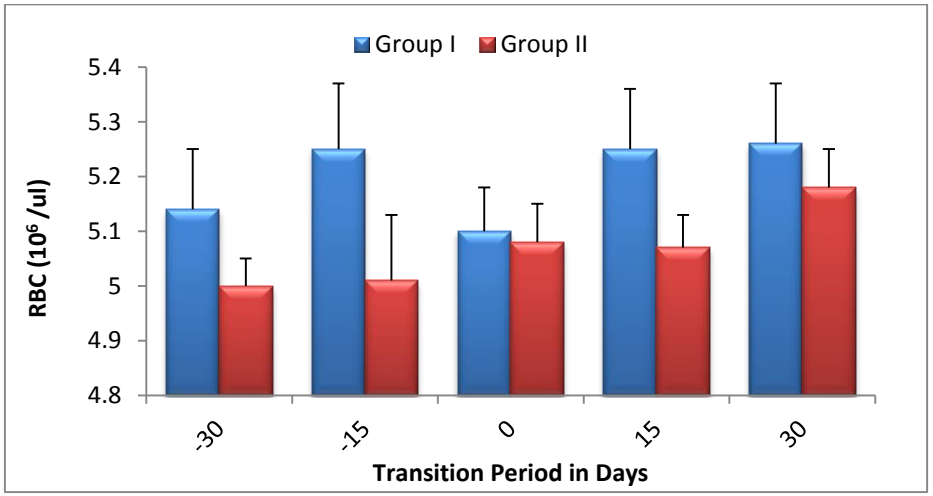


Fig 4.28: Graphical representation of changes in hematological parameters in transition feed and control group at different time interval.

Mean±SE values of lymphocytes showed significant ($p<0.05$) increase from -15 to 0 day, there after non-significant decrease ($p<0.05$) in +15 days (Table 4.16). Mean±SE values of granulocytes showed significant ($p<0.05$) increase along the period of study (-30 to +30 days) (Table 4.16). Mean±SE values of RBC's and MCH differs non-significantly ($p<0.05$) in the period of study. A significant decrease ($p<0.05$) was seen in mean±SE values of Hb (g/dl) from -30 to +15 day (10.37±0.21, 10.47±0.23, 9.83±0.10 and 9.38±0.11, respectively). There was a significant decrease ($p<0.05$) in HCT from -15 to 0 day. Moreover, there was non-significant difference among other periods (Table 4.16).

The mean±SE values of MCV increased significantly ($p<0.05$) from -30 to 0 days and thereafter non-significant difference ($p<0.05$) was observed among +15 and +30 days. In mean±SE values of MCH, there was a significant decrease ($p<0.05$) from -15 to 0 day and thereafter non significant decrease in the values were seen from +15 to +30 days (Table 4.16)

4.7.1.3 Effect of transition feed on biochemical parameters.

The mean±SE values of TNF-alpha, BHBA and IFN-γ decreased significantly ($p<0.05$) in transition feed trial group (Gp-I) than in control (Gp-II) and the values are depicted in Table 4.17.

Blood serum level of albumin **(**3.06±0.03 and 2.97±0.03), total protein (7.07±0.02 and 6.98±0.03) and A:G ratio (0.77±0.01 and 0.74±0.01)increased significantly ($p<0.05$) in Group-I as compared to Group-II, respectively. Whereas, there was non-significant difference ($p<0.05$) in the level of globulin among the groups.

Mean±SE values of glucose showed significant decrease ($p<0.05$) from -30 to +30 days (58.70^{a}±0.29, 57.40^{b}±0.31, 56.40^{c}±0.27, 54.80^{d}±0.21 and 53.35^{e}±0.18 respectively).

Mean±SE values of blood urea nitrogen (BUN) in Group-I and Group-II were11.21±0.15and11.82±0.22, respectively. The data showed a significant ($p<0.05$) decrease in Group-I as compared to group II. While, there was non-significant difference ($p<0.05$) in the creatinine level between the two groups (Table 4.17)

Table 4.17: Mean±SE values of Biochemical parameters within time period (-30, -15, 0, 15, 30 days) and between the groups (Transition feed group and control group)

Groups	Period in Days					GM
	-30	-15	0	+15	+30	
TNF-alpha (ng/ml)						
Group I	1.10^{a}±0.03	1.19^{a}±0.07	2.07^{b}±0.03	1.84^{c}±.06	1.74^{c}±0.04	**1.59^{A}±0.06**
Group II	1.54^{a}±0.02	2.05^{b}±0.07	2.27^{c}±0.07	2.25^{c}±0.12	2.07^{d}±0.12	**2.03^{B}±0.05**
PM	**1.32^{a}±0.05**	**1.62^{b}±0.11**	**2.17^{c}±0.04**	**2.05^{c}±0.08**	**1.90^{c}±0.07**	
BHBA(nmol/ml)						
Group I	298.36^{a}±4.25	293^{a}.17±3.42	300^{a}.90±3.49	315^{ab}.94±3.58	325^{b}.91±3.20	**306.86^{A}±2.33**
Group II	311.84^{a}±5.29	337.80^{b}±9.76	372.02^{c}±17.34	409.55^{d}±32.93	436.28^{e}±27.87	**373.50^{B}±11.23**
PM	**305.10^{a}±3.65**	**315.48^{a}±7.18**	**336.46^{b}±11.86**	**362.74^{c}±19.37**	**381.10^{ca}±18.62**	
INF-gamma(ng/ml)						
Group I	0.71^{a}±0.03	0.74^{a}±0.01	1.08^{b}±0.03	1.12^{bc}±0.05	1.06^{b}±0.03	**0.94^{A}±0.03**
Group II	0.83^{a}±0.02	0.92^{a}±0.06	1.14^{b}±0.04	1.14^{b}±0.05	1.09^{ba}±0.05	**1.02^{B}±0.03**
PM	**0.77^{a} ±0.02**	**0.83^{b}±0.04**	**1.11^{c}±0.03**	**1.13^{c}±0.03**	**1.07^{d}±0.03**	
Albumin(g/dl)						
Group I	3.15^{a}±0.07	3.27^{b}±0.06	3.07^{c}±0.06	2.90^{d}±0.04	2.93^{d}±0.08	**3.06^{A}±0.03**
Group II	3.16^{a}±0.07	3.09^{b}±0.06	2.96^{c}±0.07	2.79^{d}±0.04	2.84^{d}±0.05	**2.97^{B}±0.03**
PM	**3.16^{a}±0.05**	**3.18^{a}±0.05**	**3.02^{b}±0.05**	**2.85^{c}±0.03**	**2.88^{c}±0.05**	
Total protein (g/dl)						
Group I	7.13^{a}±0.05	7.20^{b}±0.05	7.04^{c}±0.05	6.91^{d}±0.04	7.07^{e}±0.04	**7.07^{A}±0.02**
Group II	7.14^{a}±0.07	7.11^{a}±0.07	6.94^{b}±0.06	6.84^{c}±0.05	6.87^{c}±0.05	**6.98^{B}±0.03**
PM	**7.13^{a}±0.04**	**7.15^{a}±0.04**	**6.99^{b}±0.04**	**6.88^{c}±0.03**	**6.97^{d}±0.04**	
Globulin(g/dl)						
Group I	3.98^{a}±0.06	3.94^{b}±0.05	3.97^{c}±0.05	4.01^{d}±0.05	4.14^{e}±0.08	**4.01^{A}±0.03**
Group II	3.97^{a}±0.03	4.02^{b}±0.03	3.98^{c}±0.04	4.05^{d}±0.03	4.03^{d}±0.02	**4.09^{A}±0.01**
PM	**3.98^{a}±0.03**	**3.98^{a}±0.03**	**3.97^{a}±0.03**	**4.03^{b}±0.03**	**4.08^{ca}±0.04**	

A:G RATIO						
Group I	0.80±0.03	0.83±0.03	0.78±0.02	0.73±0.02	0.71±0.03	**0.77^{A}±0.01**
Group II	0.80±0.02	0.77±0.02	0.75±0.02	0.69±0.01	0.71±0.02	**0.74^{B}±0.01**
PM	**0.80^{a}±0.02**	**0.80^{a}±0.02**	**0.76^{b}±0.02**	**0.71^{c}±0.01**	**0.71^{c}±0.02**	
SGOT(U/L)						
Group I	98.13±0.79	96.33±0.99	99.35±0.88	104.13±1.24	110.76±1.34	**101.74^{A}±0.87**
Group II	98.54±0.92	100.88±1.55	101.29±0.30	110.86±1.71	110.46±1.40	**104.41^{B}±0.92**
PM	**98.34^{a}±0.59**	**98.61^{a}±1.04**	**100.32^{a}±0.50**	**107.50^{b}±1.29**	**110.61^{c}±0.94**	
SGPT(U/L)						
Group I	24.43±0.55	23.76±0.34	23.03±0.22	24.97±0.59	26.47±0.48	**24.53^{A}±0.26**
Group II	25.81±0.60	24.35±0.39	23.88±0.66	25.53±0.56	26.06±0.56	**25.03^{A}±0.27**
PM	**25.12^{a}±0.43**	**24.06^{b}±0.26**	**23.45^{c}±0.35**	**25.25^{d}±0.40**	**26.27^{e}±0.36**	
GGT (U/L)						
Group I	14.72±0.37	14.32±0.33	23.97±0.33	20.97±0.69	20.75±0.42	**18.94^{A}±0.57**
Group II	16.04±0.37	16.59±0.36	24.17±0.29	21.87±0.46	21.64±0.45	**20.06^{B}±0.49**
PM	**15.38^{a}±0.30**	**15.45^{a}±0.35**	**24.07^{b}±0.21**	**21.42^{c}±0.41**	**21.19^{ca}±0.32**	
Cholesterol (mg/dl)						
Group I	76.94a±0.71	74.89^{b}±0.37	68.82±1.09	73.45±0.29	78.76±0.86	**74.57^{A}±0.58**
Group II	79.03±1.56	78.72±0.42	71.64±1.15	78.73±1.14	80.40±1.14	**77.71^{B}±0.64**
PM	**77.98^{a}±0.87**	**76.81^{a}±0.52**	**70.23^{b}±0.83**	**76.09^{c}±0.83**	**79.58^{d}±0.59**	
BUN(mg/dl)						
Group I	10.96±0.25	10.89±0.35	11.02±0.27	12.06±0.41	11.11±0.27	**11.21^{A}±0.15**
Group II	11.85±0.54	10.85±0.28	11.79±0.49	11.66±0.30	12.97±0.56	**11.82^{B}±0.22**
PM	**11.41^{a}±0.31**	**10.87^{b}±0.22**	**11.40^{c}±0.29**	**11.86^{d}±0.25**	**12.04^{d}±0.37**	
Creatinine(mg/dl)						
Group I	1.20±0.01	1.18±0.01	1.24±0.01	1.23±0.01	1.24±0.01	**1.21^{A}±0.01**
Group II	1.21±0.02	1.21±0.03	1.24±0.02	1.23±0.02	1.22±0.02	**1.22^{A}±0.01**
PM	**1.20^{a}±0.01**	**1.20^{a}±0.01**	**1.24^{b}±0.01**	**1.23^{b}±0.01**	**1.23^{b}±0.01**	
Triglyceride(mg/dl)						
Group I	25.30±0.52	25.47±0.55	22.48±0.85	15.17±0.24	14.22±0.22	**20.53^{A}±0.73**
Group II	25.25±0.65	23.13±0.58	15.37±0.37	15.13±0.33	13.82±0.23	**19.54^{A}±0.70**
PM	**$25.27±0.41^{a}$**	**24.30^{a}±0.47**	**18.93^{b}±0.93**	**15.15^{c}±0.20**	**14.02^{c}±0.16**	

HDL(mg/dl)						
Group I	41.05^{a}±0.79	39.74^{b}±0.75	38.68^{c}±0.67	43.82±0.40	45.77±0.36	**41.81^{A}±0.46**
Group II	41.49±0.69	38.86±0.44	40.35±0.69	45.04±0.48	44.41±0.29	**42.03^{A}±0.41**
PM	**41.27^{a}±0.51**	**39.30^{b}±0.44**	**39.51^{b}±0.51**	**44.43^{c}±0.33**	**45.09^{c}±0.27**	
Calcium(mg/dl)						
Group I	8.29±0.09	8.52±0.09	8.23±0.04	8.06±0.03	7.94±0.05	**8.20^{A}±0.04**
Group II	8.20±0.10	8.24±0.08	8.08±0.05	7.99±0.05	7.89±0.07	**8.08^{B}±0.04**
PM	**8.24^{a}±0.06**	**8.38^{b}±0.07**	**8.15^{c}±0.04**	**8.02^{d}±0.03**	**7.91^{e}±0.04**	
Sodium (mEq/l)						
Group I	137.85±0.77	139.12±0.76	138.97±0.44	136.57±0.55	135.84±0.56	**137.67^{A}±0.33**
Group II	137.82±0.84	136.57±0.99	138.21±0.76	135.43±0.50	134.82±0.51	**136.57^{B}±0.37**
PM	**137.83^{a}±0.55**	**137.84^{a}±0.67**	**138.59^{b}±0.44**	**136.00^{c}±0.38**	**135.33^{d}±0.39**	
Potassium(mEq/l)						
Group I	3.93±0.05	4.02±0.05	3.96±0.06	3.71±0.04	3.65±0.03	**3.85^{A}±0.03**
Group II	3.77±0.05	3.77±0.04	3.85±0.06	3.69±0.03	3.63±0.03	**3.74^{A}±0.02**
PM	**3.85^{a}±0.04**	**3.89^{a}±0.04**	**3.91^{a}±0.04**	**3.70^{a}±0.03**	**3.64^{ba}±0.02**	
Magnesium(mEq/l)						
Group I	1.20±0.03	1.22±0.03	1.17±0.01	1.18±0.02	1.15±0.02	**1.18^{A}±0.01**
Group II	1.18±0.01	1.19±0.02	1.17±0.02	1.14±0.02	1.18±0.01	**1.17^{A}±0.01**
PM	**1.19^{a}±0.02**	**1.20^{a}±0.02**	**1.17^{b}±0.01**	**1.16^{b}±0.01**	**1.17^{ba}±0.01**	
Phosphorus(mg/dl)						
Group I	4.66±0.09	4.70±0.08	4.73±0.05	4.64±0.04	4.63±0.07	**4.67^{A}±0.03**
Group II	4.90±0.03	4.85±0.03	4.93±0.03	4.69±0.04	4.78±0.03	**4.83^{B}±0.02**
PM	**4.78^{a}±0.06**	**4.77^{a}±0.04**	**4.83^{b}±0.03**	**4.66^{c}±0.03**	**4.71^{a}±0.04**	
Ca:P						
Group I	1.79±0.04	1.82±0.04	1.74±0.02	1.74±0.02	1.72±0.03	**1.76^{A}±0.01**
Group II	1.67±0.02	1.70±0.02	1.64±0.01	1.71±0.01	1.65±0.02	**1.67^{B}±0.01**
PM	**1.73^{a}±0.03**	**1.76^{b}±0.03**	**1.69^{c}±0.02**	**1.72^{d}±0.01**	**1.68^{e}±0.02**	

$^{A,B\&a,b,c,d,e}$means with different superscripts between columns and rows differs significantly ($p< 0.05$)

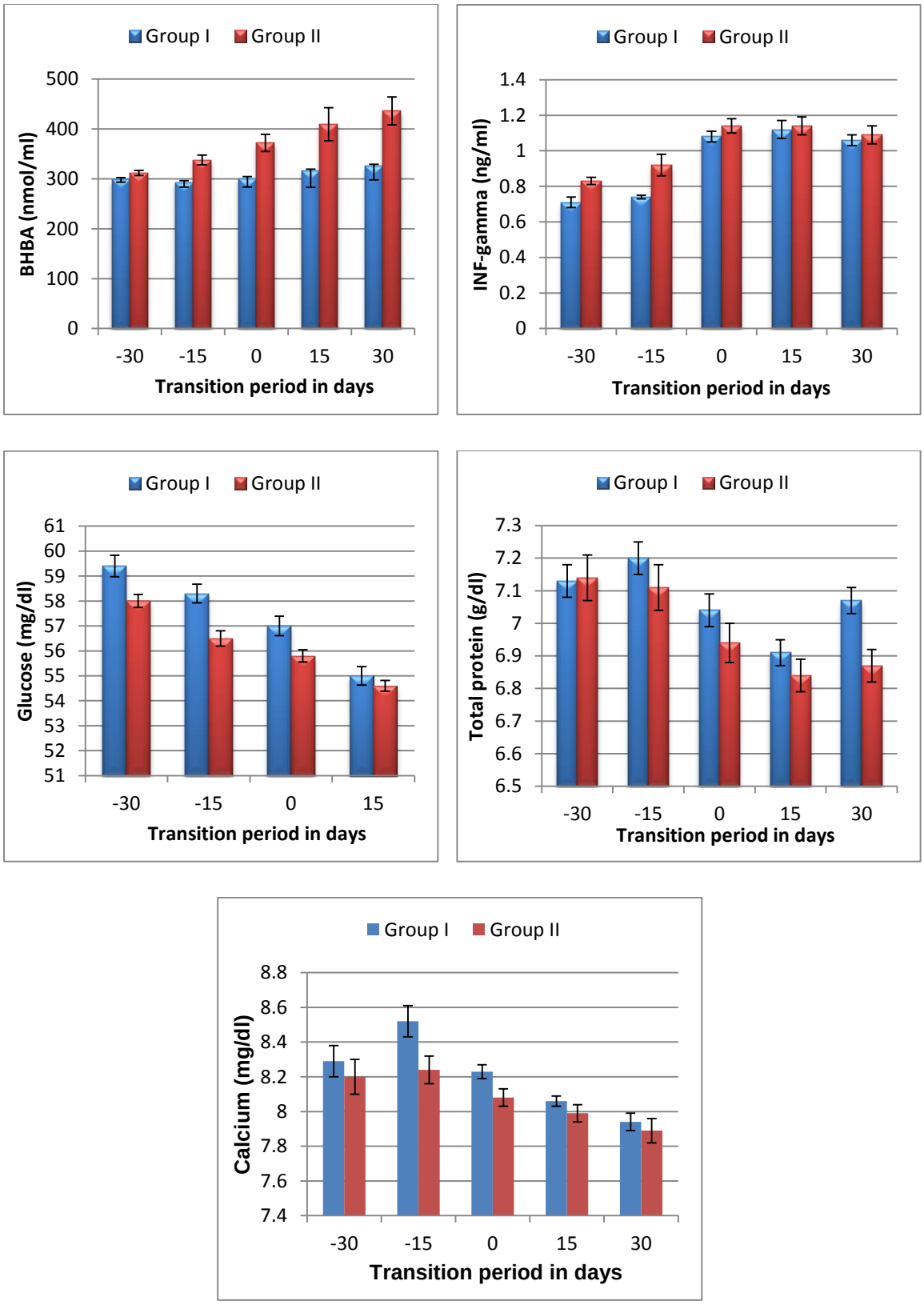


Fig. 4.29: Graphical representation of biochemical changes during transition period in feed and control groups.

Blood serum values of SGOT, GGT and cholesterol showed significant decrease ($p<0.05$) in the Group-I as compared to Group-II (Table 4.17). Similarly, there was a decrease in the level of SGPT and creatinine in feed trial group which was non-significant ($p<0.05$). The difference in the mean±SE values of triglyceride and HDL among the groups were non-significant ($p<0.05$)

Blood serum calcium level in Group-I and Group-II were 8.20±0.04 and 8.08±0.04, respectively. The data showed a significant increase (<0.05) in the calcium level in Group-I when compared with Group-II. Similar trend was seen in the Ca:P ratio among the two groups, as there was significant increase ($p<0.05$) in the Group-I, whereas, there was significant decrease ($p<0.05$) in the serum phosphorus level.

Blood serum sodium level in Group-I was significantly ($p<0.05$) higher (137.67±0.33) than Group-II (136.57±0.37).Whereas, potassium and magnesium level were non- significantly ($p<0.05$) increased in the Group-I.

Mean±SE values of BHBA (305.10±3.65, 315.48±7.18, 336.46±11.86, 362.74±19.37 and 381.10±18.62) and GGT (15.38±0.30, 15.45±0.35, 24.07±0.21, 21.42±0.41 and 21.19±0.32) showed a significant increase from -15 to +15 days of the period. The data showed a non significant increase ($p<0.05$) in the Mean±SE values of creatinine and non significant decrease ($p<0.05$) in the Mean±SE values of triglyceride along the time period.

Mean±SE values of cholesterol and HDL showed significant decrease (0.05) from Group I to Group II, then a significant increase ($p<0.05$) from 0 day upto 30 days (Table 4.17)

Mean±SE values of calcium and Ca:P ratio decreased significantly ($p<0.05$) along the period, from -30 day to 30 day (Table 4.17)

Mean±SE values of sodium showed a significant ($p<0.05$) increase from -15 to +30 days. Whereas non-significant difference ($p<0.05$) in the Mean±SE value of magnesium and potassium during the period of study.

In addition to biochemical analysis, cytokines estimation was also performed at different time intervals (-30, -15, 0, 15 and +30 days) in both the groups. Blood serum levels of TNF-α and TFN-γwere increased significantly ($p<0.05$) from -30 to 0 day of calving, and then a non-significant decreased ($p<0.05$) upto +30 days (Table 4.17).

4.7.1.4 Effect of transition feed on oxidative stress biomarkers.

The lipid peroxidation (LPO)was significantly decreased ($p<0.05$) in the transition feed trial group as compared to control group (5.31±0.04 and 5.59±0.04), respectively. Whereas, antioxidant markers viz. glutathione peroxidace (GPx), superoxide dismutase(SOD) and catalase were significantly increased ($p<0.05$) in the feed trial group than then control group. The values are depicted in Table 4.18

Mean±SE values of LPO at different time intervals of study i.e. -30, -15, 0, 15 and 30 days showed significant difference ($p<0.05$) from -30 to +30 days, with highest value at 0 day and values were above the basal level till +15 day, thereafter decreases.

Mean±SE values of GPx and catalase with the period of study showed significant decrease ($p<0.05$) from -15 to +15 days. Whereas, SOD Mean±SE values showed significant decrease ($p<0.05$) from -30 to +30 days of period (Table 4.18)

Table 4.18: Mean±SE values of oxidative biomarkers in transition feed group and control group at different time intervals of study period.

Groups	Transition Period in Days					GM
	-30	-15	0	+15	+30	
LPO						
Group I	5.37^{a}±0.21	5.09^{b}±0.07	5.66^{c}±0.07	5.31^{d}±0.05	5.12^{d}±0.04	**5.31^{a}±0.04**
Group II	5.71^{a}±0.04	5.41^{b}±0.04	5.82^{c}±0.02	5.59^{d}±0.04	5.42^{e}±0.04	**5.59^{b}±0.04**
PM	**5.54^{a}±0.11**	**5.25^{b}±0.05**	**5.74^{c}±0.04**	**5.45^{d}±0.05**	**5.27^{e}±0.04**	
GPX						
Group I	3.23^{a}±0.06	3.16^{a}±0.06	2.28^{b}±0.04	2.00^{c}±0.05	2.30^{d}±0.04	**2.59^{a}±.07**
Group II	2.99^{a}±0.04	2.88^{a}±0.03	1.96^{b}±0.12	1.84^{c}±0.04	2.04^{d}±0.03	**2.34^{b}±.07**
PM	**3.11^{a}±0.04**	**3.02^{a}±0.05**	**2.12^{c}±0.07**	**1.92^{d}±0.04**	**2.17^{b}±0.04**	
SOD						
Group I	43.42^{a}±0.84	41.69^{b}±0.60	40.95^{b}±0.40	39.28^{c}±0.45	38.30^{c}±0.72	**40.73^{a}±0.37**
Group II	42.53^{a}±0.79	39.40^{b}±0.62	37.22^{c}±0.50	35.92^{d}±0.44	34.51^{e}±0.69	**37.91^{b}±0.48**
PM	**42.97^{a}±0.57**	**40.55^{b}±0.50**	**39.08^{c}±0.53**	**37.60^{d}±0.49**	**36.40^{e}±0.65**	
Catalase						
Group I	125.61^{a}±0.37	126.66^{a}±0.37	124.59^{b}±0.67	121.10^{c}±0.79	119.12^{d}±0.54	**123.41^{a}±0.47**
Group II	123.97^{a}±0.47	125.07^{a}±0.34	123.18^{b}±0.52	119.29^{c}±0.56	117.84^{d}±0.72	**121.87^{b}±0.46**
PM	**124.79^{a}±0.34**	**125.86^{a}±0.31**	**123.88^{b}±0.44**	**120.20^{c}±0.51**	**118.48^{d}±0.46**	

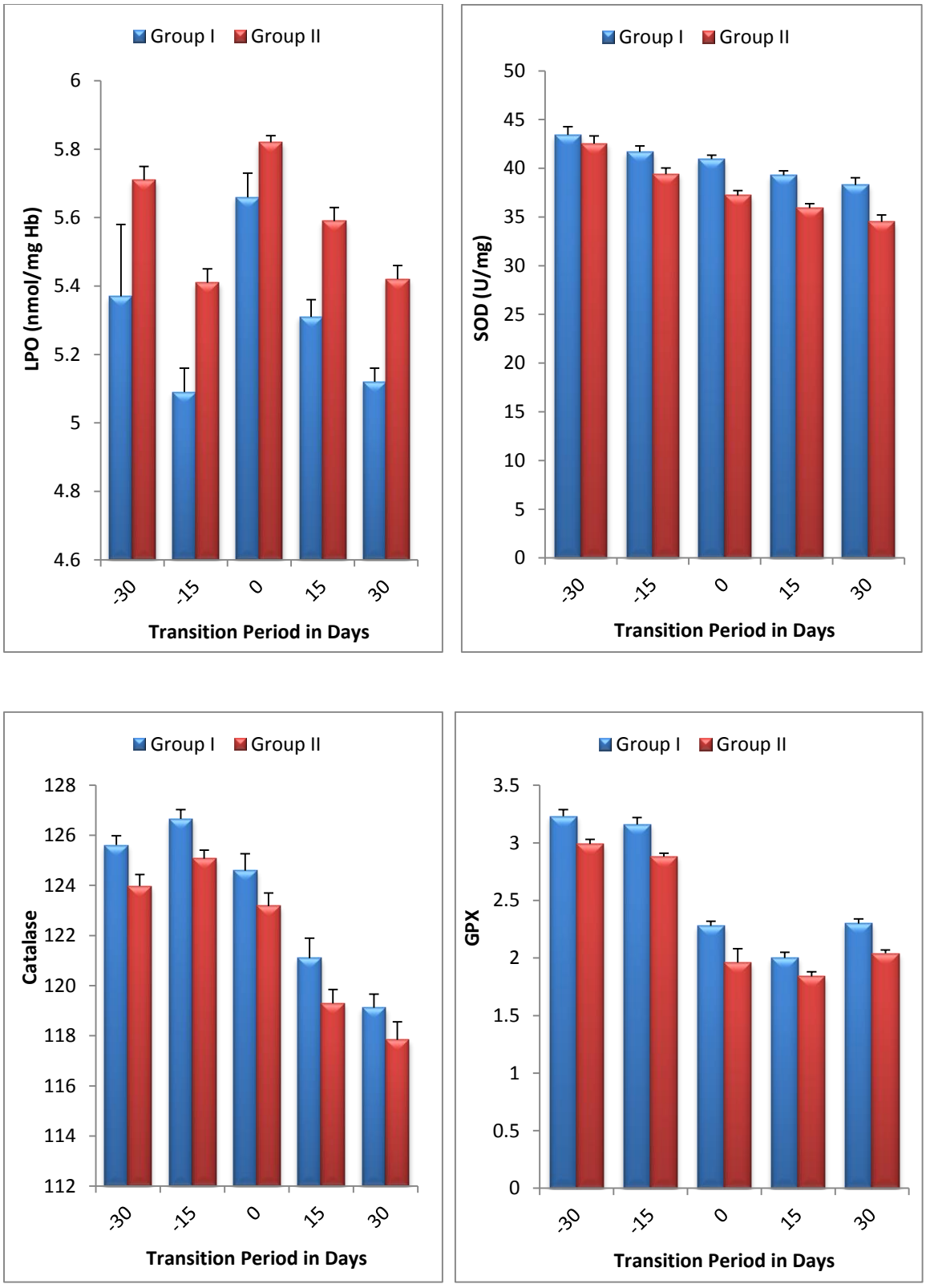


Fig. 4.30: Graphical representation of changes in oxidative stress markers during transition period in feed and control group at different time intervals.

4.7.1.5 Effect of transition feed on expression level of production diseases specific genes by qRT-PCR.

To know the effect of the transition feed given to the buffaloes on molecular level. Total RNA was isolated from blood samples of both the groups at different time intervals and subjected for cDNA synthesis for gene expression study. Up- and down-regulated genes for production diseases were identified by in-silico analysis and used for the further validation in both the groups to study the effect of transition feed at more accurate level. The changes in the expression of gene were considered between the groups majorly and within the different time period also.

4.7.1.5.1 Effect of transition feed in the expression level of hypocalcemia/milk fever specific genes.

In the present study, it was observed that there was a significant decrease ($p<0.05$) in Group Mean±SE values of expression level of gene NUAKI in transition feed group than control feed group. Along the different time interval of transition period, NUAKI got upregulated from -15 to +30 days of period significantly ($p<0.05$) in both the groups. The values of the fold change in the expression level are given in Table 4.19. NUAKI gene is upregulated in the milk fever/ hypocalcemic condition. These results showed that supplementation of transition feed reduce the probability of milk fever by low expression of NUAKI gene in the supplemented group.

Table 4.19: Difference in the fold of expression level of NUAKI gene in feed and control group at different time intervals in buffaloes.

Groups	Transition period in Days				GM
	-15	0	+15	+30	
Group-I (Supplemented)	0.908^{a}±0.03	0.937^{a} ±0.05	1.027^{b} ±0.04	1.116^{c}±0.02	**0.952^{a}±0.04**
Group-II (control)	0.918^{a}±0.04	1.083^{b}±0.02	1.032^{c}±0.01	1.179^{d}±0.15	**1.297^{b}±0.02**

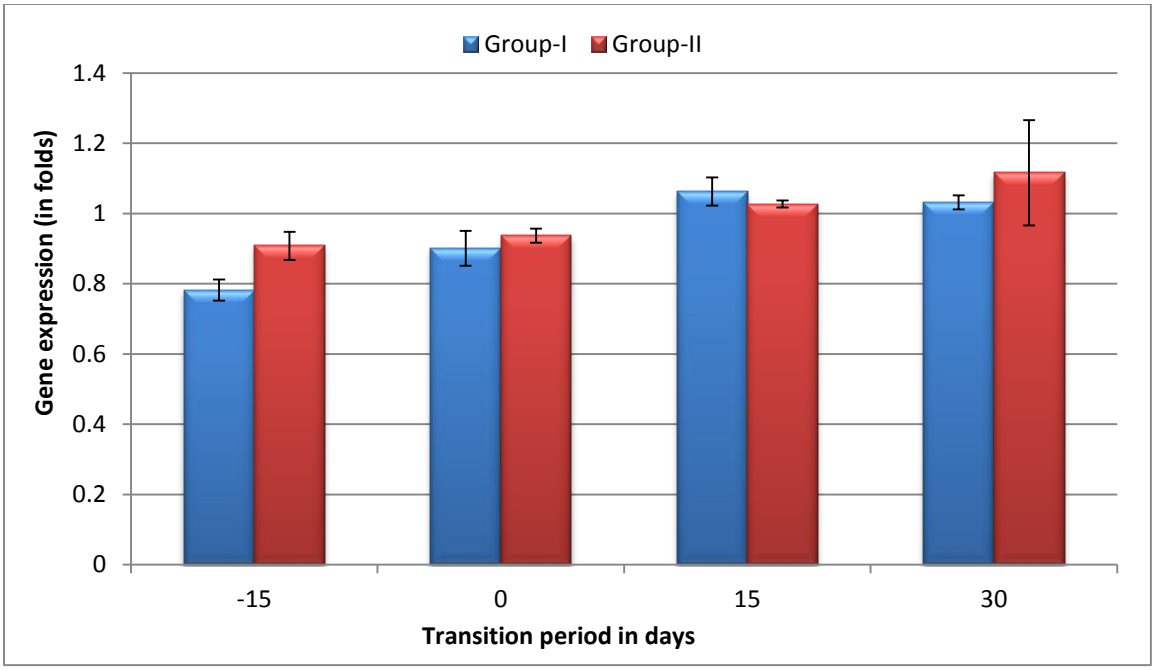


Fig. 4.31: Change in the expression level of NUAKI gene between feed and control group during transition period at different time intervals in buffaloes.

In case of downregulated gene viz. NESP55, there was a significant increase ($p<0.05$) in the expression level of NESP55 in transition feed group than the control group, which again validated that probability of milk fever/ hypocalcemic condition decreased in the supplemented group than the control group. However, along the time interval of transition period, NESP55 got downregulated from -15 to +30 days significantly ($p<0.05$) in both the groups. The values of fold change in the expression level are given in Table 4.20.

Table 4.20: Difference in the fold of expression level of NESP55 gene in feed and control group

Groups	Transition period in Days				GM
	-15	0	15	30	
Group-I (Supplemented)	0.402±0.01	0.396^{c}±0.01	0.384-b±0.1	0.332^{a}±0.02	**0.379^{a}±0.01**
Group-II (control)	0.348^{d}± 0.01	0.334^{c} ±0.01	0.318^{b}±0.01	0.303^{a}±0.01	**0.318^{b}±0.01**

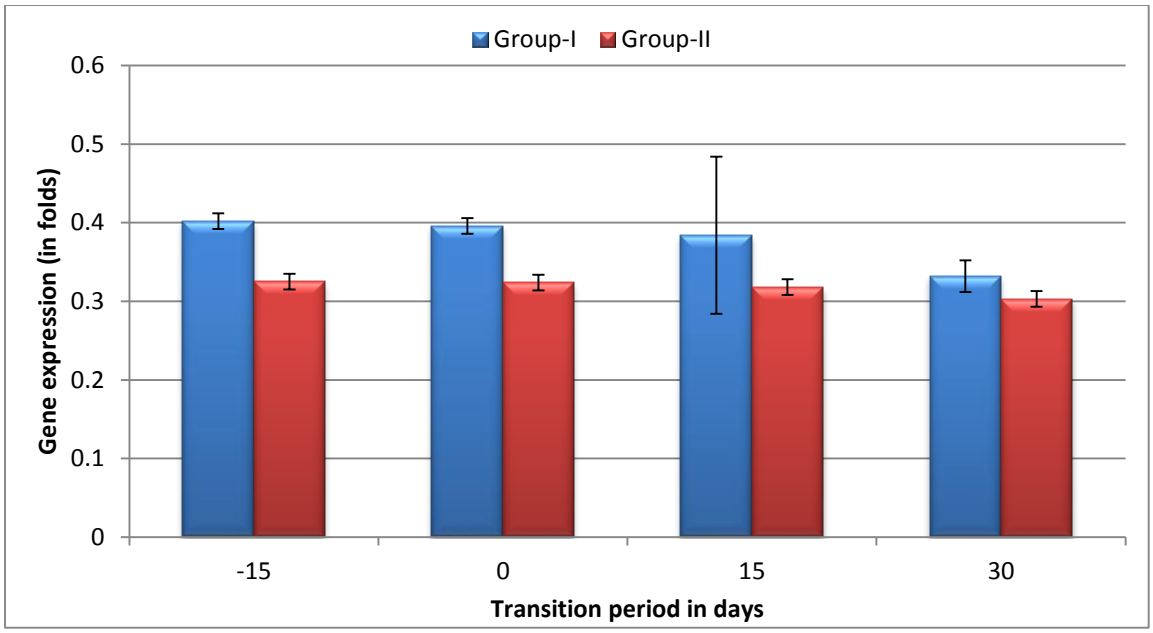


Fig 4.32: Change in the expression level of NESP55 gene between feed and control group during transition period at time intervals in buffaloes.

4.7.1.5.2: Effect of transition feed in the expression level of negative energy level/ Ketosis specific genes.

In our study, it was evident that there was a significant decrease ($p<0.05$) in the Group Mean±SE values of expression level of CPTIA (upregulated gene) in feed group as compared to control group as depicted in Table 4.21. These results suggest that supplementation of transition feed reduce the probability of negative energy balance/ ketotic condition in buffaloes. There was a significant increase ($p<0.05$) in the expression level of CPT1A as transition period progresses in both the groups.

Table 4.21: Difference in the fold of expression level of CPT1A gene in feed and control group at different time intervals in the buffaloes.

Groups	**Transition period in Days**				**GM**
	-15	0	+15	+30	
Group-I (Supplemented)	1.323^{a}± 0.02	1.335^{b}±0.02	1.353^{c}±0.02	1.489^{d}±0.11	**1.123^{A}±0.05**
Group-II (control)	1.375^{a}±0.02	1.336^{b}±0.02	1.433^{c}±0.02	1.499^{c}±0.11	**1.378^{B}±0.03**

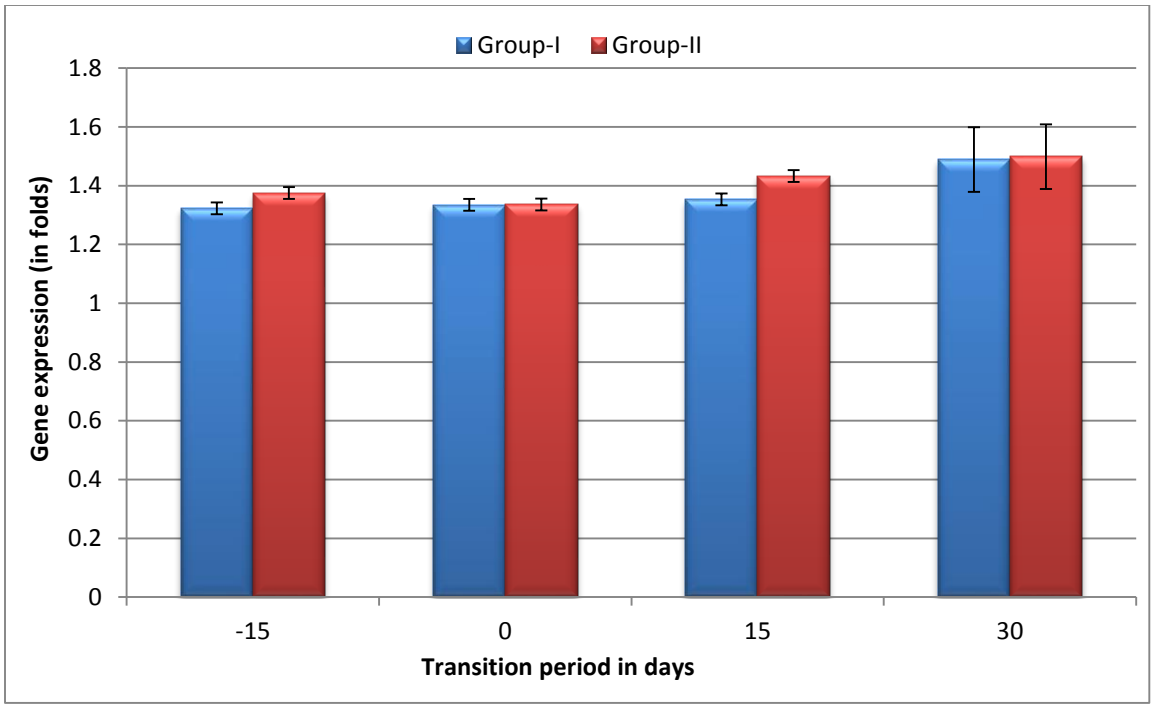


Fig 4.33: Change in the expression level of CPT1A gene between feed and control group during transition period at different intervals in buffaloes.

Similarly, there was a significant increase ($p<0.05$) in the group Mean±SE values of expression level of IGF-1 (downregulated gene) in feed group as compared to control group, which is again validated that increased expression of IGF-1in supplemented group reduce the probability of negative energy balance/ ketotic condition in buffaloes. Along the different time interval, there was significant decrease ($p<0.05$) in the expression level of IGF-1 as transition period progresses from -30 to +30 days (Table 4.22).

Table 4.22: Difference in the fold of expression level of IGF-1 gene in feed and control group

Groups	**Transition period in Days**				**GM**
	-15	0	15	30	
Group-I (Supplemented)	1.247^{c}±0.05	1.304^{c}±0.06	1.063^{b}±0.06	0.939^{a}±0.05	**0.974^{A}±0.06**
Group-II (control)	1.203^{d}±0.05	1.214^{c}± 0.01	1.008^{b}± 0.03	0.838 ± 0.15	**0.905^{A}±0.04**

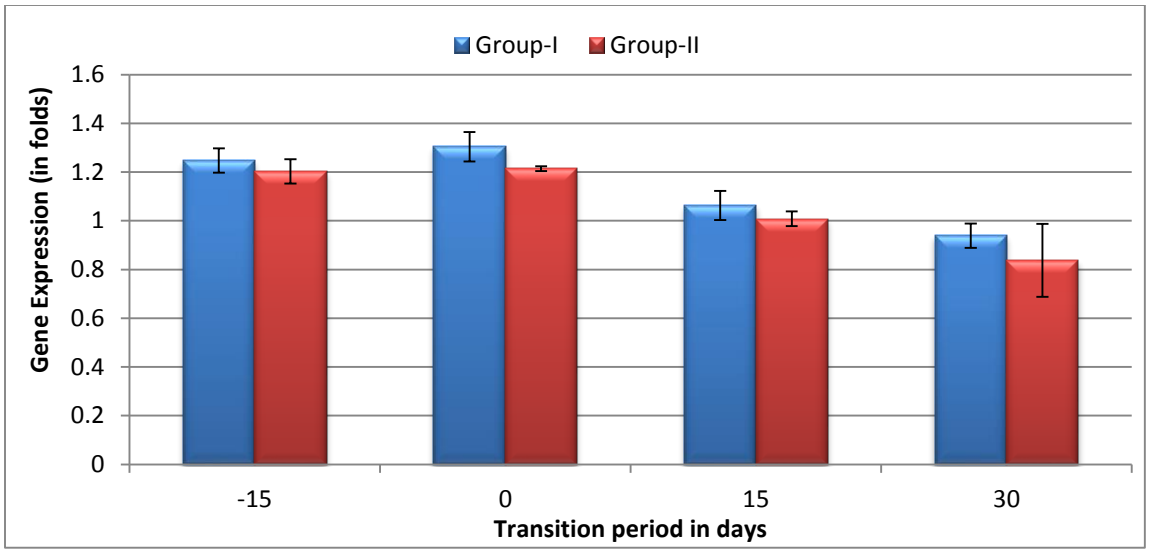


Fig 4.34: Change in the expression level of IGF-1 gene between feed and control group during transition period at different intervals in buffaloes.

4.7.1.5.3: Effect of transition feed in the expression level of mastitis/ inflammations specific genes.

It is well established that mastitis causes inflammation and increased the expression of inflammatory markers e.g. TNF-α, IFN-γ etc. Our study revealed a significant decrease ($p<0.05$) in the Group Mean±SE values of expression level of TNF-α **(**upregulated gene for inflammation) in the feed group as compared to control group (Table 4.23). Although there was a significant increase ($p<0.05$) in the expression level of TNF-α as progression of transition period occurred i.e from -15 to +30 days. Similar trend was seen with the expression level of INF-gamma with feed and control group as depicted in Table 4.24.

Table 4.23: Difference in the fold of expression level of TNF-α gene in feed and control group at different time intervals in buffaloes.

Groups	Transition period in Days				GM
	-15	0	15	30	
Group-I (Supplemented)	2.658^{a}±0.09	3.195^{b}±0.08	2.802^{c}±0.06	2.757^{c}±0.09	**2.853 ± 0.06**
Group-II (control)	3.028^{a}±0.05	3.512^{b}±0.12	3.275^{c}±0.18	3.227^{c}±0.12	**3.274 ±0.07**

Table 4.24: Difference in the fold of expression level of INF-gamma gene in feed and control group at different time intervals in buffaloes.

Groups	-15	0	15	30	GM
Group-I (Supplemented)	$2.645^{a}\pm0.06$	$2.715^{a}\pm0.07$	$3.108^{b}\pm0.08$	$2.702^{c}\pm0.06$	**2.840 ± 0.04**
Group-II (control)	$3.015^{a}\pm0.09$	$3.107^{a}\pm0.16$	$3.43^{b}\pm0.10$	$3.113^{c}\pm0.10$	**3.120±0.06**

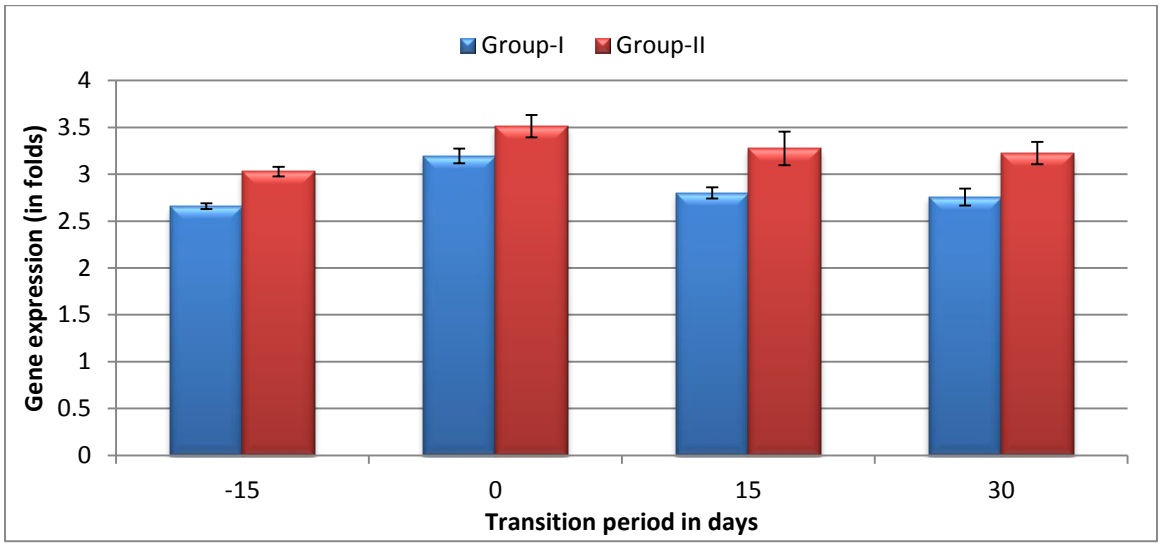


Fig 4.35: Change in the expression level of TNF-α gene between feed and control group during transition period at different intervals in buffaloes.

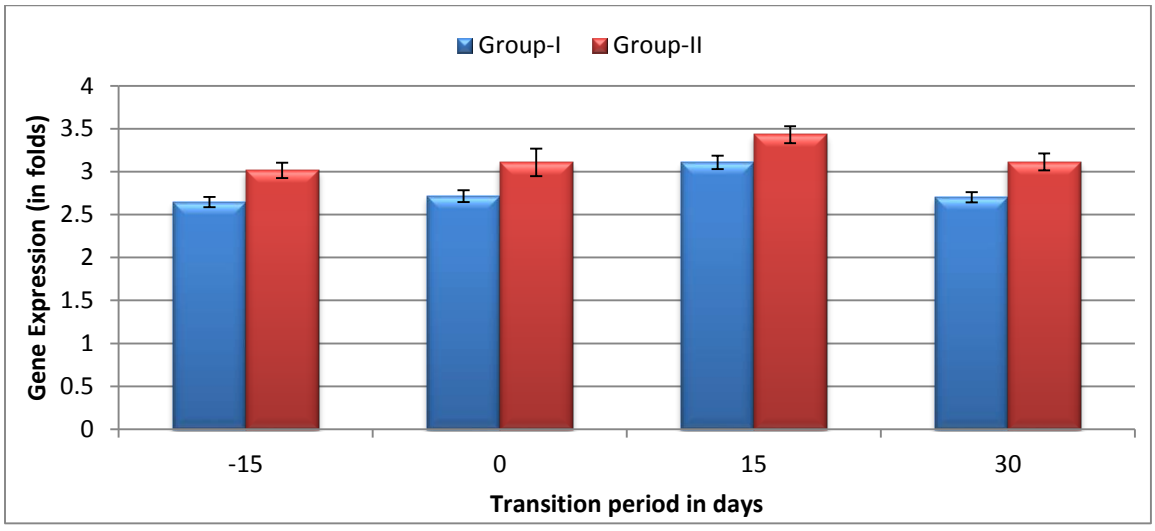


Fig 4.36: Change in the expression level of INF-γ gene between feed and control group during transition period at different intervals in buffaloes.

4.7.1.5.4: Effect of transition feed on the milk production and occurrence of mastitis.

The average milk production by the buffaloes of feed supplemented group was $8.35^{a}\pm0.183$ kg/day where as in control group it was found to be $6.25^{b} \pm 0.2$ kg/day. There was a significant increase ($p<0.05$) in the daily milk production in the feed group.

The analysis of milk revealed that more number of animals were positive for mastitis by MCMT(T/+) in control group (n=4) as compared to feed group (n=2) with average somatic cell count of 284 ±14.8 (10^3/ml) and 264 ± 7.08 (10^3/ml), respectively. However, MCMT score T/+ is negligible.

Chapter-V

Discussion

CHAPTER-5

DISCUSSION

Transition period is considered to occur from 3 weeks prepartum to 3 weeks postpartum period. It is a critical period in sense of significant metabolic and physiological changes that occurs as a result of adaptation to it. Dairy cattle undergo major changes during this time frame like negative energy balance, drop in milk production, lipolysis, and catabolism of body reserve in order to meet the requirement of the lactation in postpartum period. These changes encompass various alterations in the physiological processes that make dairy cattle prone to infectious and metabolic diseases. And these diseases cause major production losses in dairy industries. The factors causing severe production loss in dairy cattle are diagnosed at much later stage of the disease. Even after the treatment animal is unable to recover its full production yield.Therefore a scientific approach for the early diagnosis of the metabolic and production disease in clinically healthy animal is a need of an hour. Many studies have been conducted during transition period in dairy cows. But limited data is available on the early diagnosis of metabolic changes that occurred during this period in buffaloes. Keeping in view about the importance of early diagnosis of production diseases, we designed our study to establish a baseline study with haemato-biochemical parameters as well as diagnosis of production diseases by the analysis of selected genes with the aid of qRT-PCR (real time) in buffaloes particularly in transition period.

5.1 Prevalence of the milk fever, ketosis and mastitis in buffaloes during transition period

In our present study, it was seen that there was no evidence of clinical cases of milk fever/typical hypocalcemia in buffaloes during transition period when subjected for biochemical analysis.Our findings were in agreement with the findings of Khan *et al.* (2015), who screened 607 buffaloes during a period of time and prevalence for milk fever was found to be 3.6% in buffaloes. Similar finding was seen in the study of Thirunavukkarasu *et al.* (2010), whichhas done random screening in 557 buffaloes during

the period of 2005-2008 and 6.3% of them were found positive for milk fever condition. Khatri *et al.* (2013) screened 1000 pregnant buffaloes for parturition in Pakistan and the incidence of milk fever was found to be 6.7%. All these findings shows low rate of prevalence of milk fever/hypocalcemic condition in buffaloes particularly when analysed biochemically. But 17.14% animals in Group-I (n=70), 38% animals in Group-II (n=70) and 45% animals in Group-III (n=70) were found to have hypocalcemic condition when subjected to molecular level diagnosis via quantification of selected genes (NUAK1; upregulated and NESP55; downregulated). Our findings showed significant agreement with the findings of Sasaki *et al.* (2014) who generated a microarray data for gene expression of selected genes in PBMC of cows particularly for milk fever/hypocalcemia condition. They selected 5 genes viz protein kinase (cAMP-dependent, catalytic) inhibitor β (PKIB); DNA-damage-inducible transcript 4 (DDIT4); period homolog 1 (PER1); NUAK family, SNF1-like kinase, 1 (NUAK1); expressed sequence tag (BI537947) and neuroendocrine secretory protein 55(NESP55) that were highly correlated with milk fever condition and showed significant change in the fold expression. The cows showed hypocalcemia condition got an upregulation of gene NUAK1 and down regulation of NESP55 (Saski *et al.*, 2014). Similar findings were found in our study with upregulation of NUAK1 (0.901±0.04, 1.084±0.05 and 1.032±0.02) and downregulation of NESP55 during -30, 0 and +30 days (0.402±0.01, 0.383±0.01 and 0.332±0.01 respectively)in buffaloes having calcium level 8-8.5mg/dl with no clinical evidences of the condition. From the study of (Inazuka *et al.*, 2012) it was found that NUAK1 has a role in the suppression of glucose uptake via negative regulation of insulin pathway in the rat and in hypocalcemic condition there is reduced secretion of insulin and this leads to increased concentration of glucose in the blood (Larsen *et al*,. 2001). Here the role of NUAK1 comes into play and it gets upregulated in hypocalcemic condition. Even it has been seen that for proper functioning of immune cells viz lymphocytes, a proper regulation of glucose uptake by them is a necessity for homeostasis (Maclver *et al.*, 2008)

The clinical cases of ketosis in three groups (Group I to Group III) of buffaloes were not seen in our study. There was no positive case found in Rothera's test and urine strips used for detection of ketone bodies. The value of BHBA was also under the normal range (<1200nmol/ml) in all the three groups (Gp-I to Gp-III; 313.96±2.81nmol/ml,

397.36±1.69nmol/ml and 483.69±1.98nmol/ml respectively) of our study. Similar findings were seen in the study of Anantwar *et al.*(1993) which screened 3395 she buffaloes for Rothera's detection of ketone bodies and found only 2.85% positive cases. Singh *et al.* (1988) screened 500 buffaloes in period of two years and found 7.3% of positive cases of ketosis in buffaloes. Even though, a study was conducted by Kumar *et al.* (2015) on 145 buffaloes and got 24 cases positive for primary clinical ketosis in them. The screening was done on the basis of rothera's test and Keto-diastix strip test. The cases were present with typical clinical signs viz sudden drop in milk production, hypoglycemia, ketonuria, atony of rumen and anorexia. A similar study was conducted by Walia *et al.*(2017) on 159 buffaloes and found 18.9 % of positive cases of ketosis in buffaloes which were in parity 5^{th} to 8^{th}. Krishna *et al.* (2014) carried out a study on the prevalence of subclinical ketosis on 900 buffaloes and 179 were screened, out of which 20.67% of buffaloes showed positive results. Majority of the buffaloes were high yielders (>10 kg/day) and in 4^{th} parity. In our study, cDNA samples from all the buffaloes of three groups were subjected to analysis of genes for negative energy balance condition; CPT1A and IGF-1. CPT1A showed upregulation during the progression of transition period. CPT1A (Carnitine palmotyl transferase A) are the carriers of free fatty acid into the mitochondria for their β oxidation (Voet and Voet, 1995). During post calving period, there is increase in the hepatic fatty acid oxidation to cover up the demand of energy from body reserves (Graber *et al.* 2010). Meanwhile, we found a down regulation of IGF-1 expression level along the transition period (-30 to +30). The demands in early lactation are met by physiological alteration with hormones involved in it. Amongst the hormones, growth hormone plays pivotal role in partitioning of nutrition and metabolism of carbohydrate and lipid (Lucy *et al.*, 2001). The transcription of GHR mRNA is mainly done by GHR1A promoters, which are found in bulk in liver of adult animal (Hauser *et al.*, 1990). The decrease in the GHR1 mRNA expression in liver during early lactation leads to decrease in the synthesis of growth hormone dependent IGF-1 secretion (Vicni *et al.*, 1991). The nutrition plays an important role in the regulation of growth hormone secretion from liver. During periparturient period, dairy cattle undergo several metabolic changes to accommodate for lactation and lead to under nutrition of proteins and

carbohydrates. This causes marked reduction in the synthesis of GHR and subsequent decrease in the synthesis of IGF-1 (Breier *et al.*, 1999).

The prevalence of mastitis among buffaloes, animals from Gp-III were examined for MCMT and SCC, 62.85% were MCMT negative and had an average of 168.41±0.8 ($\times 10^3$/ml) somatic cell count whereas 37% were CMT (T/+) with SCC of 278 ± 0.67 ($\times 10^3$/ml). Our result showed agreement with the findings of Dhakal *et al.* (2006) which screened 60 clinically healthy buffaloes for CMT and SCC. They found that 94% of the buffaloes with CMT (1+ or trace positive) were having SCC >2, 00,000/ml of milk. On a contrary, others had mentioned 15.20% prevalence of subclinical mastitis was recorded in buffaloes out of 593 screened buffaloes (Hussain *et al.*, 2001). Meanwhile, the prevalence of subclinical mastitis in buffaloes was comparatively less than cows during early lactation as seen in the findings of Khan *et al.* (2005) with 20% and 36% prevalence in buffaloes and cows respectively. The major isolates found in buffaloes were *Staphylococcus aureus*(45%), *Streptococcus agalactiae* (23%), *E. coli* (18%) *and Bacillus spp*. (14%)(Khan *et al.*, 2005).

5.2 Changes occur in body condition score in buffaloes during transition period

Present study showed a marked reduction in BCS score from group I to group III during the progression of transition period. Also, buffaloes with greater loss in BCS during postpartum group (Gp-III) had a higher level of BHBA (483.69±1.98) than other two groups. Similar findings were found in the study of Bernabucci *et al.* (2005) with greater reduction in BCS score during first 30 days in milk in cows with good or high BCS during prepartum period. Barletta *et al.*(2017) observed that concentration of BHBA after calving differed ($p<0.01$) for cows that lost and maintained BCS from 3 weeks prepartum to 3 weeks post partum period (0.73±0.02 and 0.70±0.02, respectively) in 232 Holstein cows. Previous studies showed that dry matter intake during last 3 weeks of prepartum has been reduced to 10-30% and there is a sharp change in the feed of animal mainly from forage based to concentrate rich diet (Bell *et al.*, 1995). This period is marked by marked adipose tissue lipolysis, increase in concentration of BHBA, NEFA and catabolism of body reserves along with decrease in body condition reserve (Esposito *et al.*, 2013). During negative energy balance there is uncoupling of growth hormone and

insulin growth factor -1 axis which leads to marked reduction of insulin growth factor-1, high insulin resistance and increase in the level of growth hormone, particularly responsible for direct catabolism of lipid reserve in the body (Lucy *et al.,* 2009). Increased lipolysis results in high concentration of BHBA (beta hydroxy butyric acid; ketone bodies) and NEFA (non esterfied fatty acid) and low glucose level in blood (Grummer, 1995; Duffield *et al.*, 2000; Melendez *et al.*, 2006).

In our study we found that with greater loss of BCS, there was an increase in the level of oxidative stress markers and reduction in the anti-oxidant defense mechanism of the body. Our results were in agreement to the results of Zhang *et al.* (2011) in which NEFA production leads to increase in reactive oxygen species and development of oxidative stress in subclinical ketosis condition in dairy cattle. Ster *et al.*, 2012 reported that NEFA and BHBA had negative effect on the oxidative burst capability of phagocyte cells and the anti-oxidants markers.

5.3 Effect of dry period length on daily milk yield of the buffaloes

In our study, buffaloes with dry period shorter than 44.21±0.59 were having milk yield about 6.81±0.10 kg compared to those having dry period length above 53.83±0.48 i.e 7.92±0.87kg. Similar findings were seen in study of Weber *et al.* (2015) with three periods of dry period (28 days, 56 days and 90 days). The milk yield was significantly ($p<0.05$) increased in 56d-DP with respect to 28d and 90 days DP. Pinedo *et al.* (2011) showed in their study of dairy cows that short dry periods (<45 days) were negatively related to lactation yield and 305 day milk yield whereas, extended dry period length (>90 days) increased the culling rates in herd with reference to standard length (60 days) due to high incidence of clinical mastitis. Atashi *et al.* (2013) observed that shorter dry periods (0 to 35 d and 36 to 50 d) were associated with lower initial milk yield and declining slopes of the lactation curve, compared with dry period length of 51 to 60 d. Our study were in broader agreement to the findings of Watter *et al.* (2008) who did study on the effect o dry period length on HF cows (n=781); 34 days and 56 days dry period. Cows with 56 days dry period produced more milk than the former one. It has been seen in many studies that continuous milking and shortened dry period length inhibits the renewal of mammary epithelial cells required for lactogenesis prior to

parturition (Smith *et al.*1967). Some reports suggest that DNA content of mammary gland, parenchyma content and proliferation of mammary epithelial cells found in greater quantity in cows with 60 days traditional dry period rather than in shortened one. This was suggestive of slow or decreased proliferation of mammary epithelial cells (Capuco *et al.*,2001). In this pivotal time, there is increase in incorporation of [3H]-uridine and hydroxyproline content in dried glands, suggestive of remodelling of extracellular matrix which are foundation of mammary epithelial cells turnover in later stage of dry period (Bachman *et al.*, 2002). This encompasses the benefits of dry period for senescence and renewal of cells in mammary gland for subsequent lactation.

5.4 Cytokines level in buffaloes during transition period

In our study of 210 buffaloes during the transition period (-30, 0 and +30 days), we found that level of TNF-alpha and IFN-γwere raised significantly as the period progresses with highest value in group II. Thereafter, a significant decrease ($p<0.05$) in the level has been seen from 0 day to 4 weeks postpartum period. Similar results were seen in the study of El-Deeb *et al.* (2017), in which 25 HF cows during post partum period showed significant ($p<0.05$) increase in the level of BHBA, NEFA and pro-inflammatory cytokines (TNF –alpha, IL-6, INF-gamma) when compared to healthy cows. Kushibiki *et al.*(2011) observed that NEFA during post partum period showed high level of TNF-alpha and interferon gamma secreted from macrophages. El-Deeb *et al.*(2017) observed a strong correlation between BHBA, NEFA, acute phase proteins and cytokines in dairy cattle during post partum period. Acute phase proteins and cytokines could be use as a biomarker for negative energy level in cows.

Buffaloes from all the three groups when subjected to qRT- PCR for genes of inflammatory condition (TNF-alpha and interferon gamma) showed increase fold of expression in both the genes from -30 to +30 days of transition period. Lange *et al.*(2016) reported a study on immunological studies with the aid of qRT-PCR during the transition period in dairy cattle. They found an increase in the expression level of TNF-alpha, Interferon –gamma and IL-17 from pre partum to +2 weeks and thereafter decrease in the expression level from +4 weeks was seen. Heiser *et al.* (2015) reported a study on the decreased expression level of TNF-alpha, interferon gamma and increased level of

interleukins in grazing cows in 2 weeks post calving. Interferon- gamma and TNF-alpha are crucial components of innate and adaptive immunity against infectious pathogen and is an important activator of macrophages (Schroder *et al.,* 2004). The relative number of T-cells, natural killer cells and monocytes in peripheral blood increases during post calving period (Locksley *et al.*, 2001).

5.5 Haematological and biochemical changes during the transition period

In present study there was a significant increase ($p<0.05$) in the level of WBC (10^3/ul) with the progression of transition period, along with lymphocytes and granulocytes at the time of calving. Similar findings were found in the study of Preisler *et al.* (2000) with increased amount of WBC, lymphocytes and neutrophil due to rise in concentration of cortisol at the time of calving. Cortisol is suppose to cause demargination of neutrophil from endovascular linings eventually leads to increase concentration of neutrophil in peripheral blood circulation (Shoenfeld *et al.*, 1981).

Significant decrease ($p<0.05$) in the level of RBC, Hb and HCT was seen in our study during the calving time and postpartum period. Our results showed agreement to the findings of Gavan *et al.* (2010) who observed a marked reduction in the values of RBC, Hb and HCT in periparturient period. (Kumar and pachauri, 2000) observed that Hb concentration in blood decreases during the calving time that might be due to decreased erythropoiesis in periparturient buffaloes. (Chikazawa and Dunning, 2016) suggested that during time of parturition, there is increased in level of pro-inflammatory cytokines (TBF-alpha and interferon's) which are inhibitors of erythropoiesis through the action on erythroid precursors in bone marrow. HCT level in prepartum group was significantly ($p<0.05$) higher than calving day. Daramola *et al.*(2004) observed low level of Hb and PCV/HCT during the calving period suggesting the anemia condition attributed due to stress in parturition. On a contrary, Ate *et al.*(2009) did not find any significant ($p<0.05$) difference in the mean values of Hb, RBC and PCV during peri parturient period.

In our study, biochemical parameters like albumin, total protein and A:G ratio showed significant ($p<0.05$) decrease from -30 to +30 days of parturition. In last trimester

of pregnancy with prevailing endocrine situations, there is insulin resistance and decreased level in insulin growth factor-1 which causes net mobilisation of amino acid reserves and decreases the plasma level of albumin and protein (Block *et al.*, 2001). Albumin is considered as negative acute phase protein and its level decreased during transition period which encounters high inflammatory responses (Bionaz *et al.,* 2007)

The blood glucose level decreased significantly ($p<0.05$) as transition period progresses. Hagwane *et al.* (2009) observed that glucose level in dry buffaloes was greater than in early lactating buffaloes (52.72±4.22 mg dLG1 48.23±3.44 mg dLG1 respectively). There are reports of exponential decrease in the level of glucose in plasma and serum when dairy cattle approach towards calving signifying rapid utilisation of glucose by the growing foetus (Elitok *et al.*2006). Nale *et al.* (2003) reported that low level of glucose is attributed to increase lactose synthesis during early lactation period. There are evidences of insulin resistance during early parturition which contributes to low level of glucose in blood of periparturient cattle (Mir *et al*, 2008)

There was significant ($p<0.05$) increase in the level of SGOT (U/l) from -30 to +30 days and in BUN (mg/dl) from 0 day to +30 days. Roubies *et al.(*2006) observed that level of BUN are influenced by many actors viz dietary intake, protein quantity in feed, rumen degradability and catabolism of body reserved protein. Similar findings were seen in study of Kulkarni *et al.* (2010), as in late gestation, availability of glucose is supplemented by increased catabolism of amino acid reserve of the body at the expense of protein production. Mills *et al.*(1896) observed high level of SGOT during calving and postpartum period indicating of stressed hepatic metabolism and pronounced catabolism of body reserves. Similar findings were found in the study of Abdulkareem *et al.*(2013), with higher level (96.06±8.75-102.61±11.62 Unit LG1) during postpartum periods in comparison with its value at calving (90.06±8.04 Unit LG1)

There was significant decrease ($p<0.05$) in the level of cholesterol and triglyceride along the transition period. Similar findings were found in study of Karapehlivan *et al.* (2007) with low level of triglyceride as lactation progresses as triglyceride are used by the mammary glands for production of milk fat. Total cholesterol reflects the indirect availability of exogenous source and hepatic functionality (Grum *et al.* 1996) and Kweon

et al. (1986) observed that higher rate of disease occurrence in cattle with low cholesterol level at the time of calving. The level of serum cholesterol (mg/dl) decreased during calving and got build but as lactation progresses as a physiological adaptation to lactation requirement. In ruminants, cholesterol and triglyceride are transported from liver via lipoproteins and these lipoproteins get decreased during periparturient period (Katoh *et al.*2002) which eventually leads to decrease level of cholesterol and triglyceride around calving (Akamatsu *et al.*, 2007).

Calcium level along the transition period decreased significantly ($p<0.05$) in our study. Similar findings were found in Kronqvist *et al.* (2011) study in which calcium level decreased from 0 day, 2, 4 and 7 days after calving till peak lactation attains. Parathyroid hormone regulates the level of calcium via stimulation of osteocytic osteolysis mechanisms that releases calcium from lacunae to blood stream (Le *et al.,* 2020*).* During the lactation, calcium homeostasis becomes less effective in high yielding cattle (Kovacs *et al.,* 2011). Wu *et al.* (2008) observed in a study that calcium level of plasma on the day of calving was significant lower than the prepartum period (8.33 vs 9.30 mg/dl).

Significant decrease ($p<0.05$) in the level of sodium and potassium from calving to +30 day of transition period was seen in our study. The decrease in the potassium level during postpartum period was seen in study of Jacob *et al.* (2011) suggesting of transfer of the cation in the milk during lactation. Similar findings were found in study of Deshpande *et al.*(1998) related to sodium level during postpartum period.

5.6 Oxidative stress markers during transition period in buffaloes

Oxidative stress occurs during the period of high metabolic demands which leads to imbalance of anti-oxidant defence markers and reactive oxygen species (Sordillo *et al.,* 2013).The reactive oxygen species progressively increased from late lactation to post parturient period, even higher in peak lactation (Abuelo *et al.*, 2013).

In our study we found significant increase ($p<0.05$) in level of LPO during calving and postpartum period and decreased level o SOD, GPx and catalase. Similar findings were found in the study of Singh *et al.* (2015) which encounters a great level of

lipid peroxidation during fresh period (around calving) (272.11±5.38) as compared to other period (236.96 ± 4.56) and SOD, Gpx during calving (51.08±1.23 and 1.94±0.05U/mg of Hb) with respect to other periods (67.54±1.33 and 2.29±0.04 U/mg of Hb). Dimri *et al.* (2009) observed a significantly higher level of LPO in erythrocytes during pregnancy and first day of calving with respect to mid pregnancy (0.325±0.79 and 0.106±0.73 respectively). Hanafi *et al.* (2008) increased MDA ($p< 0.01$) and decreased NO ($p<0.01$),CAT ($p<0.01$) and total anti-oxidant capacity in periparturient buffaloes in Egypt. The adaptation to negative energy balance during transition period in various tissues, primarily NEFA leads to oxidation in intensified manner which eventually result into formation of reactive oxygen species (Herdt, 2000). The intermediate product of lipid peroxidation, MDA level is increased during this period suggesting higher level of lipid peroxidation (Aviram *et al.*1999). The SOD activity declines with the progression of parturition and slight increase has been noted from 45 days postpartum (0.23±0.006 and 0.28±0.06 µmoles/mg of Hb). Superoxide dismutase is considered as first line of defense against oxidative stress radicals, by converting superoxide radical to hydrogen peroxide and is considered as important antioxidant marker (Adela *et al.*, 2010). A significant decrease in superoxide dismutase is evident of higher oxidative stress during periparturient period in dairy cattle (Castillo *et al.*¸2006). Konvicna *et al.*(2015) observed that mean values of GPx was lower in first week postpartum than mild- lactating cattle. Glutathione peroxidase plays an important role in anti oxidant defense mechanism by reducing the hydrogen peroxide into water and alcohol. Glutathione is used as a reducing agent in the reaction (Adela *et al.*, 2010). From the findings of level of GPx during calving and immediate post- partum time, one can say that animal is having marked reduction in antioxidant markers and increase in reactive oxygen species during transition period.

5.7 Effect of transition feed on the metabolic profile of buffaloes.

In our study, transition feed (crushed maize, wheat bran, rice bran, oil cakes and essential vitamins viz A, D and E etc) was given to a group of buffaloes (n=10) and the comparison in their metabolic profiles and genes expression was done with control group.

5.7.1 Effect of feed on body condition score and milk yield of buffaloes during transition period.

Our study showed non-significant difference ($p<0.05$) in BCS of buffaloes between feed and control group. Similar findings were seen in the study of Uddin *et al.* (2013) which gave concentrate feed (comprises of cotton seed cake and essential components) to the transition cows and had non-significant difference among the groups. But the rate of BCS loss between predefined intervals was less than control group. Present study was in greater agreement to these findings.

Milk yield was significantly ($p<0.05$) greater in feed group (8.35±0.183 kg/day) as compared to control group (6.25 ± 0.2 kg/day). Our findings were in agreement with findings of Jabbar *et al*. (2008), milk yield was increased significantly ($p<0.05$) with inclusion of cotton seed cake in the concentrate diet given to the cows. The increase in the milk production is related to high intake of rumen bypass protein present in concentrate diet (Kunju *et al.,* 1992).

5.7.2 Effect of transition feed on haemato-biochemical parameters of buffaloes.

In our study, there was significant increase ($p<0.05$) in the level of total WBC, lymphocytes, RBC and Hb in feed group in comparison to control group. In findings of Sadeghian *et al*. (2012), there was proliferation of lymphocytes seen on supplementation of vitamin E and selenium in ration of dairy cattle. Selenium is considered as an important factor in proliferation of lymphocytes and transferin receptors (Phigetti *et al.,* 1998). Meglia *et al.* (2018) conducted a study on HF cows during transition period provided with ration of grass silage, concentrates and hay. The total white blood cell counts (WBC), RBC and haemoglobin were significantly ($p < 0.05$) higher during calving than before and after calving in feeding group as compared to control group. Little *et al*. (2016) suggested that neutrophil from cows having silage plus concentrate diet having higher phagocytic index at 1 and 2 week postpartum than those provided with silage diet only.

Tienken *et al*. (2015) observed that group of cows provided with high concentrate level in the basic ration consumed more DM, had higher energy intakes and a more

positive calculated energy prepartum and therefore less increase in the level of NEFA, BHBA postpartum.

Reist *et al*. (2003) did a study on the effect of 30% and 50% concentrate diet of total dry matter intake in dairy cattle during the peri-parturient period. Glucose level decreased and NEFA and BHBA level were increased in groups given 30% of concentrate diet than 50% diet. But cholesterol level maintained in higher percentage of concentrate diet. Depression in calcium level was less in 50% concentrate group. Our findings were similar to the findings of Reist *et al*. (2013) where the level of BHBA was significantly ($p<0.05$) less than control group (306.86±2.33 and 373.50±11.23 respectively). Calcium (8.20±0.04; feed and 8.08±0.04; control) and glucose level (56.58±0.36; feed and 56.58±0.36; control) are significantly ($p<0.05$) higher than control group along the time period of transition time.

Goff *et al.* (2008) suggested that supplementation of dry cow with 20–30,000 IU Vitamin-D/day in the diet helps in prevention of milk fever and increases the intestinal absorption of calcium. Our formulated diet contained required amount of vitamin-D which were given during transition period. And it showed significant increase ($p<0.05$) in the level of calcium in feed group when compared to control one. Negative Dietary cation and anion difference helps in prevention of milk fever and is maintained when more percentage of anion salts (chloride, sulphur) and less percentage of (cation) sodium and potassium are present in feed (Goff *et al*., 2008). Our feed formulated with greater percentage of anion salts (magnesium sulphate and sodium chloride). Martens *et al*. (2000) suggested that magnesium plays an important role in the activity of parathyroid hormone which eventually controls the level of calcium in plasma. Therefore, magnesium does play a vital role in prevention of milk fever in dairy cattle.

5.7.3 Oxidative profile in feeding and control groups during transition period.

In our feed, there were appropriate amount of vitamin (E, A and D) and essential minerals (copper, zinc, calcium etc) present. The oxidative stress parameter (MDA) was significantly less in feed group than ($p<0.05$) control group (5.31±0.04 and 5.59±0.04 respectively). Similarly the anti-oxidants were significantly increased ($p<0.05$) in feed

group (SOD; 40.73±0.37 and 37.91±0.48), showing great defence against reactive oxygen species. Similar findings were seen in the study of Dimri *et al*. (2010), showed significantly ($p<0.05$) less lipid peroxidation during the transition period in water buffaloes and increased level of superoxide dismutase enzymes in group of cattle supplemented with vitamin and selenium along with essential trace minerals.

Superoxide dismutase is copper and zinc dependent enzyme which causes desmutation of reactive hydrogen peroxide radical and act as first line of defense against reactive oxygen species (Adela *et al*., 2010). Cristol *et al.* (1997) reposted that exogenous administration of Vitamin E restored the low level of RBC vitamin E towards the normal level. Vitamin E is considered as lipophilic chain breaking antioxidant that breaks lipid peroxidation reaction (Cristol *et al.*, 1997)

5.7.4 Assaying of cytokines during transition period in feed and control groups.

In our study, there was significant decrease ($p<0.05$) in the level of pro-inflammatory cytokines in feed group with respect to control group. Similar findings were seen in the study of Kokou *et al*. (2017) in which there was increase in the level of anti-inflammatory mediators in the group of cattle given graded from of soyabean concentrate diet. Galyean *et al.* (1999) reported that copper and zinc supplemented cattle during pregnancy had higher level of TNF-alpha level in plasma than those who were on basal diet.

Wang *et al*. (2020) did a trial on the effect of replacement of protein source with soya protein and observed that the mRNA level of anti-inflammatory cytokines (TGF-β, 43 IL-10, epinecidin, MHCIIβ and hepcidin) were upregulated and pro-inflammatory cytokines were downregulated (TNF-alpha, IL-10, INF-gamma, IL-12P40). Similar findings were present in our study, there was comparatively less upregulation of TNF-alpha and INF-gamma expression level in feed group when compared with control one.

5.7.5 Effect of plane of nutrition in the expression level of hepatic based genes during transition period in buffaloes

Our study showed a marked decrease in the expression level of hepatic based genes during transition period in buffaloes. The feeding group showed significant less

expression level ($p<0.05$) than in control group. Similar findings were found in the study of Loor *et al*. (2006), in which the comparison between two groups of cattle were made ad-libtum and feed restricted diet. Feed restricted group has got more upregulation of (CPT1A, ADIPOR2), gluconeogenesis (PC), and cholesterol synthesis (SC4MOL) genes, indicating of more hepatic oxidation of free fatty acid. Thus more stress on the hepatic metabolism. Douglas *et al.*(2006) did a study on supplementation of moderate grain- fat supplementation at ad libtum or restricted form to cows during -21 days of pre-calving to calving. They observed a higher activity of CPT (Carnitine palmotyl transferase) in mitochondria of liver of previously fed restricted diet.CPT activity declined more rapidly in cows fed ad libtum during pre-calving time, indicating of stable hepatic metabolism and less stress during post calving time.

Khan *et al*. (2012) did a study on the mRNA expression level of lipogenic genes ((PCK1, FASN, DGAT2, SCD) and lipolytic genes ((LIPE, PNPLA2, MGLL, ADRB2, ADFP, ABHD5) in liver at -14, 7, 14, and 30 d around parturition from cows fed a control (CON; NEL = 1.34 Mcal/kg) or moderate-energy (OVER; NEL = 1.62 Mcal/kg) diet during the dry period. Cows with moderate energy based diet had lower expression of lipogenic genes after parturition. The expression decreased at day 7 after parturition which gradually increased to day 28 and substantially lowest at day 56.

From all the above mentioned studies, one can say that plane of nutrition had a great impact on the expression level of hepatic based genes and their change in the expression level can play a pivotal role in early diagnosis of the disease condition even in clinically healthy animal. These studies can make advancement in early detection of production diseases and thus prevention in the production loss to the farmers.

Chapter-VI

Summary, Conclusion and Suggestions for Future

CHAPTER-6

SUMMARY AND CONCLUSION

In our present study, it was observed that there was no evidence of clinical cases of milk fever/typical hypocalcemia in buffaloes during transition period when subjected for biochemical analysis. All these findings shows low rate of prevalence of milk fever/hypocalcemic condition in buffaloes particularly when analyzed biochemically. But 17.14% animals in Group-I (n=70), 38% animals in Group-II (n=70) and 45% animals in Group-III (n=70) were found to have hypocalcemic condition when subjected to molecular level diagnosis via quantification of selected genes (NUAK1; upregulated and NESP55; downregulated).

The clinical cases of ketosis in all three groups (Group I to Group III) of buffaloes were not found in our study. There was no positive case found in Rothera's test and urine strips used for detection of ketone bodies. The value of BHBA was also under the normal range (<1200nmol/ml) in all the three groups (Gp-I to Gp-III; 313.96±2.81nmol/ml, 397.36±1.69nmol/ml and 483.69±1.98nmol/ml respectively) of our study. cDNA samples from all the buffaloes of three groups were subjected for quantification of selected genes (Up- and down-regulated) for negative energy balance and ketotic condition. The genes for negative energy balance/ketotic condition viz CPT1A (Upregulated) in buffaloes showed a significant ($p<0.05$) increased expression from day -30 to +30 days and IGF-1 (Downregulated) showed significant ($p<0.05$) increase in downregulation from -30 to 0 days and non-significant difference was observed between 0 day and +30 days. The percentage of animals that showed positive result for negative energy balance via fold change in the expression of CPT1A and IGF-1 were 24%, 47% and 62% in group I, II and III

The prevalence of mastitis among buffaloes, animals from Gp-III were examined for MCMT and SCC, 62.85% were MCMT negative and had an average of 168.41±0.8 ($\times 10^3$/ml) somatic cell count whereas 37% were CMT (T/+) with SCC of 278±0.67 ($\times 10^3$/ml).Meanwhile, the prevalence of subclinical mastitis in buffaloes was

comparatively less than cows during early lactation .Blood samples from buffaloes (n=25) with CMT (T/+) and SCC of 278±0.67 ($\times 10^3$/ml) when subjected to qRT-PCR for genes (TNF-α and interferon gamma) regulated for inflammation condition. They showed upregulation of both the genes (3.372±0.20 and 3.09±0.3891 respectively). Whereas, buffaloes (n=45) with MCMT negative showed significantly less upregulation of TNF-alpha and interferon gamma (2.96±0.20 and 2.94±0.24) when compared to positive results.

Present study showed a marked reduction in BCS score from group I to group III during the progression of transition period. Also, buffaloes with greater loss in BCS during postpartum group (Gp-III) had a higher level of BHBA (483.69±1.98) than other two groups. With greater loss of BCS, there was an increase in the level of oxidative stress markers and reduction in the anti-oxidant defense mechanism of the body. Buffaloes with dry period shorter than 44.21±0.59 were having milk yield about 6.81±0.10 kg compared to those having dry period length above 53.83±0.48 i.e 7.92±0.87kg.

In study of 210 buffaloes during the transition period (-30, 0 and +30 days), we found that level of TNF-alpha and interferon gamma were raised significantly as the period progresses with highest value in group II. Thereafter, a significant decrease ($p<0.05$) in the level has been seen from 0 day to 4 weeks postpartum period. Buffaloes from all the three groups when subjected to qRT-PCR for genes of inflammatory condition (TNF-alpha and interferon gamma) showed increase fold of expression in both the genes from -30 to +30 days of transition period.

In haemotology, there was a significant increase ($p<0.05$) in the level of WBC (10^3/ul) with the progression of transition period, along with lymphocytes and granulocytes at the time of calving. Significant decrease ($p<0.05$) in the level of RBC, Hb and HCT was seen in our study during the calving time and postpartum period.

In biochemical analysis, parameters like albumin, total protein, glucose and A:G ratio showed significant ($p<0.05$) decrease from -30 to +30 days of parturition. There was significant ($p<0.05$) increase in the level of SGOT (U/l) from -30 to +30 days and in

BUN (mg/dl) from 0 day to +30 days. Calcium level along the transition period decreased significantly ($p<0.05$) in our study.

Oxidative stress occurs during the period of high metabolic demands which leads to imbalance of anti-oxidant defense markers and reactive oxygen species. In our study we found significant ($p<0.05$) decreased level of SOD (43.0±0.39, 39.10±0.45 and 37.97±0.36), GPx (3.16±0.03, 2.07±0.02 and 2.21±0.03) and catalase. It showed significant ($p<0.05$) increase in the mean±SE values of malondialdehyde (MDA) (nmoles of MDA produced/ g of Hb /hr) in Group-II (7.01±0.08) in comparison to Group-I and -III (6.09±0.10 and 6.31±0.13).

Effect of transition feed on buffaloes was evaluated on the basis of various parameters such as body condition score, haemato-biochemical changes, oxidative markers and molecular markers for production diseases. Blood samples were collected at different time intervals viz. -30 days, -15 days, 0 day, +15 days and +30 days of calving for analysis various blood parameters and gene expression studies to find out the impact of transition feed. Milk yield was significantly ($p<0.05$) greater in feed group (8.35±0.183 kg/day) as compared to control group (6.25±0.2 kg/day).There was significant increase ($p<0.05$) in the level of total WBC, lymphocytes, RBC and Hb in feed group. The level of BHBA was significantly ($p<0.05$) less than control group (306.86±2.33 and 373.50±11.23respectively). Calcium (8.20±0.04; feed and 8.08±0.04; control) and glucose level (56.58±0.36; feed and 56.58±0.36; control) are significantly ($p<0.05$) higher than control group along the time period of transition time.

The lipid peroxidation (LPO) was significantly decreased ($p<0.05$) in the transition feed trial group as compared to control group (5.31±0.04 and 5.59±0.04), respectively. Whereas, antioxidant markers viz. glutathione peroxidase (GPx), superoxide dismutase(SOD) and catalase were significantly increased ($p<0.05$) in the feed trial group than then control group.

The changes in the expression level of diseases specefic genes were seen. Upregulated genes viz NUAK1, CPT1A showed less expression level in the feed group

than the control one. Similarly downregulated genes viz (NESP55 and IGF-1) showed more expression level in the feed group.

Thus, the present study generated for diagnosis of production diseases in buffaloes by the expression level of selected genes (Milk fever, ketosis and mastitis)with the aid of qRT-PCR in addition to hemato-biochemical and oxidative stress analysis during transition period. Parameters studied in different time interval of transition period to make a study on the dynamics of the metabolic and physiological changes that occur during this period. Also, the nutritional intervention that can be done to elevate the metabolic adaptation of buffaloes for the subsequent lactation.

CONCLUSION

- Prevalence of milk fever was found to be17.14% in pre-calving time, 38% buffaloes during/near calving time and 45% animals in post calving time when subjected to milk fever specefic genes (NUAK1, NESP55). Although, prevalence rate from biochemical analysis was negligible in buffaloes.

- Prevalence of ketosis/ negative energy balance was found to be 66% (pre-calving), 87% (calving) and 92% (post-calving) when detected with the ketosis specefic genes (CPT1A, IGF-1).

- Animals from post-partum group showed 62.85% (MCMT negative) and had an average of 168.41 ± 0.8 ($\times 10^3$/ml) somatic cell count whereas 37% were CMT (T/+) with SCC of 278 ± 0.67 ($\times 10^3$/ml).Buffaloes (n=25) with CMT (T/+) and SCC of 278 ± 0.67 ($\times 10^3$/ml) when subjected to qRT-PCR for genes (TNF-αand interferon gamma) regulated for inflammation condition. They showed upregulation of both the genes (3.372^a ± 0.20 and 3.09^a ± 0.3891 respectively).

- Body condition score decreases as transition period progresses with greater loss of BCS during post partum period.

- Dry period length affects the milk yield of the buffaloes; shortened dry length decreases the milk yield.

- Hematological parameters like Hb and PCV show a significant decline during transition period.
- Biochemical parameters like TPP, Albumin and Glucose decrease significantly after parturition whereas BHBA mark a significant increase.
- Oxidative stress parameters like GSH and SOD decrease as transition period progresses, whereas, LPO increases significantly during calving day and post partum
- Cytokines like TNF-alpha and INF-gamma are significantly higher during calving time than any other period.
- Impact of nutritional interventions during the transition period has been seen with significant increase level of WBC, granulocytes, RBC, Hb, total protein, albumin, glucose , calcium and sodium in feed group. Whereas, decrease in the level of SGOT, GGT, BUN, Globulin has been noticed.
- Transition feed group had lower level of TNF-alpha and INF-gamma when analyzed both through ELISA and their expression level of genes.
- Impact of nutritional intervention has been seen on the molecular expression level of disease specefic genes. NUAK1 and CPT1A (upregulated genes) was less expressed and downregulated genes (NESP55 and IGF-10) were more expressed in feed group than control group.

References

REFERENCES

Abd-Elrahman, A.H., 2013. Mastitis in housed dairy buffaloes: incidence, etiology, clinical finding, antimicrobial sensitivity and different medical treatment against E. coli mastitis. *Life Science Journal*, **10** :.532-538.

Abdulkareem, T.A., 2013. Some hematological and blood biochemical attributes of Iraqi riverine buffaloes (Bubalus bubalis) around calving and postpartum periods. *Al-Anbar J. Vet. Sci*, **6**(1): 143-150.

Abuelo, A., Hernández, J., Benedito, J.L. and Castillo, C., 2013. Oxidative stress index (OSi) as a new tool to assess redox status in dairy cattle during the transition period. *Animal*, **7(**8): 1374-1378.

Adela, P., DUMITRIŢA, R., Raluca, P. and Carmen, S., 2010. Catechin-rich green tea extract modulates the oxidative status of human retinal pigment epithelial cells. *Romanian Biotechnological Letters*, **15**(1):4965.

Aebi, H., 1984. [13] Catalase in vitro. *Methods in enzymology*, **105:** 121-126.

Afshar-Hamidi, B., Maheri-Sis, N., Sadegipanah, A., Asri-Rezaei, S. and Gorbani, A., Effects of body condition score and parity on oxidative stress indicators of periparturient Iranian dairy buffaloes. *Indian J Dairy Sci*, **72**(4): 390-396.

Aggarwal, A., Chandra, G. and Singh, A.K., 2012. Heat shock protein 70, oxidative stress, and antioxidant status in periparturient crossbred cows supplemented with α-tocopherol acetate. *Tropical animal health and production*, **45**(1): 239-245.

Akamatsu, H.; Saitoh, Y.; Serizawa, M.; Miyake, K.; Ohba, Y.; Nakashima, K., 2007: Changes of serum 3-methylhistidine concentration and energy-associated metabolites in dairy cows with ketosis. Journal of Veterinary Medical Science **69**: 1091–1093

Almeida, P.E., Weber, P.S., Burton, J.L., Tempelman, R.J., Steibel, J.P. and Zanella, A.J. 2007. Gene expression profiling of peripheral mononuclear cells in lame dairy cows with foot lesions. *Veterinary Immunology and Immuno pathology*, **120(**3-4):234-245.

Ametaj, B.N., Bradford, B.J., Bobe, G., Nafikov, R.A., Lu, Y., Young, J.W. and Beitz, D.C., 2005. Strong relationships between mediators of the acute phase response and fatty liver in dairy cows. *Canadian Journal of Animal Science*, **85**(2):165-175.

Anantwar LG, Singh B. (1993) Epidemiology, clinic-pathology and treatment of clinical ketosis in buffaloes (Bubalus bubalis). Indian Vet J 1993; **70**:152-156.

Anitha, A., Rao, K.S., Suresh, J., Moorthy, P.S. and Reddy, Y.K., 2011. A body condition score (BCS) system in Murrah buffaloes. *Buffalo Bull*, **30**(1): 79-96.

Annen, E.L., Collier, R.J., McGuire, M.A. and Vicini, J.L., 2004. Effects of dry period length on milk yield and mammary epithelial cells. *Journal of Dairy Science*, **87**: E66-E76.

Antunović, Z., Novoselec, J., Šperanda, M., Steiner, Z., Ćavar, S., Pavlović, N., Lendić, K.V., Mioč, B., Paćinovski, N. and Klir, Ž., 2017. Monitoring of blood metabolic profile and milk quality of ewes during lactation in organic farming.*Mljekarstvo/Dairy*, **67**(4).

Ashmawy, N.A., 2015. Blood metabolic profile and certain hormones concentrations in Egyptian buffalo during different physiological states. *Asian Journal of Animal and Veterinary Advances*, **10**(6): 271-280.

Atashi, H., Zamiri, M.J. and Dadpasand, M., 2013. Association between dry period length and lactation performance, lactation curve, calf birth weight, and dystocia in Holstein dairy cows in Iran. *Journal of dairy science*, **96**(6):.3632-3638.

Ate, I.U., Rekwot, P.I., Nok, A.J. and Tekdek, L.B., 2009. Haematological values of cows during third trimester of pregnancy and early lactation in settled cattle herds

in Zaria, Northern Nigeria. *African Journal of Biomedical Research*, **12**(3):..225-231.

Aviram, M., Rosenblat, M., Billecke, S., Erogul, J., Sorenson, R., Bisgaier, C., Newton, R., La Du, B., 1999. Human serum paraoxonase (PON1) is inactivated by oxidized low density lipoprotein and preserved by antioxidants. Free Radic. Biol. Med. **26**: 892–904

Bachman, K. C. 2002. Milk production of dairy cows treated with estrogen at the onset of a short dry period. J. Dairy Sci. **85**:797–803

Barletta, R.V., Maturana Filho, M., Carvalho, P.D., Del Valle, T.A., Netto, A.S., Rennó, F.P., Mingoti, R.D., Gandra, J.R., Mourão, G.B., Fricke, P.M. and Sartori, R., 2017. Association of changes among body condition score during the transition period with NEFA and BHBA concentrations, milk production, fertility, and health of Holstein cows. *Theriogenology*, **104**:30-36.

Bell, A.W., 1995. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J. Anim. Sci. **73**: 2804

Bernabucci, U., Ronchi, B., Lacetera, N. and Nardone, A., 2005. Influence of body condition score on relationships between metabolic status and oxidative stress in periparturient dairy cows. *Journal of dairy science*, **88**(6): 2017-2026.

Bertoni, G., Trevisi, E. and Lombardelli, R., 2009. Some new aspects of nutrition, health conditions and fertility of intensively reared dairy cows. *Italian Journal of Animal Science*, **8(***4):491-518.

Bhatti, S.A., Sarwar, M., Khan, M.S. and Hussain, S.M.I., 2007. Reducing the age at first calving through nutritional manipulations in dairy buffaloes and cows: a review. *Pakistan Veterinary Journal*, **27**(1): 42.

Bhimte, A., Singh, L.K., Balamurugan, B., Lakhani, N., Maurya, V.P., Bharti, M.K., Kumar, A., Singh, G. and Sarkar, M., 2018. Amelioration of transition stress by

supplementation of antioxidant (Vitamin E), trace minerals (Selenium, copper, zinc) with increased energy allowance in crossbred cows.

Bionaz, M., Trevisi, E., Calamari, L., Librandi, F., Ferrari, A. and Bertoni, G. (2007): Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. J. Dairy Sci. **90**: 1740–1750.

Block, S.S., Butler, W.R., Ehrhardt, R.A., Bell, A.W., Van Amburgh, M.E. and Boisclair, Y.R., 2001. Decreased concentration of plasma leptin in periparturient dairy cows is caused by negative energy balance. *Journal of Endocrinology*, **171**(2):339-348.

Breier, B.H., 1999. Regulation of protein and energy metabolism by the somatotropic axis. *Domestic animal endocrinology*, **17**(2-3):.209-218.

Capuco, A. V., D. L. Wood, R. Baldwin, K. McLeod, and M. J. Paape. 2001. Mammary cell number, proliferation, and apoptosis during a bovine lactation: Relation to milk production and effect of bST. J. Dairy Sci. **84**:2177–2187

Castillo, C., Hernandez, J., Valverde, I., Pereira, V., Sotillo, J., Alonso, M.L. and Benedito, J.L., 2006. Plasma malonaldehyde (MDA) and total antioxidant status (TAS) during lactation in dairy cows. *Research in veterinary Science*, **80**(2): 133-139.

Chikazawa, S. and Dunning, M. D. (2016): A review of anaemia of inflammatory disease in dogs and cats. J. Small Anim. Pract**. 57**:348–353.

Colakoglu, H.E., Yazlik, M.O., Kaya, U., Colakoglu, E.C., Kurt, S., Oz, B., Bayramoglu, R., Vural, M.R. and Kuplulu, S., 2017. MDA and GSH-Px activity in transition dairy cows under seasonal variations and their relationship with reproductive performance. *Journal of veterinary research*, **61**(4): 497-502.

Cristol, J. P., J. Y. Bosc, S. Badiou, M. Leblanc, R. Lorrho, B. Descomps, and B. Canaud. "Erythropoietin and oxidative stress in haemodialysis: beneficial effects of vitamin E supplementation." *Nephrology, dialysis, transplantation: official*

publication of the European Dialysis and Transplant Association-European Renal Association 12, no. **11** (1997): 2312-2317.

Dang, A.M.A., 2017. Blood neutrophil dynamics in periparturient Murrah buffaloes. *Indian J Dairy Sci*, **70**(5): 571-576.

Daramola, J.O.; Adeloye, A.A. and Soladoye, O. (2004): Changes in some hematological and biochemical parameters during pre-partum and postpartum periods in female West African dwarf goats. J. Agric. Sci. **10**: 53-60

Delfino, N.C., de Aragão Bulcão, L.F., Alba, H.D.R., da Silva Oliveira, M.X., de Queiroz, F.P.S., de Carvalho, G.G.P., Rennó, F.P. and de Freitas, J.E., 2018. Influence of body condition score at calving on the metabolic status and production performance of Murrah buffaloes (Bubalus bubalis) during the transition period. *Asian-Australasian journal of animal sciences*, **31**(11): 1756.

Deshpande S M, Mantri A M, Talvelkar B A and Deshmukh B T. 1998.Studies on microelements during gestation and early postpartum period in Gir and crossbred cows. Indian Journal of Dairy Science **51**(5): 275- 79

Dhakal, I.P., 2006. Normal somatic cell count and subclinical mastitis in Murrah buffaloes. *Journal of Veterinary Medicine, Series B*, **53**(2):.81-86.

Dimri, U., Ranjan, R., Sharma, M.C. and Varshney, V.P., 2010. Effect of vitamin E and selenium supplementation on oxidative stress indices and cortisol level in blood in water buffaloes during pregnancy and early postpartum period. *Tropical animal health and production*, **42**(3):405-410.

Douglas, G. N.; Overton, T. R.; Bateman, H. G.; Dann, H. M.; Drackley, J. K., 2006: Prepartal plane of nutrition, regardless of dietary energy source, affects periparturient metabolism and dry matter intake. Journal of Dairy Science **89**: 2141–2157

Drackley, J. K., 1999: Biology of dairy cows during the transition period: the final frontier? Journal of Dairy Science **82**: 2259–2273

Duffield, T., 2000. Subclinical ketosis in lactating dairy cattle. *Veterinary Clinics of North America: Food Animal Practice*, **16**(2):231-253.

El-Deeb, W.M. and El-Bahr, S.M., 2017. Biomarkers of ketosis in dairy cows at postparturient period: acute phase proteins and pro-inflammatory cytokines. *Veterinarski arhiv.*, **87**(4): 431-440.

Elitok, B., M. Kabu and O.M. Elitok, 2006. Evaluation of liver function tests in cows during periparturient period. F U Saglik Bil Dergisi, **20**: 205-209.

Elsayed, D.H., Abdelrazek, H.M., El Nabtiti, A.A., Mahmoud, Y.K. and Abd El-Hameed, N.E., 2019. Associations between metabolic profiles, post-partum delayed resumption of ovarian function and reproductive performance in Egyptian buffalo: Roles of IGF-1 and antioxidants. *Animal reproduction science*, **208**: 106134.

Emig D, Salomonis N, Baumbach J, Lengauer T, Conklin BR, Albrecht M., 2017 AltAnalyze and DomainGraph: analyzing and visualizing exon expression data. Nucleic acids research. 29;**38** :W755-62.

Enevoldsen, C., 1993. Nutritional risk factors for milk fever in dairy cattle: Meta-analysis revisited. *Acta Veterinaria Scandinavica. Supplementum (Denmark)*.

Erb, H.N. and Grohn, Y.T., 1988. Epidemiology of metabolic disorders in the periparturient dairy cow. *Journal of Dairy Science*, **71**(9):2557-2571.

Esposito, G., Irons, P.C., Webb, E.C. and Chapwanya, A., 2014. Interactions between negative energy balance, metabolic diseases, uterine health and immune response in transition dairy cows. *Animal reproduction science*, **144**(3-4): 60-71.

Fayed, H., Ghanem, M.M., Abdel-Raof, Y.M. and El-Attar, H.M., 2018. Oxidative stress and antioxidant activity in buffaloes with postparturient hemoglobinuria. *Benha Veterinary Medical Journal*, **34**(3):57-63.

Fendri K, Patten SA, Kaufman GN, Zaouter C, Parent S, Grimard G, Edery P, Moldovan F., 2013 June 1. Microarray expression profiling identifies genes with altered

expression in Adolescent Idiopathic Scoliosis. European Spine Journal. 1; **22** (6):1300-11.

Fikadu, W., Tegegne, D., Abdela, N. and Ahmed, W.M., 2016. Milk fever and its economic consequences in dairy cows: a review. *Global Vet*, **16,** 441-452

Fiore, E., Arfuso, F., Gianesella, M., Vecchio, D., Morgante, M., Mazzotta, E., Badon, T., Rossi, P., Bedin, S. and Piccione, G., 2018. Metabolic and hormonal adaptation in Bubalus bubalis around calving and early lactation. *PloS one*, **13(**4).

Fiore, E., Gianesella, M., Arfuso, F., Giudice, E., Piccione, G., Lora, M., 2014. Glucose infusion response on some metabolic parameters in dairy cows during transition period. *Archives Animal Breeding*, **57:** 1–9.

Galyean, M.L., Perino, L.J. and Duff, G.C., 1999. Interaction of cattle health/immunity and nutrition. *Journal of Animal Science*, **77**(5):1120-1134.

Găvan, C., Retea, C. and Motorga, V., 2010. Changes in the hematological profile of Holstein primiparous in periparturient period and in early to mid lactation. *Scientific Papers Animal Science and Biotechnologies*, **43**(2):244-246.

Ghadge, H.R., Bhikane, A.U., Awaz, K.B. and Syed, A.M., 2010. MINERALS PROFILE OF LACTATING MARATHWADI BUFFALOES. *Veterinary Practitioner*, **11**(2): 156-157.

Gobiraju, S., Vasan, P., Purushothaman, M.R., Rajendran, K., Senthilkumar, S., Sakthivel, P.C., Gomathi, G. and Ajantha, A., Dietary Cation–Anionic Balance (DCAB) to Prevent Milk Fever.

Goff, J.P. and Horst, R.L.,1997. Physiological changes at parturition and their relationship to metabolic disorders1, 2. *Journal of Dairy Science*, **80**(7):1260-1268

Goff, J.P., 2008.Transition period management and nutrition problems - A few solutions. In *Proceedings of the High Plains Dairy Conference, Albuquerque, NM* (123-138).

Golla, N., Chopra, A., Boya, S., Kumar, T.V.C., Onteru, S.K. and Singh, D., 2019. High serum free fatty acids and low leptin levels: Plausible metabolic indicators of negative energy balance in early lactating Murrah buffaloes. *Journal of cellular physiology*, **234**(6): 7725-7733.

González Maldonado, J., 2018. Impacts of the injections of vitamins C and E on reproductive events in Holstein dairy cattle during a synchronized estrus.

Graber, M., S. Kohler, T. Kaufmann, M. G. Doherr, R. M. Bruckmaier, and H. A. van Dorland. 2010. A field study on characteristics and diversity of gene expression in the liver of dairy cows during the transition period. J. Dairy Sci. **93**:5200–5215.

Grum, D. E., Drackley, J. K., Younker, R.S., LaCount, D. W. and Veenhuizen, J. J., 1996. Nutrition during the dry period and hepatic lipid metabolism of periparturient dairy cows, Journal of Dairy Science, **79**: 1850 – 1864.

Grummer, R. R., 1995: Impact of changes in organic nutrient metabolism on feeding the transition dairy cow. Journal of Animal Science **37**: 2820–2833.

Gustafsson, A.H., Andersson, L. and Emanuelson, U., 1995. Influence of feeding management, concentrate intake and energy intake on the risk of hyperketonaemia in Swedish dairy herds. *Preventive Veterinary Medicine*, **22**(4): 237-248.

Hafeman, D.G., Sunde, R.A. and Hoekstra, W.G., 1974. Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat. *The Journal of nutrition*, **104**(5):580-587.

Hagawane, S.D., Shinde, S.B. and Rajguru, D.N.,2009. Haematological and blood biochemical profile in lactating buffaloes in and around Parbhani city. *Veterinary World*, **2**(12):467-469

Hanafi, E.M., Ahmed, W.M., Abd El Moez, S.I., El Khadrawy, H.H. and Abd El Hameed, A.R., 2008. Effect of clinical endometritis on ovarian activity and oxidative stress status in Egyptian buffalo-cows. *Am-Eurasian J Agric Environ Sci*, **4:**530-536.

Hartwiger, J., Schären, M., Frahm, J., Kersten, S., Hüther, L., Sauerwein, H., Meyer, U., Breves, G. and Dänicke, S., 2019. Effects of a change from an indoor-based total mixed ration to a rotational pasture system combined with a moderate concentrate feed supply on immunological cell and blood parameters of dairy cows.*Veterinary sciences*, **6**(2): 47.

Hassan, F.A., Ali, M.A. and El-Tarabany, M.S., 2017. Economic impacts of calving season and parity on reproduction and production traits of buffaloes in the sub-tropics. *Environmental Science and Pollution Research*, **24**(11): 10258-10266.

Hauser, S. D., M. F. McGrath, R. J. Collier, and G. G. Krivi. 1990. Cloning and in vivo expression of bovine growth hormone receptor mRNA. Mol. Cell Endocrinol. **72**:187-200.

Heiser, A., McCarthy, A., Wedlock, N., Meier, S., Kay, J., Walker, C., Crookenden, M.A., Mitchell, M.D., Morgan, S., Watkins, K. and Loor, J.J., 2015. Grazing dairy cows had decreased interferon-γ, tumor necrosis factor, and interleukin-17, and increased expression of interleukin-10 during the first week after calving. *Journal of dairy science*, **98**(2): 937-946.

Herdt, T.H., 2000. Ruminant adaptation to negative energy balance: influences on the etiology of ketosis and fatty liver. Vet. Clin. North Am. Food Anim. Pract. 16, 215–231.*l*, **7**(8):1374-1378.

Hernández-Castellano, L.E., Hernandez, L.L. and Bruckmaier, R.M., 2020. Endocrine pathways to regulate calcium homeostasis around parturition and the prevention of hypocalcemia in periparturient dairy cows. **14**(2):330-338.

Holcomb, C.S., Van Horn, H., Head, H., Hall, M., Wilcox, C. 2001. Effects of prepartum dry matter intake and forage percentage on postpartum performance of lactating dairycows. *Journal of Dairy Science*, **84**: 2051–2058.

Hulin A, Hortells L, Gomez-Stallons MV, O'Donnell A, Chetal K, Adam M, Lancellotti P, Oury C, Potter SS, Salomonis N, Yutzey KE., 2012. Maturation of heart valve cell populations during postnatal remodeling.Development.; **146** (12):dev173047.

Hussain S, Muhammad A, Saeed A, et al., 2001. Serum electrolytes in buffaloes during late pregnancy, parturition and postpartum periods. Pak Vet J; **21**:175-179.

Inazuka, F., N. Sugiyama, M. Tomita, T. Abe, G. Shioi, and H. Esumi. 2012. Muscle-specific knock-out of NUAK family SNF1-like kinase 1 (NUAK1) prevents high fat diet-induced glucose intolerance. J. Biol. Chem. **287**:16379–16389

Ingvartsen, K.L., Dewhurst, R.J. and Friggens, N.C., 2003. On the relationship between lactational performance and health: is it yield or metabolic imbalance that cause production diseases in dairy cattle? A position paper. *Livestock Production Science*, **83**(2-3), 277-308.

ISHIKAWA, Y., NAKADA, K., HAGIWARA, K., KIRISAWA, R., IWAI, H., MORIYOSHI, M. and SAWAMUKAI, Y., 2004. Changes in interleukin-6 concentration in peripheral blood of pre-and post-partum dairy cattle and its relationship to postpartum reproductive diseases. *Journal of Veterinary Medical Science*, **66**(11): 1403-1408.

Jabbar, M.A., S. Ahmad and S. Riffat, 2008. Effect of Replacing Cotton Seed Cake with Sunflower Meal in the Ration of Lactating Crossbred Cows J. Vet. Anim. Sci., **1**: 11-13

Jacob S K, Philomina P T and Ramnath.V. 2011. Electrolyte and erythrocyte profile during pregnancy and early lactation in crossbred heifers. Journal of Indian Veterinary Association **9**(2): 27-31

Jonsson, N.N., Fortes, M.R.S., Piper, E.K., Vankan, D.M., de Cisneros, J.P.J. and Wittek, T., 2013. Comparison of metabolic, hematological, and peripheral blood leukocyte cytokine profiles of dairy cows and heifers during the periparturient period. *Journal of dairy science*, **96**(4): 2283-2292.

Kabir, A., Uroog, L., Ahmad, N., Ahmad, F., Saqib, M., Badshah, N. and Khan, T.A., 2019. Etio-prevalence of environmental bacterial species causing subclinical mastitis in a cohort of buffaloes at Khyber Pakhtunkhwa. *Veterinary Sciences: Research and Reviews*, **5**(1): 25-32.

Kamath-Rayne BD, Du Y, Hughes M, Wagner EA, Muglia LJ, DeFranco EA, Whitsett JA, Salomonis N, Xu Y. Systems biology evaluation of cell-free amniotic fluid transcriptomeof term and preterm infants to detect fetal maturity. BMC medical genomics. 2015 Dec; **8** (1):67.

Karapehlivan, M., Atakisi, E., Atakisi, O., Yucayurt, R. and Pancarci, S.M., 2007. Blood biochemical parameters during the lactation and dry period in Tuj ewes. *Small Ruminant Research*, **73(**1-3):267-271.

Katoh, N., 2002: Relevance of apolipoproteins in the development of fatty liver and fatty liver-releted peripartum diseases in dairy cows. Journal of Veterinary Medical Science **64**: 293–307.

Khan, A., Mushtaq, M.H., Khan, A.W., Chaudhry, M. and Hussain, A., 2015. Descriptive epidemiology and seasonal variation in prevalence of milk fever in KPK (Pakistan). *Global Veterinaria*, **14**(4): 472-477.

Khan, A.Z. and Muhammad, G., 2005. Quarter-wise comparative prevalence of mastitis in buffaloes and crossbred cows. *Pakistan Veterinary Journal*, **25**(1):9-12.

Khan, H.M., Mohanty, T.K., Bhakat, M., Gupta, A.K., Tyagi, A.K. and Mondal, G., 2015. Effect of vitamin E and mineral supplementation on biochemical profile and reproductive performance of buffaloes. *Buffalo Bull*, **34**(1): 63-72.

Khan, M., 2012. Transition cows: hepatic endocannabinoid system gene expression in response to prepartal plane of nutrition and the effects of genetic merit on genes of lipid metabolism in adipose tissue.

Khatri P, Das D, Kaka I, et al., 2013. Influence of environmental temperature on postpartum reproductive potential of Kundhi buffaloes. J Vet Adv; **3**:139-145

Kokou, F., Sarropoulou, E., Cotou, E., Kentouri, M., Alexis, M. and Rigos, G., 2017. Effects of graded dietary levels of soy protein concentrate supplemented with methionine and phosphate on the immune and antioxidant responses of gilthead sea bream (Sparus aurata L.). *Fish & shellfish immunology*, **64**:111-121.

Koller, A., Reist, M., Blum, J.W. and Küpfer, U., 2003.Time empty and ketone body status in the early postpartum period of dairy cows. *Reproduction in Domestic Animals*, **38(**1):41-49.

Konvičná, J., Vargová, M., Paulíková, I., Kováč, G. and Kostecká, Z., 2015. Oxidative stress and antioxidant status in dairy cows during prepartal and postpartal periods. *Acta Veterinaria Brno*, **84**(2):133-140.

Koujalagi, S., Chhabra, S., Randhawa, S.N.S. and Singh, R., 2018. Effect of herbal bio choline supplementation on oxidative stress and biochemical parameters in transition dairy cows. *The Pharma Innovation J*, **7**(4): 842-847.

Kovacs, CS 2011. Calcium and bone metabolism disorders during pregnancy and lactation. *Endocrinology and Metabolism Clinics of North America* **40**: 795–826

Krishna, K.S., Suresh, K., Sreenu, M. and Sundar, N.S., 2014. PREVALENCE OF SUBCLINICAL HYPOCALCAEMIA AND SUBCLINICAL KETOSIS IN BUFFALOES. *Buffalo Bulletin (March 2014)*, **33**(1).

Kronqvist, C., Emanuelson, U., Spörndly, R. and Holtenius, K., 2011. Effects of prepartum dietary calcium level on calcium and magnesium metabolism in periparturient dairy cows. *Journal of dairy science*, **94**(3):1365-1373.

Kulkarni S S, Gaikwad N Z and Bapat S T. 2010. Effect of summer season on certain biochemical parameters in Deccani sheep. Indian Veterinary Journal **87**: 729-30.

Kulkarni, A.V., Nagvekar, A.S., Ingole, S.D., Bharucha, S.V., Kekan, P.M., Kharde, S.D. and Dagli, N.R., 2019. Interleukin 1β (IL-1 β) and protein profile during different stages of gestation in buffalo. *Buffalo Bulletin*, **38**(1): 41-48.

Kumar, A., Sindhu, N., Kumar, P., Kumar, T. and Charaya, G., 2015. Incidence and clinical vital parameters in primary ketosis of Murrah buffaloes. *Veterinary world*, **8**(9):1083.

Kumar, B. and Pachauri, S.P. (2000). Haematological profile of crossbred dairy cattle to monitor herd health status at medium elevation in Central Himalayas. Research in Veterinary Science, ***69***: 141–145.

Kumar, V., Rana, Y.S., Kumar, P., Jhambh, R. and Jain, V.K., 2018. Assessment of metabolic parameters of dairy buffaloes from an organized dairy farm during different phases of lactation. *parameters (Jain et al., 2009 and Roubies et al., 2006)*, **32**(46).

Kunju, P.J.G., A.K. Mehta and M.R. Garg, 1992. Feeding of bypass protein to crossbred cows in India on straw based ration. AJAS, **5**: 107-112

KUSHIBIKI, S. (2011): Tumor necrosis factor-α- induced inflammatory responses in cattle. Anim. Sci. J. **82**: 504-511.

Kweon, O.K., Ono, H., Osasa, K., Onda, M., Oboshi, K., Uchisugi, H. H., Kurosawa, S., Yamashina, H. and Kanagawa, H. 1986., Factors affecting serum cholesterol level of lactating Holstein cows, Japan. Journal of Veterinary. Science, **48**: 481–486.

Lange, J., McCarthy, A., Kay, J., Meier, S., Walker, C., Crookenden, M.A., Mitchell, M.D., Loor, J.J., Roche, J.R. and Heiser, A., 2016. Prepartum feeding level and body condition score affect immunological performance in grazing dairy cows during the transition period. *Journal of dairy science*, ***99***(3): 2329-2338.

Larsen, T., G. Møller, and R. Bellio. 2001. Evaluation of clinical and clinical chemical parameters in periparturient cows. J. Dairy Sci. **84:**1749–175

LeBlanc, S.,2010. Monitoring metabolic health of dairy cattle in the transition period. *Journal of Reproduction and Development*, **56**(S):S29-S35.

Li, Y., Ding, H.Y., Wang, X.C., Feng, S.B., Li, X.B., Wang, Z., Liu, G.W. and Li, X.W., 2016. An association between the level of oxidative stress and the concentrations of NEFA and BHBA in the plasma of ketotic dairy cows. *Journal of animal physiology and animal nutrition*, **100**(5): 844-851.

Little, M.W., O'Connell, N.E., Welsh, M.D., Barley, J., Meade, K.G. and Ferris, C.P., 2016. Prepartum concentrate supplementation of a diet based on medium-quality grass silage: Effects on performance, health, fertility, metabolic function, and immune function of low body condition score cows. *Journal of Dairy Science,* **99**(9):7102-7122.

Locksley, R. M., N. Killeen, and M. J. Lenardo. 2001. The TNF and TNF receptor superfamilies: Integrating mammalian biology. Cell **104**:487–501.

Lockstone HE., 2011. Exon array data analysis using Affymetrix power tools and R statistical software.Briefings in bioinformatics.;**12** (6):634-44.

Loor, J.J., Dann, H.M., Guretzky, N.A.J., Everts, R.E., Oliveira, R., Green, C.A., Litherland, N.B., Rodriguez-Zas, S.L., Lewin, H.A. and Drackley, J.K., 2006. Plane of nutrition prepartum alters hepatic gene expression and function in dairy cows as assessed by longitudinal transcript and metabolic profiling. *Physiological genomics*, ***27***(1).29-41.

Lucy, M.C., Jiang, H. and Kobayashi, Y., 2001. Changes in the somatotrophic axis associated with the initiation of lactation. *Journal of Dairy Science*, **84**:.E113-E119.

MacIver, N. J., S. R. Jacobs, H. L. Wieman, J. A. Wofford, J. L. Coloff, and J. C. Rathmell. 2008. Glucose metabolism in lymphocytes is a regulated process with significant effects on immune cell function and survival. J. Leukoc. Biol. **84**:949–957.

Mandali GC, Dave MR, Raval SK., 1993. Clinico pathological studies in post parturient paresis in buffaloes. Indian Vet J; **70**:739-740.

Marklund, S. and Marklund, G., 1974. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European journal of biochemistry*, **47**(3): 469-474.

Martens, H., Schweigel, M., 2000. Pathophysiology of grass tetany and other hypomagnesemias. Implications for clinical management. Vet. Clin. North Am. Food Anim. Pract. **16**: 339–368

Meglia, G.E., Johannisson, A., Petersson, L. and Waller, K.P., 2018. Investigation in blood Leukocytes and Neutrophils in Periparturient Dairy Cow. *Sci J of Ani and Vet Sci*, **1**(1):001-006.

Melendez, P.; Gonzalez, G.; Aguilar, E.; Loera, O.; Risco, C.; Archbald, L. F., 2006: Comparison of two estrus-synchronization protocols and timed artificial insemination in dairy cattle. Journal of Dairy Science **89**: 4567–4572.

Mili, B., Pandita, S. and Mohini, M., 2015. Effect of vitamin E supplementation on antioxidant status and selective humoral and cellular immune responses in periparturient buffaloes. *Indian Journal of Animal Sciences*, ***85*(**8): 853-855.

Miller, J.K., Brzezinska-Slebodzinska, E. and Madsen, F.C., 1993. Oxidative stress, antioxidants, and animal function. *Journal of dairy science*, **76(**9): 2812-2823.

Mills, S. E.; Beitz, D. C. & Young, J. W. 1896. Evidence for impaired metabolism in liver during induced lactation ketosis of dairy cows. J. Dairy Sci., **69**: 362-370.

Miltenburg, C., 2015. *Management of peripartum dairy cows for metabolic health and immune function* (Doctoral dissertation).

Mir, M.R., Z.A. Pampori, S. Iqbal, J.I.A. Bhat, M.A. Pal and M.A. Kirmani, 2008. Hemato-biochemical indices of crossbred cows during different stages of pregnancy. Int. J. Dairy. Sci., **3**: 154-159.

Mohamed, G.A., Abd-Elnaser, E.M. and Elsayed, H.K., 2015. Preliminary study on lipid profile with relation to total antioxidant capacity and some hematological and biochemical changes of pre-post-partum buffalo heifers at Assiut city. *Assiut Vet. Med. J*, **61**(144): 159-165.

Mushtaq, A., Qureshi, M.S., Khan, S., Habib, G., Swati, Z.A. and Rahman, S.U., 2012. Body condition score as a marker of milk yield and composition in dairy animals. *J Anim Plant Sci*, **22**(3): 169-173.

Nale, R.A., 2003. Metabolic profiling in buffaloes before and after parturition. M.V.Sc. Thesis, Maharashtra Animal and Fishery Sciences University, Nagpur.

Nava-Trujillo, H., Escalona-Muñoz, J., Carrillo-Fernández, F. and Parra-Olivero, A., 2018. Effect of Parity on Productive Performance and Calving Interval in Water Buffaloes. *Journal of Buffalo Science*, **7**(1); 13-16.

Nava-Trujillo, H., Valeris-Chacin, R., Quintero-Moreno, A. and Escalona-Muñoz, J., 2020. Milk yield at first lactation, parity, and season of calving affect the reproductive performance of water buffalo cows. *Animal Production Science*,**60**(8):1073-1080.

Nazifi, S., Ahmadi, M. R., and Gheisary, H. R. (2008). Hematological changes of dairy cows in postpartum period and early pregnancy. Comparative clinical pathology, **17**(3): 157-163.

O'Hara, E.A., Omazic, A., Olsson, I., Båge, R., Emanuelson, U. and Holtenius, K., 2018. Effects of dry period length on milk production and energy balance in two cow breeds. *animal*, **12**(3): 508-514.

Oetzel, G.R., 1991. Meta-analysis of nutritional risk factors for milk fever in dairy cattle. *Journal of Dairy science*, **74**(11) :3900-3912.

Omur, A., Kirbas, A., Aksu, E., Kandemir, F., Dorman, E., Kaynar, O. and Ucar, O., 2016. Effects of antioxidant vitamins (A, D, E) and trace elements (Cu, Mn, Se, Zn) on some metabolic and reproductive profiles in dairy cows during transition period. *Polish journal of veterinary sciences*, **19**(4).

Pacheco, H.A., da Silva, S., Sigdel, A., Mak, C.K., Galvão, K.N., Texeira, R.A., Dias, L.T. and Peñagaricano, F., 2018. Gene mapping and gene-set analysis for milk fever incidence in Holstein dairy cattle. *Frontiers in genetics*, **9**: 465.

Paiano, R.B., Lahr, F.C., Silva, L.S., Marques, D.S., Ferreira, C.A., Birgel, D.B., Bisinotto, R.S. and Birgel Junior, E.H., 2019. Haematological and biochemical profiles during the puerperium in dairy cows–Short communication. *Acta Veterinaria Hungarica*, **67**(3): 377-384.

Pande, N., Agrawal, R., Shrivastava, O.P. and Swamy, M., 2016. Alterations in haemato-metabolic status and body condition score of buffaloes during the transition period. *Journal of Livestock Science (ISSN online 2277-6214)*, **7**: 122-125.

Parmar, V.L., Panchasara, H.H., Sutaria, P., Sutaria, T.V., Sadhu, D.B. and Prajapati, H.B., 2015. Synergism of vitamin E and selenium on oxidative stress during transition period in Kankrej cattle. *The Indian Journal of Veterinary Sciences and Biotechnology*, **10**(03): 01-03.

Pathan, M.M., Latif, A., Das, H., Siddiquee, G.M., Chaudhary, S.S. and Vadodaria, V.P., 2010. Plasma trace Element changes in periparturient Mehsana buffaloes. *Indian Journal of Animal Nutrition*, **27**(2): 134-137.

Patra, M.K., Kumar, H. and Nandi, S., 2013. Neutrophil functions and cytokines expression profile in buffaloes with impending postpartum reproductive disorders. *Asian-Australasian journal of animal sciences*, **26**(10): 1406.

Pedernera, M., Celi, P., García, S.C., Salvin, H.E., Barchia, I. and Fulkerson, W.J., 2010. Effect of diet, energy balance and milk production on oxidative stress in early-lactating dairy cows grazing pasture. *The Veterinary Journal*, **186**(3): 352-357.

Piccione, G., Messina, V., Marafioti, S., Casella, S., Giannetto, C. and Fazio, F., 2012. Changes of some haematochemical parameters in dairy cows during late gestation, post partum, lactation and dry periods. *Vet Med Zoot*, **58**(1): 59-64.

Pighetti, G. M., Eskew, M. L., Reddy, C. C. & Sordillo, L. M. (1998). Selenium and vitamin E deficiency impair transferrin receptor internalization but not IL-2, IL-2 receptor, or transferrin receptor expression. *Journal of Leukocyte Biology*, **63(**1): 131-137.

Pinedo, P., Risco, C. and Melendez, P., 2011. A retrospective study on the association between different lengths of the dry period and subclinical mastitis, milk yield, reproductive performance, and culling in Chilean dairy cows. *Journal of dairy science*, **94**(1):.106-115.

Praveen, S. and Dhaarani, C.2018. Managemental approach of the cow during transition period. *International Journal of Science, Environment and Technology*, **7**(3): 2018, 950 – 954

Preisler, M.T.; Weber, P.S.; Tempelman, R.J.; Erskine, R.J.; Hunt, H. and Burton, J.L. (2000): Glucocorticoid receptor downregulation in neutrophils of peri-parturient cows. Am J Vet Res. **61**: 14-19

Purohit, G.N., Gaur, M., Saraswat, C.S. and Bihani, D.K., 2013. Metabolic disorders in the parturient buffalo. *Bubaline theriogenology*.

Radcliff, R.P., McCormack, B.L., Crooker, B.A. and Lucy, M.C., 2003. Growth hormone (GH) binding and expression of GH receptor 1A mRNA in hepatic tissue of periparturient dairy cows. *Journal of dairy science*, **86**(12): 3933-3940.

Randhawa, C.S., Randhawa, S.S. and Uppal, S.K., 2009. Plasma mineral status of buffaloes in Punjab. *Indian Journal of Animal Sciences*, **79**(10): 1024-1027.

Rautmare, S.S. and Anantwar, L.G., 1993. Prevalence, biochemistry and treatment of clinical ketosis in buffaloes. *Buffalo Bull*, **12**(4), 81-83

Rehman, S.U., 1984. Lead induced regional lipid peroxidation in brain. *Toxicology Letters*, **21** :333-337.

Reist, M., Erdin, D., Von Euw, D., Tschuemperlin, K., Leuenberger, H., Delavaud, C., Chilliard, Y., Hammon, H.M., Kuenzi, N. and Blum, J.W., 2003. Concentrate feeding strategy in lactating dairy cows: metabolic and endocrine changes with emphasis on leptin. *Journal of Dairy Science*, ***86***(5):1690-1706.

Roche, J.R., Friggens, N.C., Kay, J.K., Fisher, M.W., Stafford, K.J. and Berry, D.P., 2009. Invited review: Body condition score and its association with dairy cow productivity, health, and welfare. *Journal of Dairy Science*, **92**(12):5769-5801

Roubies N, Panouis N, Fytianou A, Katsoulos P.D, Giadinis N and Karatzias H. 2006. Effects of age and reproductive stage on certain serum biochemical parameters of Chios sheep under greek rearing conditions. Journal of Veterinary Medicine **53**: 277– 81.

Ruprechter, G., de Lourdes Adrien, M., Larriestra, A., Meotti, O., Batista, C., Meikle, A. and Noro, M., 2018. Metabolic predictors of peri-partum diseases and their association with parity in dairy cows. *Research in veterinary science*, **118**: 191-198.

Sadeghian, S., Kojouri, G. A. & Mohebbi, A. (2012). Nanoparticles of selenium as species with stronger physiological effects in sheep in comparison with sodium selenite. *Biological Trace Element Research*, **146***(*3): 302-308

Sarwar, A. and Chaudhry, M.N., 2001. Influences of Lactation and Pregnancy on Physio-Chemical Properties of Buffalo Blood: Red and White Cell Indices.*Sciences*,**4**(8): 1050-1051.

Sasaki, K., Yamagishi, N., Kizaki, K., Devkota, B. and Hashizume, K., 2014. Microarray-based gene expression profiling of peripheral blood mononuclear cells in dairy cows with experimental hypocalcemia and milk fever. *Journal of dairy science*, **97**(1): 247-258.

Sathya, A., Prabhakar, S., Arora, A.K. and Ghuman, S.P.S., 2010. Alterations in polymorphonuclear leukocyte functions during periparturient period in buffaloes. *Indian Journal of Animal Sciences*, **80**(1): 12-16.

Schroder, K., P. J. Hertzog, T. Ravasi, and D. A. Hume. 2004. Interferon-gamma: An overview of signals, mechanisms and functions. J. Leukoc. Biol. **75**:163–189.

Shahzad, M.A. and Sarwar, M., 2008. Influence of varying dietary cation anion difference on serum minerals, mineral balance and hypocalcemia in Nili Ravi buffaloes. *Livestock science***, 113**(1): 52-61.

Sharma, N., Singh, N.K., Singh, O.P., Pandey, V. and Verma, P.K., 2011. Oxidative stress and antioxidant status during transition period in dairy cows. *Asian-Australasian Journal of Animal Sciences*, **24**(4):479-484.

Shoenfeld, Y., Gurewich, Y.G.L.A., Gallant, L.A. and Pinkhas, J., 1981. Prednisone-induced leukocytosis: influence of dosage, method and duration of administration on the (5):773-778. degree of leukocytosis. *The American journal of medicine*, **71**

Singh B, Kasaralikar VR. Prevalence, biochemistry and treatment of clinical ketosis in buffaloes. Proc II World Buffalo Congress New Delhi 1988; IV:138-141.

Singh M, Randhawa SS and Randhawa CS (1994). Clinical symptoms and epidemiological studies on parturient paretic buffaloes in the state of Punjab. Indian Journal of Dairy Science **46**:564-567

Singh, R., 2015. *Prediction and management of metabolic diseases in dairy cows and buffaloes* (Doctoral dissertation, Ph. D. Dissertation. Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, India).

Singh, R., Randhawa, S.N.S. and Randhawa, C.S., 2017. Oxidative stress, Hemato-biochemical and plasma mineral profile in transition buffaloes. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences*, **87**(4): 1091-1099

Singh, R., Randhawa, S.N.S. and Randhawa, C.S., 2019. Propylene glycol as a prophylactic agent for prevention of sub clinical ketosis in buffaloes.

Singh, S.P., Mehla, R.K. and Singh, M., 2012. Plasma hormones, metabolites, milk production, and cholesterol levels in Murrah buffaloes fed with Asparagus racemosus in transition and postpartum period. *Tropical animal health and production*, **44**(8): 1827-1832.

Singh, V.K., Singh, S.K., Tripathi, A.K., Nakade, U.P., Choudhury, S., Yadav, B. and Garg, S.K., 2019. Evaluation of immuno-regulatory cytokines and negative energy balance markers drift of zebu cows during the transition period. *Biological Rhythm Research*, 1-7

Smith, A., J. V. Wheelock, and F. H. Dodd. 1967. Effect of milking throughout pregnancy on milk secretion in the succeeding lactation. J. Dairy Res. **34**:145–150.

Solangi, M.H.A., 2004. Study of the blood biochemical values during parturient paresis in buffaloes and their relationship with age, pregnancy and lactation.

Sordillo, L.M. and Raphael, W., 2013. Significance of metabolic stress, lipid mobilization, and inflammation on transition cow disorders. *Veterinary Clinics: Food Animal Practice*, **29**(2): 267-278.

Spears, J.W. and Weiss, W.P., 2008. Role of antioxidants and trace elements in health and immunity of transition dairy cows. *The Veterinary Journal*, **176**(1):70-76

Ster, C., Loiselle, M.C. and Lacasse, P., 2012. Effect of postcalving serum nonesterified fatty acids concentration on the functionality of bovine immune cells. *Journal of Dairy Science*, **95**(2):708-717.

Sucupira, M.C.A., Nascimento, P.M., Lima, A.S., Márcia de Oliveira, S.G., Della Libera, A.M.M.P. and Susin, I., 2019. Parenteral use of ADE vitamins in prepartum and its influences in the metabolic, oxidative, and immunological profiles of sheep during the transition period. *Small ruminant research*, **170**: 120-124.

Thirunavukkarasu, M., Kathiravan, G., Kalaikannan, A. and Jebarani, W., 2010. Prevalence of ketosis in dairy farms–A survey in Tamil Nadu.

Tienken, R., Kersten, S., Frahm, J., Hüther, L., Meyer, U., Huber, K., Rehage, J. and Dänicke, S., 2015. Effects of prepartum dietary energy level and nicotinic acid supplementation on immunological, hematological and biochemical parameters of periparturient dairy cows differing in parity. *Animals*, **5**(3):.910-933.

Trevisi, E., Jahan, N., Bertoni, G., Ferrari, A. and Minuti, A., 2015. Pro-inflammatory cytokine profile in dairy cows: consequences for new lactation. *Italian Journal of Animal Science*, **14**(3): 3862.

Tripathi, P.M., Ingole, S.D., Deshmukh, B.T., Nagvekar, A.S. and Bharucha, S.V., 2010. Serum lipid profile during lactation in buffalo. *Indian Journal of Animal Sciences*, **80**(3), 217-219.

Uddin, PH, Rahman, A, Khan, J, khan, BD, Muhammad, D, Farid, K, Anwar, K, Ullah, S e Ali, Z, 2013. Effect of Cotton Seed Cake on Cattle Milk Yield and Composition at Livestock Research and Development Station Surezai, Peshawar, Pakistan. Pakistan Journal of Nutrition, 12, 468-475.

Vala, K.B., Dhami, A.J., Kavani, F.S., Parmar, S.C. and Pathan, M.M., 2018. Effect of Peripartum Nutritional Supplementation on Plasma Profiles of Macro Minerals and Postpartum Fertility in Jaffarabadi Buffaloes. *THE INDIAN JOURNAL OF VETERINARY SCIENCES AND BIOTECHNOLOGY*, **14(**01): 22-27.

Van Hoeij, R.J., Dijkstra, J., Bruckmaier, R.M., Gross, J.J., Lam, T.J.G.M., Remmelink, G.J., Kemp, B. and Van Knegsel, A.T.M., 2017. The effect of dry period length and postpartum level of concentrate on milk production, energy balance, and plasma metabolites of dairy cows across the dry period and in early lactation. *Journal of dairy science*, **100**(7): 5863-5879

Vanholder, T., Papen, J., Bemers, R., Vertenten, G. and Berge, A.C.B.,2015. Risk factors for subclinical and clinical ketosis and association with production parameters in dairy cows in the Netherlands. *Journal of Dairy Science*, **98**(2):880-888.

Vicini, J. L., F. C. Buonomo, J. J. Veenhuizen, M. A. Miller, D. R. Clemmons, and R. J. Collier. 1991. Nutrient balance and stage of lactation affect responses of insulin, insulin-like growth factors I and II, and insulin-like growth factor-binding protein 2 to somatotropin administration in dairy cows. J. Nutr. **121**:1656-1664.

Voet, D., and J. G. Voet. 1995. Biochemistry. 2nd ed. John Wiley and Sons, New York, NY

Waliya, P., Gupta, D.K., Mourya, A., Baghel, R.P.S., Shukla, P.C., Sharma, V. and Pathak, S., Epidemiological study of ketosis in lactating buffaloes in Jabalpur district.

Wang, Junxian, Dazhi Liang, Qihui Yang, Beiping Tan, Xiaohui Dong, Shuyan Chi, Hongyu Liu, and Shuang Zhang., 2019."The effect of partial replacement of fish meal by soy protein concentrate on growth performance, immune responses, gut morphology and intestinal inflammation for juvenile hybrid grouper (Epinephelus fuscoguttatus♀× Epinephelus lanceolatus♂)." *Fish & shellfish immunology* **98** :(619-631.

Wathes, D.C., Cheng, Z., Chowdhury, W., Fenwick, M.A., Fitzpatrick, R., Morris, D.G., Patton, J. and Murphy, J.J., 2009. Negative energy balance alters global gene expression and immune responses in the uterus of postpartum dairy cows. *Physiological genomics*, **39**(1): 1-13.

Watters, R.D., Guenther, J.N., Brickner, A.E., Rastani, R.R., Crump, P.M., Clark, P.W. and Grummer, R.R., 2008. Effects of dry period length on milk production and health of dairy cattle. *Journal of Dairy Science*, **91**(7): 2595-2603.

Weber, C., Hametner, C., Tuchscherer, A., Losand, B., Kanitz, E., Otten, W., Sauerwein, H., Bruckmaier, R.M., Becker, F., Kanitz, W. and Hammon, H.M., 2013. Hepatic gene expression involved in glucose and lipid metabolism in transition cows: Effects of fat mobilization during early lactation in relation to milk performance and metabolic changes. *Journal of dairy science*, **96**(9): 5670-5681.

Weber, C., Losand, B., Tuchscherer, A., Rehbock, F., Blum, E., Yang, W., Bruckmaier, R.M., Sanftleben, P. and Hammon, H.M., 2015. Effects of dry period length on milk production, body condition, metabolites, and hepatic glucose metabolism in dairy cows. *Journal of dairy science*, **98**(3);1772-1785.

Wildman, E.E., Jones, G.M., Wagner, P.E., Boman, R.L., Troutt Jr, H.F. and Lesch, T.N., 1982. A dairy cow body condition scoring system and its relationship to selected production characteristics. *Journal of dairy science*, **65**(3): 495-501.

Wu, W.X., Liu, J.X., Xu, G.Z. and Ye, J.A., 2008. Calcium homeostasis, acid–base balance, and health status in periparturient Holstein cows fed diets with low cation–anion difference. *Livestock Science*, **117**(1):7-14.

Youssef, M.A., El-Khodery, S.A., El-deeb, W.M. and El-Amaiem, W.E.A., 2010. Ketosis in buffalo (Bubalus bubalis): clinical findings and the associated oxidative stress level. *Tropical Animal Health and Production*, **42**(8); 1771-1777.

Zambon AC, Gaj S, Ho I, Hanspers K, Vranizan K, Evelo CT, Conklin BR, Pico AR, Salomonis N., 2012. GO-Elite: a flexible solution for pathway and ontology over-representation. Bioinformatics.; **28** (16):2209-10.

Zhang, Z. G.; Li, X. B.; Gao, L.; Li, Y. F.; Liu, G. W.; Wang, H. B.; Wang, Z., 2011: Serum antioxidant capacity of dairy cows with subclinical ketosis. The Veterinary Record **168** : 2820–2833

Vita

VITA

Name of the student	:	Savleen Kour
Father's name	:	Mr. Dilbagh Singh
Mother's name	:	Mrs. Jagjeet Kour
Nationality	:	Indian
Date of birth	:	11-06-1995
Permanent home address	:	Sector 7, GTB Nagar, Channi Rama, Jammu.
Mobile No.	:	9419231030
Email	:	savleenkour.j13bv833@gmail.com

EDUCATIONAL QUALIFICATION

Degree	**Year**	**OGPA**	**University**
B.V.Sc. and A.H.	2018	7.54	Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Jammu
M.V.Sc. (Veterinary Medicine)	2021	8.44	Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, Jammu

Title of Master's Thesis : **Molecular diagnostic markers for blood immune-metabolic indices and oxidative stress in production diseases during transition period and its management in buffaloes**

CERTIFICATE-IV

Certified that all the necessary corrections as suggested by the external examiner/evaluator and the advisory committee have been duly incorporated in the thesis entitled "**Molecular diagnostic markers for blood immune-metabolic indices and oxidative stress in production diseases during transition period and its management in buffaloes**" submitted by **Ms. Savleen Kour,** Regd. No. **J-18-MV-538.**

Dr. Neelesh Sharma
Major advisor and Chairman
Advisory Committee

No. AUJ/FVSc/VMD/2020-21/848
Date: 29-03-2021

Forwarded to Dean PGS for necessary action

(Dr. Rajiv Singh)
Professor and Head
Division of Veterinary Medicine