

Identification and validation of putative biomarkers by in silico analysis, mRNA expression and oxidative stress indicators for negative energy balance in buffaloes during transition period

Savleen Kour¹, Neelesh Sharma^{1,*}, Praveen Kumar Guttula², Mukesh Kumar Gupta², Marcos Veiga dos Santos³, Goran Bacic⁴, Nino Macesic⁴, Anand Kumar Pathak⁵, and Young-Ok Son^{6,*}

*** Corresponding Authors:**

Neelesh Sharma

Tel: +91-94192-48061,

E-mail: drneesh_sharma@yahoo.co.in

Young-Ok Son

E-mail: sounagi@jejunu.ac.kr

¹ Division of Veterinary Medicine, Faculty of Veterinary Sciences & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu, R.S. Pura, Jammu, UT of J&K 181 102, India

² Department of Biotechnology and Medical Engineering, National Institute of Technology Rourkela, Odisha 769 008, India

³ Department of Animal Sciences, School of Veterinary Medicine and Animal Sciences, University of São Paulo, Pirassununga, SP 13635-900, Brazil

⁴ Clinic for Reproduction and Theriogenology, Faculty of Veterinary Medicine, University of Zagreb, Zagreb 100 00, Croatia

⁵ Division of Animal Nutrition, Faculty of Veterinary Sciences & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu, R.S. Pura, Jammu, UT of J&K 181 102, India

⁶ Department of Animal Biotechnology, Faculty of Biotechnology, College of Applied Life Sciences and Interdisciplinary Graduate Program in Advanced Convergence Technology and Science, Jeju National University, Jeju 690756, Korea

ORCID

Savleen Kour

<https://orcid.org/0000-0002-8375-2536>

Neelesh Sharma

<https://orcid.org/0000-0003-3932-4064>

Praveen Kumar Guttula

<https://orcid.org/0000-0002-9612-8097>

Mukesh Kumar Gupta

<https://orcid.org/0000-0002-0125-0381>

Marcos Veiga dos Santos

<https://orcid.org/0000-0002-4273-3494>

Goran Bacic

<https://orcid.org/0009-0005-9484-5534>

Nino Macesic

<https://orcid.org/0000-0003-4156-9431>

Anand Kumar Pathak

<https://orcid.org/0000-0001-7214-0086>

Young-Ok Son

<https://orcid.org/0000-0003-0154-4061>

Submitted Aug 2, 2023; Revised Nov 7, 2023;

Accepted Nov 20, 2023

Objective: Transition period is considered from 3 weeks prepartum to 3 weeks postpartum, characterized with dramatic events (endocrine, metabolic, and physiological) leading to occurrence of production diseases (negative energy balance/ketosis, milk fever etc). The objectives of our study were to analyze the periodic concentration of serum beta-hydroxy butyric acid (BHBA), glucose and oxidative markers along with identification, and validation of the putative markers of negative energy balance in buffaloes using in-silico and quantitative real time-polymerase chain reaction (qRT-PCR) assay.

Methods: Out of 20 potential markers of ketosis identified by in-silico analysis, two were selected and analyzed by qRT-PCR technique (upregulated; acetyl serotonin o-methyl transferase like and down regulated; guanylate cyclase activator 1B). Additional two sets of genes (carnitine palmitoyl transferase A; upregulated and Insulin growth factor; down-regulated) that have a role of hepatic fatty acid oxidation to maintain energy demands via gluconeogenesis were also validated. Extracted cDNA (complementary deoxyribonucleic acid) from the blood of the buffaloes were used for validation of selected genes via qRT-PCR. Concentrations of BHBA, glucose and oxidative stress markers were identified with their respective optimized protocols.

Results: The analysis of qRT-PCR gave similar trends as shown by in-silico analysis throughout the transition period. Significant changes ($p < 0.05$) in the levels of BHBA, glucose and oxidative stress markers throughout this period were observed. This study provides validation from in-silico and qRT-PCR assays for potential markers to be used for earliest diagnosis of negative energy balance in buffaloes.

Conclusion: Apart from conventional diagnostic methods, this study improves the understanding of putative biomarkers at the molecular level which helps to unfold their role in normal immune function, fat synthesis/metabolism and oxidative stress pathways. Therefore, provides an opportunity to discover more accurate and sensitive diagnostic aids.

Keywords: Beta-hydroxy Butyric Acid (BHBA); Buffaloes; Gene Expression; In-silico; Ketosis; Oxidative Stress Markers

INTRODUCTION

Immediately before calving and during first few weeks of lactation, there is increased mammary gland activity and lipid mobilization in the body which eventually causes energy deprivation in dairy cattle [1]. Despite having homeostatic mechanism to maintain the basal levels of metabolic parameters, changes in their concentration and activity occur because of pregnancy and lactation demand. These changes are not necessarily indicative of disease condition but makes cattle physiologically unstable during transition period. This instability makes cattle susceptible to several metabolic disorders which eventually

compromises their productivity [2]. Dairy animals face 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.) [3]. With the progress of pregnancy, lipid peroxidation (LPO) level (oxidative stress) increases slowly and increases marginally during initial days of postpartum period [4]. Metabolic demands during transition period increase the concentration of reactive oxidative species and immune cells are very sensitive to peroxidation [5]. Natural immunosuppression occurs in most cattle during transition period, exaggerated by the factors like negative energy balance, hypocalcemia, and increased cortisol level around calving [6]. The balance to maintain the levels of peroxidation are done primarily by antioxidants viz. catalase and superoxide dismutase (SOD). Therefore, estimation of their concentration and imbalances during this period provides affirmation of tissue or membrane damage.

Dairy cattle during lactation period depend upon body reserve to meet their demands but a prolonged negative energy imbalance (NEB) is associated with the development of ketosis [7]. The subclinical form of ketosis is a common condition seen in high producing dairy cattle (SCK; 1.2 to 2.9 mmol of beta-hydroxybutyric acid (BHBA)/L of serum) [8]. 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 BHBA [9]. Concentration of liver fat after calving is an indicator of fat mobilization and may affect hepatic gene expression of enzymes involved in gluconeogenesis and fatty acid oxidation [10]. Genes associated with β -oxidation of fatty acids are carnitinepalmitoyl-transferase 1A (*CPT1A*); acyl-CoA synthetase, long chain (*ACSL1*) and acyl-CoA dehydrogenase, very long chain (*ACADVL*). Data related to response of buffaloes to the changes in metabolic demand during this period have been documented by many authors [11,12] in the form of various hemato-biochemical and oxidative parameters. Only a low incidence level of metabolic diseases has been documented in buffaloes as compared to other ruminants. However, some authors [13,14] still consider ketosis/negative energy balance as one of major metabolic disease in buffaloes during lactation. The concentration of blood metabolites like glucose, BHBA and insulin provides some information of metabolic status of the buffalo and can be used as a surveillance tool [15]. Ketosis can be monitored using blood, urine, or milk samples from transition buffaloes [16]. Urine or milk testing in a herd periodically is the easiest and cost-effective way for qualitative detection of ketone bodies but plasma BHBA concentrations and changes in mRNA expressions of selected markers associated with negative energy balance provides better understanding of

dynamic regulation of metabolism during this period [17].

Despite having documented data of various biochemical markers used as indicators of ketosis/negative energy balance condition in dairy cattle and buffaloes, genomic technologies may help in understanding of the involvement of different metabolic pathways of liver that are sensitive to nutrient partitioning and balancing during transition period. The hypothesis tested was that the concentrations of blood metabolites and mRNA expressions of selected markers indicative of negative energy balance are different between time points during transition period.

MATERIALS AND METHODS

Animals, diet and experimental design

A total of 210 she buffaloes during their transition period (-30 to +30 days) were selected randomly from dairy farms and the veterinary referral hospital, Faculty of Veterinary Sciences and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu, R.S. Pura, UT of Jammu & Kashmir, India. Animal experiment was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animal (CPCSEA), New Delhi (No.: 25/15/2018/CPCSEA). These buffaloes were categorized into three groups, on the basis of days of their transition period: Gp-I (-30 days), Gp-II (near parturition), Gp-III (+30 days). Buffaloes selected for this study had a body weight of 724.00 kg (± 60), previous lactation length of 286 days (± 7) previous lactation milk yield 1,800 to 2,058 kg (± 125), body condition score of group-I; 3.04 (± 0.04), group II; 2.914 (± 0.038); and group III; 2.8 (± 0.029) and with parity between 3rd-6th. The buffaloes were given stall feeding (containing 25 to 30 kg of dry fodder, salt lick and *ad libitum* water provided).

Sample collection

Blood samples were collected from each buffalo of all the groups on pre-decided days (a month pre-partum, close to partum and one-month post-partum) in ethylenediamine tetra-acetic acid (EDTA) contained vacutainer; VACUETTE (Cat. 455036, Greiner bio-one, Austria) for extraction of total ribonucleic acid (RNA) from peripheral blood mononuclear cells and in serum clot activator vacutainers; VACUETTE (Cat. XLGA-C5; Greiner Bio-One India Pvt. Ltd., Noida, India) for estimation of BHBA levels using enzyme linked immunosorbent assay (ELISA) kits (Immunotag; GBiosciences, St Louis, MO, USA). Blood samples collected in heparin containing vacutainers VACUETTE (Cat. 455051; Greiner bio-one, Austria) were used for estimation of oxidative biomarkers; glutathione peroxidase (GPX), LPO, SOD, and catalase. Buffaloes in mid-lactation period were considered a control group for estimation of differential gene expression

of interest. Glucose concentration in whole blood (mg/dL) was estimated with Accu-check glucometer immediately with fresh blood.

Urine collection and analysis

Urine samples from the buffaloes were collected in sterile Uricol (Cat. PW016; Himedia, Mumbai, India). Then urine analysis strips (SD Urocolor, Standard diagnostics, INC, Kyonggi, Korea) were dipped in urine for seconds and compared with corresponding color chart on the bottle label and results were read immediately. Immediately after collection, Rothera's test was performed with 1 to 2 gm of ammonium sulphate added in 5 mL of urine, 5% solution of sodium nitroprusside and concentrated ammonium hydroxide was layered over the sample to obtain purple ring within 2 to 3 min.

RNA extraction from whole blood and its gel documentation

Total RNA was extracted from EDTA preserved blood by trizol method (Invitrogen Life Technologies, Carlsbad, CA, USA). Trizol reagent (Cat. 15596018; Thermo Fisher Scientific, Ahmedbad, India) which contains various components viz guanidine isothiocyanate, phenol and isoamyl which facilitate the extraction of RNA with high yield. EDTA contained blood was diluted in phosphate buffer solution (PBS) and then layered over the HiSep LSM-1077 (Cat. LS001; HiMedia, India). Subsequent centrifugation at 2,400 rpm for 24 min at 4°C was done in a refrigerated centrifuge (Centrifuge 5430R; Eppendorf India Ltd., Chennai, India). After separation of buffy coat in 1× PBS and its centrifugation at 17,800 rpm for 10 min thrice, a clear pellet was obtained at the bottom. TRIzol (Cat. 15596026; Thermo Fisher Scientific, India) was added after separation of supernatant and mixed properly with pellet. After a 30 to 40 min. at -20°C incubation period a TRIzol, chloroform (Cat. MB109; Himedia, India) and isopropanol treatment was applied to the pellet. The pellet was subsequently washed three times with ethanol and collected by centrifuging at 7,500 rpm for 5 min. Following air drying of the pellet, 20 µL of diethyl pyrocarbonate (DEPC) treated water (Cat. R0601; Thermo Fisher Scientific, India) was added. Optical density (OD) of the final pellet was taken spectrophotometrically (Biospectrometer; Eppendorf India Ltd., India) to determine concentration and purity. Cut-off point of RNA concentration (µg/mL) recommended for cDNA synthesis was >1.0 µg/mL with ratio of 260/280 nm >1.5. Gel electrophoresis with 1% agarose of molecular grade containing 1 µg/mL of Ethidium bromide (Cat. H5041; Promega, New Delhi, India) was made with 1× TAE (Tris-acetate-EDTA) buffer (40× molecular grade, Cat. V4281; Promega, USA) treated in DEPC water (Cat. MB076; Himedia, India). Voltage 1 to 5 V/cm was applied

across the gel until the bromophenol blue migrated to appropriate distance. Resultant band (28s band and 18s) in the gel was seen with gel documentation system (Vilber; Eppendorf India Ltd., India), under the illumination of UV light and confirmed the presence of RNA.

cDNA synthesis and its validation

Total isolated RNAs were subjected to cDNA synthesis using Hi-cDNA synthesis kit (Cat. MBT076-100R; Himedia, India). A reaction mixture of 1 µg of RNA template, 2 µL of oligo-dtprimer (oligonucleotides that contained a segment of repeating deoxythymidines) (10 pmol) and 7 µL of nuclease free water in sterile polymerase chain reaction (PCR) tubes was made. This mixture was incubated for 5 min at 65°C in a thermal cycler (BioRad T100 Thermal cycler, Gurugram, India). Other components: 4 µL of RT buffer, 2 µL of 10× solution, 10 mM dNTP (Deoxynucleoside triphosphates) 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 final volume of 20 µL. This mixture was subjected to PCR conditions; one cycle of 42°C for 60 min, 70°C for 5 min and hold at 4°C in a thermal cycler. A negative control reaction mixture was made, containing all the kit components except RNA template.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a house keeping gene for validation of cDNA; forward primer (AAGGCCATCACCATCTTCCA) and reverse primer (CCACTACATACTCAGCACCAGCAT). The reaction mixture totaled 20 µL containing 10 µL of PCR pre-mix (Thermo Scientific, India), 2 µL of cDNA, 0.3 to 0.5 µL of GAPDH forward and reverse primer (10 pm each) and nuclease free water to make the final volume in sterile PCR tubes. These reactions were subjected to PCR cyclic conditions and gel electrophoresis for the validation of cDNA products when compared with negative control (Table 1).

Microarrays and quantitative real-time reverse transcription polymerase chain reaction

In-silico analysis of molecular markers for ketosis

i) Datasets 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

Table 1. PCR cycling condition for validation of cDNA with GAPDH primer

Step	Temperature (°C)	Time	Cycle
Initial denaturation	94	3 min	1
Denaturation	94	30 s	35
Annealing	58.9	30 s	35
Extension	72	45 s	35

PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

liver of healthy cows of early postpartum and 14 samples from cows in early postpartum ketosis 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), their 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 [18].

Gene expression and clustering analysis

The analyses of gene expression levels and gene ontology (GO) annotation were carried out with GO-Elite software, using default options [19]. Microarray expression values were reported as log₂ values. 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 [20]. Differentially expressed genes were subjected to hierarchical clustering to identify gene clusters within the control and test groups [21]. Gene-set enrichment analysis and comparison was executed using the GO-Elite in AltAnalyze, where only terms with an false discovery rate adjusted enrichment p<0.05 was considered for further evaluation.

Identification 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 [22].

Quantitative real time PCR for quantification of expression of gene interest

Real time PCR helps in the amplification of the targeted molecule in real time. It was conducted by quantitative real time PCR cyclo- MyGo mini (MyGo 002). A reaction mixture with 10 µL Hi-SYBr master mix (Cat. MBT074-100R; Himedia, India), 0.5 µL of forward and reverse primer of

Table 2. qRT-PCR cycling conditions

Steps	Temperature (°C)	Cycles	Time
Pre incubation	95	1	5 min
Amplification (3 steps)	95	40	20 s
Annealing	Varied by gene of interest	40	15 s
	72	40	15 s
Pre melt	95	1	5 s
Melting	70	1	1 min
Melting	95	1	40 s
Cooling	40	1	30 s

qRT-PCR, quantitative real time-polymerase chain reaction.

gene of interest and nuclease free water up to 20 µL volume was made and subjected to qRT-PCR reaction condition (Table 2). For calculation of C_q values (quantification cycles), comparative CT method also referred to as the 2^{-ΔΔC_t} method was used with internal gene control; GAPDH and buffaloes in mid-lactation as control group [23].

Oxidative stress markers

Various oxidative stress markers such as SOD, GPx, LPO, and catalase were evaluated from transition buffaloes (Group-I, II, and III). For evaluation, 5 mL of blood in heparin containing vacutainer was taken and centrifuged at 3,000 rpm for 10 min. After that, plasma was stored and left over red blood cells (RBC) lysate was used to determine the anti-oxidant status. Washing of RBC lysate was done with normal saline by diluting in a ratio of 1:1 and centrifuged for 10 min at 3,000 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.

Lipid peroxidation

Lipid peroxidation in erythrocytes was determined by the evaluation of malondialdehyde (MDA) production by the method of [24]. In brief, 1 mL of 33% RBC haemolysate was taken and added to 1 mL of 10% w/v of trichloroacetic acid. After thorough mixing, mixture was centrifuged at 3,000 rpm for 10 min and supernatant was extracted. To 1 mL of the extracted supernatant added 1 mL of 0.67% w/v of thio-barbituric acid and kept in water bath for 10 min, cooled and diluted with 1 mL of distilled water. The same reagents were used except the haemolysate for control samples. Absorbance was noted at 535 nm in the spectrophotometer (Biospectrometer; Eppendorf India Ltd., Country).

Calculation

Calculations were done using the extinction co-efficient (EC, 13,100 M⁻¹cm⁻¹) and results expressed in mM MDA per mL of blood, using the following formula.

$$\text{LPO} \left(\text{mM} \frac{\text{MDA}}{\text{mL}} \right) = \frac{\text{OD}}{\text{EC}} \times \frac{\text{Total volume of reaction mixture} \times 1,000 \times \text{DF}}{\text{Amount of sample taken}}$$

Catalase

The plasma catalase activity was determined by the method described by Marklund and Marklund [25]. 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°C and the decrease in the absorbance was observed at every 10 s interval for 1 min at 240 nm OD. 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 .

Superoxide dismutase

The activity of erythrocytic SOD was determined by method of Hafeman et al [26]. In brief, 1.5 mL of 100 mM tris HCl 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 s up to 4 min. For the test, an 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 SOD was expressed as SOD units/mg protein.

Calculation:

SOD activity (U/mg of Hb)

$$= \frac{(\Delta E_0 - \Delta E)}{\Delta E_0} \times \frac{1}{2} \times \frac{1}{\text{g of protein in 0.01 mL}}$$

ΔE_0 = change of absorbance of pyrogallol; ΔE = change of absorbance of sample.

Glutathione peroxidase

The activity of GPx in erythrocytes was performed as per method of Loor [27]. In brief, 0.1 mL of erythrocytes was taken. To this was added 1 mL of glutathione peroxidase, 1 mL 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. The solution was incubated for 5 min. After the incubation, 1 mL of 125 mM hydrogen peroxidase added and incubation continued for about 3 min. 4 mL of meta-phosphoric acid was added to 1 mL of liquid from the incubation mixture. Then, 2 mL of this solution was added with 2 mL of 0.4 M sodium hypo-phosphate (NaHPO_4) and 1 mL of DTNB (5,5'-dithio-bis-2-nitrobenzoic acid) reagent. For the 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.

Calculation:

$$\text{Activity of GPx} = 10 \log \frac{C_0}{C}$$

Statistical analysis

All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) statistical software version 16 (IBM SPSS Statistics 16). Data were analyzed using analysis of variance and the Duncan's multiple range test was performed to provide significance levels ($p < 0.05$) for the difference between the means.

RESULTS

Urine analysis for detection of ketone bodies

Urine sample analysis of the selected buffaloes showed negative result for Rothera's test and urine strips (SD Urocolor) for ketone bodies when compared with their positive control (Figure 1).

Metabolic measurements

Serum level of BHBA (nmol/mL) increased significantly ($p < 0.05$) from pre-partum to post-partum period; Gp-I to Gp-III (313.96 \pm 2.81 nmol/mL, 397.36 \pm 1.69 nmol/mL, and 483.69 \pm 1.98 nmol/mL, respectively) and glucose concentrations showed significant decline ($p < 0.05$) from far off dry period to early lactation (Gp-I, 65.31 \pm 3.01; Gp-II, 59.44 \pm 1.78; and Gp-III, 53.08 \pm 1.74), although buffaloes in these groups were non-ketotic (Figure 2).

Differential expression of genes by microarray assay and qRT-PCR

In-silico analysis for ketosis markers: The distribution of the probe intensities varied from 0 to 8,000 for ketosis and 0 to 12,000 observations for healthy samples (Figure 3).

Differentially expression analysis, clustering, and principal component analysis

Compared with normal non-ketosis samples, 2,121 genes were found to be differentially expressed in the ketosis samples. Among these 1,053 were found to be upregulated and 1,068 were found to be downregulated. Principal component analysis of the two groups revealed that all ketosis samples were sharing one component, and all healthy samples were sharing other principal components along with the heat map showing log fold changes of gene and hierarchical clustering of various healthy and ketosis samples (Figures 4 and 5).

Gene ontology and pathway analysis

Gene ontology analysis revealed that some of the genes were

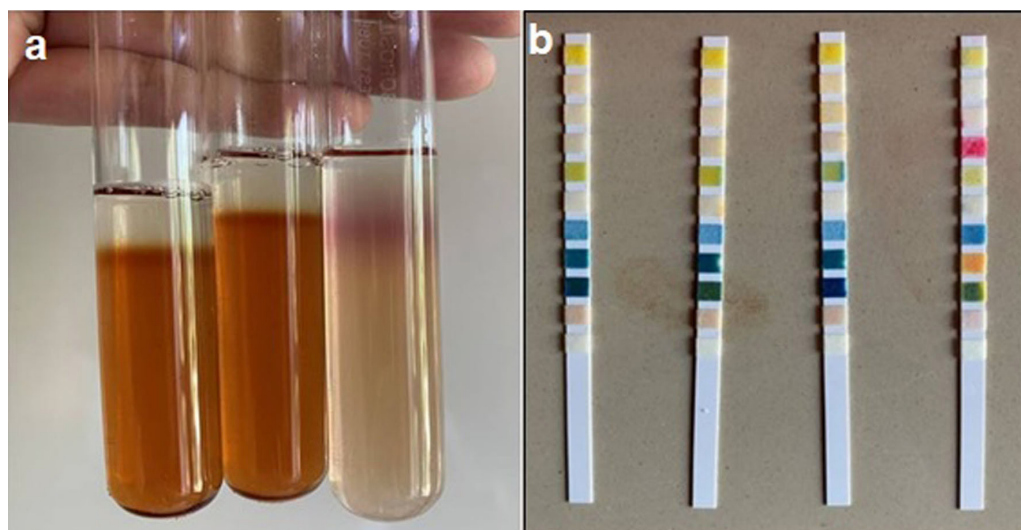


Figure 1. Urine analysis (a) Rothera's test: Negative Rothera's test result of urine samples with positive control in third test tube; (b) SD Urocolor strips for detection of ketone bodies: negative result of ketone bodies detection in urine strips (SD Urocolor) in first three strips from left to right with positive control (4th block with pink colour).

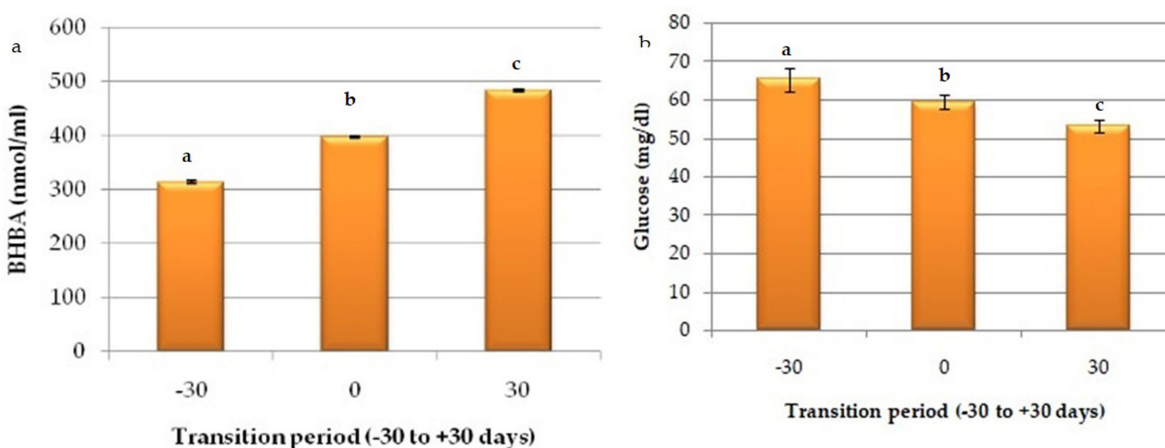


Figure 2. Graphs depicting trends of (a) beta-hydroxy butyric acid (BHBA, nmol/mL) and (b) glucose (mg/dL) during transition period (GP-I, -30 days; GP-II, near parturition; and GP-III, +30 days) in buffaloes. ^{a,c} Means with different superscripts between rows differs significantly ($p < 0.05$).

directly related to the immune response, inflammatory response, and chemotaxis and leukocyte cell-cell adhesion. Upregulated genes included interleukin-6 (*IL6*), interleukin-3 (*IL3*), C-C chemokine receptor type 1 (*CCR1*), interferon beta 1 (*IFNB1*), acetylserotonin O-methyltransferase like (*ASTML*), interleukin-12 receptor, beta 1 (*IL12RB1*), and leptin (*LEP*). Pathway analysis of the up-regulated genes was related to the immune response, with most genes involved in the cytokine-cytokine receptor interaction (Figure 6), induction, regulation of the local inflammatory response and oxidative stress related mechanisms. The group of down-regulated genes in the ketosis guanylate cyclase activator 1B (*GUCA1B*), general receptor for phosphoinositides-1 (*GRTPI*) found to involve in Glycosphingolipid biosynthesis (Figure

7), fat digestion and absorption pathways and in fatty acid β oxidation.

Marker genes identification for ketosis

A total of 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 cluster (Figure 8). The top 20 genes identified as markers in Ketosis include *IL6*, *IL3*, *CCR1*, *IFNB1*, *IL12RB1*, *ASTML*, and *LEP*. On the other end, the top 10 genes identified as marker genes in healthy samples were beta-1,3-N-acetylglucosaminyltransferase (*B3GNT5*), beta-1,4-galactosyltransferase (*B4GALT3*), and fucosyltransferase 9 (*FUT9*) (Table 3).

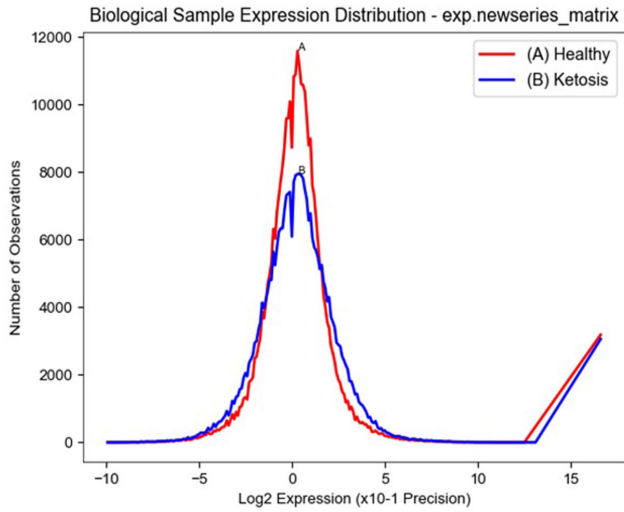


Figure 3. Distribution of microarray probe intensities in ketosis and healthy samples.

qRT-PCR analysis

Out of 20 potential markers of ketosis, two were selected (ASTML; upregulated and GUCA1B; downregulated) and analysed by qRT-PCR technique (Table 4). Additional two sets of genes (*CPT1A*; upregulated and *IGF-1*; downregulated) that were not a part of microarray assay were also subjected to qRT-PCR cycling condition (Table 4). They have role in

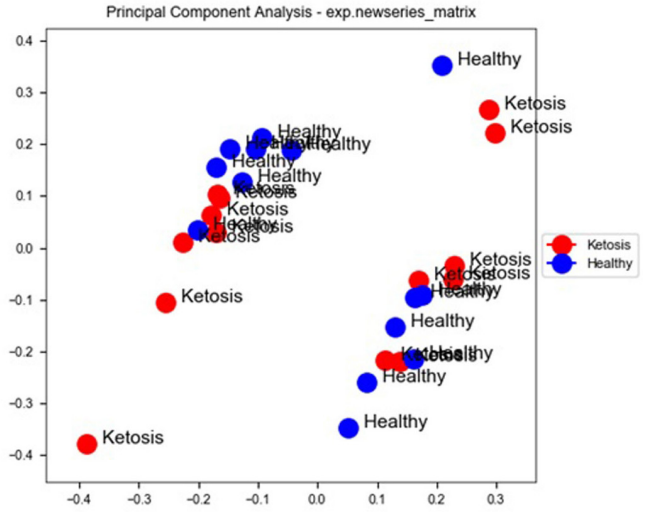


Figure 4. Principal component analysis (PCA) of ketosis and healthy samples sharing different principal components. Red spheres represent ketosis samples whereas blue spheres represent healthy samples.

β -oxidation during post-partum stimulation of hepatic fatty acid oxidation to maintain energy demand via gluconeogenesis [28] and uncoupling of growth hormone-insulin growth factor-1 (GH-IGF-1) axis causing downregulation of liver growth hormone receptor-1A (GHR 1A) and IGF-1 level,

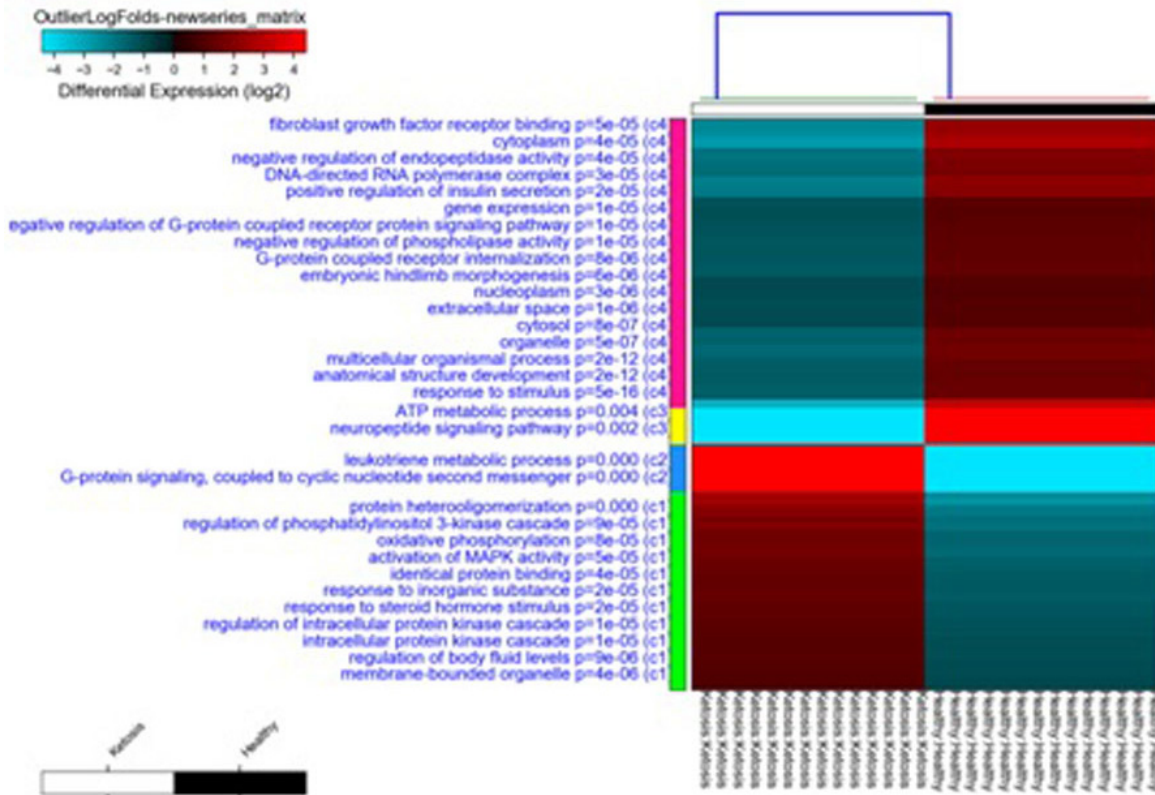


Figure 5. Heatmap showing the log-fold changes of genes and hierarchal clustering of various Ketosis and Healthy samples.

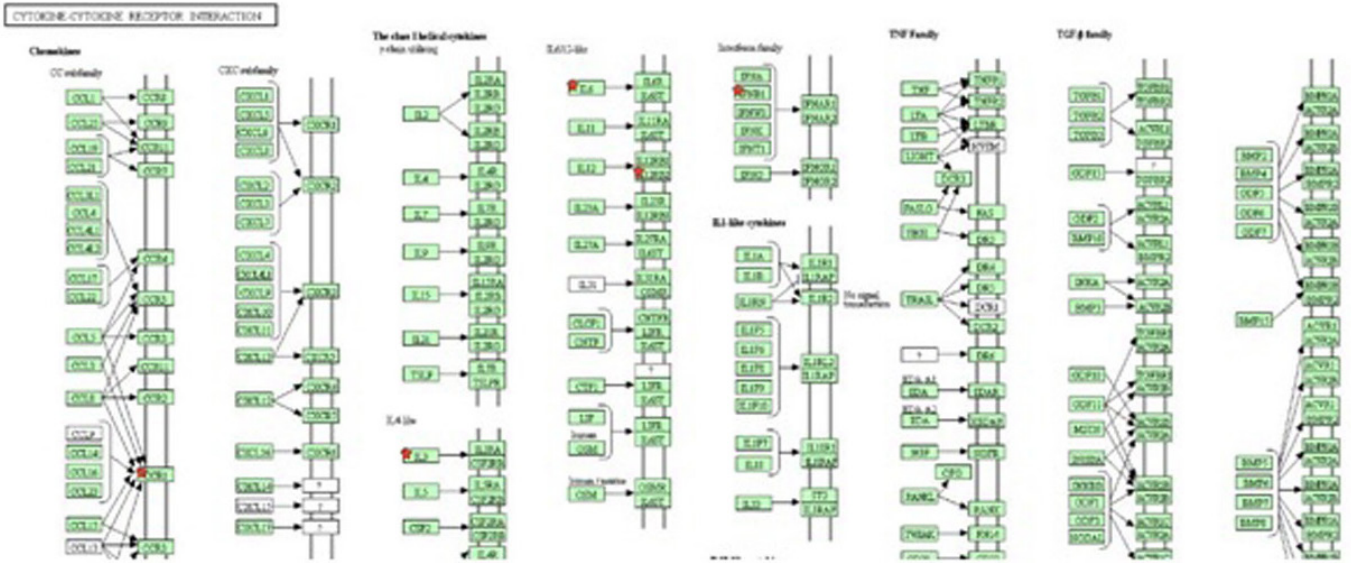


Figure 6. Participation of up-regulated genes in cytokine receptor signaling pathways. Genes in star marked were found to be up-regulated in ketosis samples.

GLYCOSPHINGOLIPID BIOSYNTHESIS - LACTO AND NEO-LACTO SERIES

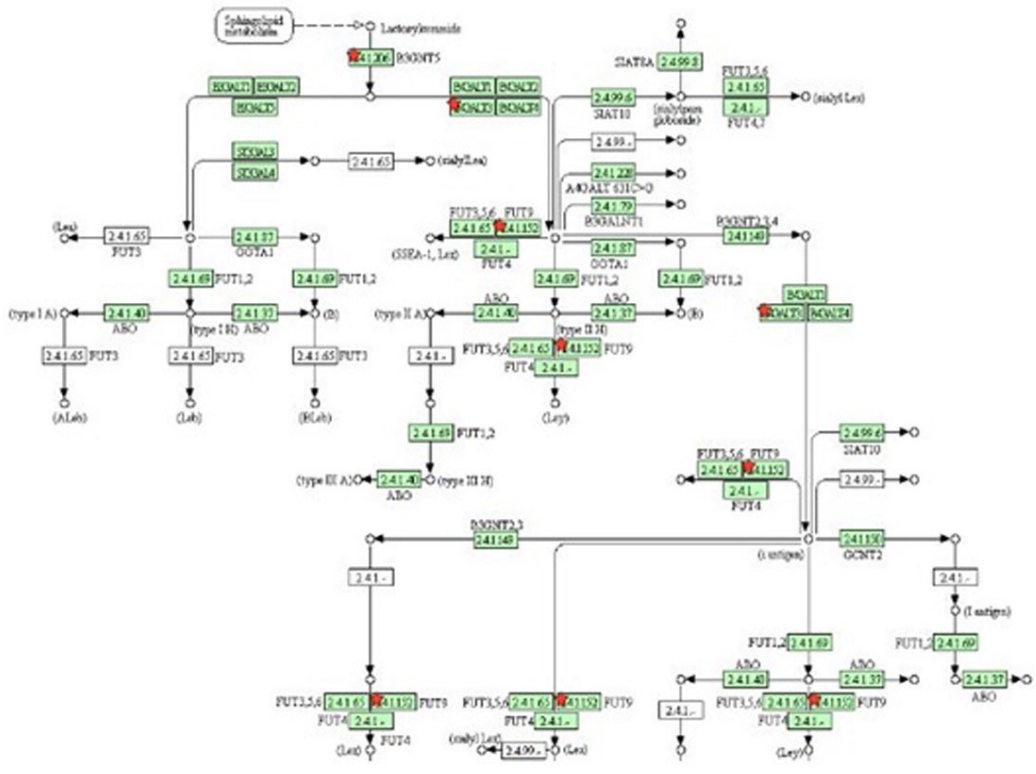


Figure 7. Participation of down-regulated genes in glycosphingolipid biosynthesis. Genes in star marked were found to be down-regulated in ketosis samples.

ultimately elevation in the growth hormone concentration that antagonizes the function of insulin and causes lipolysis and gluconeogenesis in early lactation [29] respectively. Relative mRNA expression of marker ASTML showed

significant ($p < 0.05$) upregulation as transition period progressed from prepartum period (Gp-I) to calving (Gp-II) and post-partum period (Gp-III); 0.836 ± 0.02 , 1.268 ± 0.03 and 1.624 ± 0.01 respectively (Figure 8). The same trend in

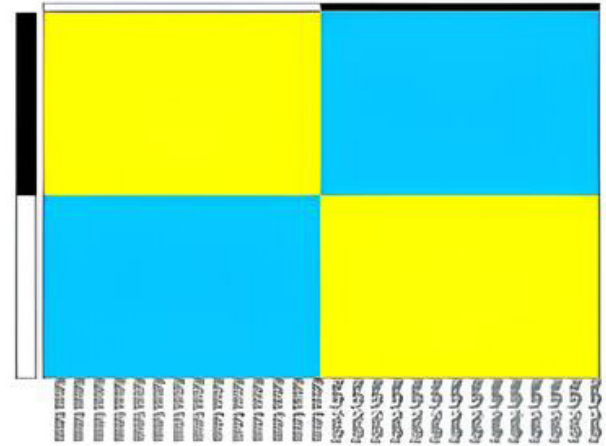


Figure 8. ICGS analysis of transcriptomes for finding markers of ketosis and health.

Table 3. 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

GUCA1B, guanylate cyclase activator 1B; GRTP1, growth hormone regulated TBC protein 1; ASMTL, acetylserotonin O-methyltransferase like; SNCA, acetylserotonin O-methyltransferase like; AEBP1, AE binding protein 1; CLPP, caseinolytic mitochondrial matrix peptidase proteolytic subunit.

the expression level of CPT1A was seen from Gp-I to Gp-III (0.96±0.06, 1.336±0.02, and 1.372±0.02) (Figure 9). Whereas downregulation in the expression level of GUCA1B was seen from pre-partum period (1.548±0.05) to calving (1.474±0.08) and post-partum period (1.014±0.04) (Figure 10). The expression fold of IGF-1 marker showed a significant (p<0.05) trend of downregulation from pre-calving (1.063±0.05) to near calving (0.839±0.02) term thereafter, gradual increase

Table 4. Primer sequences and annealing temperatures of genes of interest

Genes	Annealing temperature (°C)	Size (bp)	Sequence
CPT1A	58.7	292	F:TTATGTGAGCGACTGGTGGG R:GTGCTGGATGGTGTCTGTCT
ASTML	67.3	215	F:CTCATTGCGCCACCCCGCA R:CGCTGTGCTCCTTCCCAC
GUCA1B	65.5	253	F:CTCTGCTCCCTGCCGTCC R:GGTGTGTCCCCATTCGTGT
IGF-1	59.6	140	F:CATCCTCCTGCGATCTTTC R:GAAATAAAGCCCTGTCTCC

CPT1A, carnitinepalmitoyl-transferase 1A; ASTML, acetylserotonin O-methyltransferase like; GUCA1B, guanylate cyclase activator 1B; IGF-1, insulin growth factor-1.

in the concentration as post-calving period progressed (1.123±0.02).

Changes in oxidative stress markers during transition period in buffaloes.

The imbalance between oxidative marker and antioxidant markers level causes oxidative stress. On analysis of these antioxidant and oxidative markers, our results showed a significant difference (p<0.05) amongst these groups providing sufficient evidence about oxidative biomarkers and antioxidants production during transition period in buffaloes.

Our study showed significant (p<0.05) increase in the mean±standard error (SE) values of MDA (nmoles of MDA produced/g of Hb/h) in Group-II (near parturition) with comparison to Group-I (-30 days) and Group-III (+30 days). Whereas, mean±SE values of SOD (U/mg of Hb) and catalase (CAT; µmoles) showed a significant decrease (p<0.05) in their values from Group-I (-30 days) to Group-III (+30

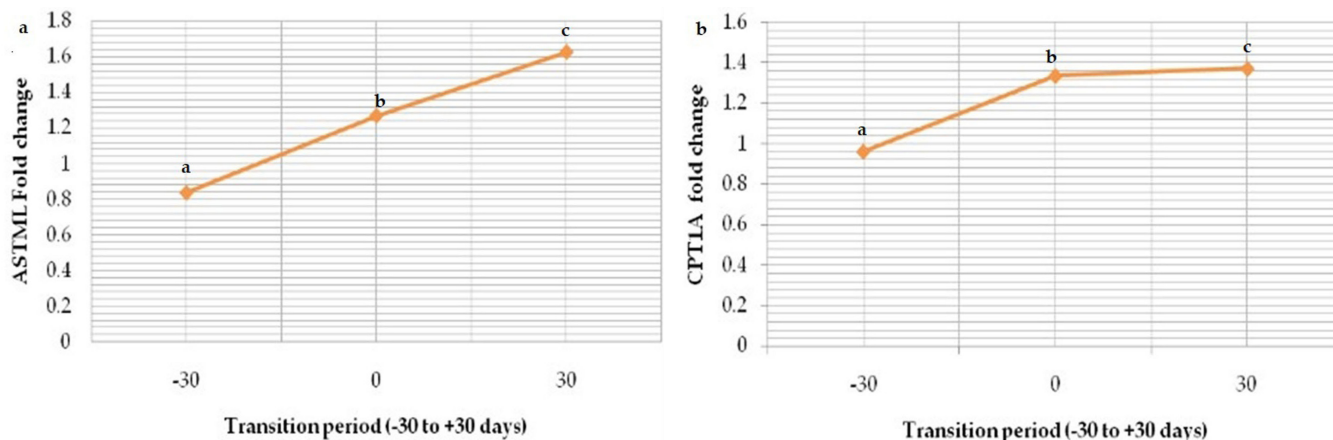


Figure 9. Graphs depicting expressions of upregulated genes during transition period (GP-I; -30 days, GP-II; near parturition and GP-III; +30 days) in buffaloes; (a) *ASTML* (b) *CPT1A*. ^{a-c} Means with different superscripts between rows differs significantly ($p < 0.05$).

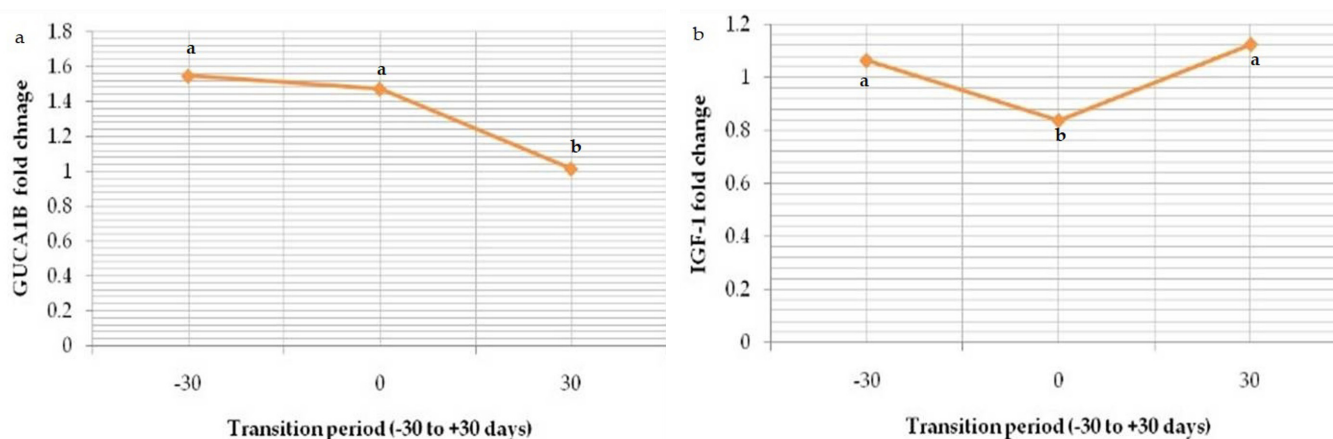


Figure 10. Graphs depicting expressions of downregulated genes during transition period (GP-I, -30 days; GP-II, near parturition; and GP-III, +30 days) in buffaloes; (a) *GUCA1B* (b) *IGF-1*. ^{a-c} Means with different superscripts between rows differs significantly ($p < 0.05$).

days) (Table 5). Blood GPx (mg/Hb) levels showed trend of significant decrease ($p < 0.05$) from pre-partum period (-30 days) to near parturition time. Although, a significant increase ($p < 0.05$) in the levels of GPx was seen in buffaloes when transition period progressed to +30 days (Table 5).

DISCUSSION

Energy and nitrogen demand by the conceptus in late pregnancy are mostly met by dam's glucose and amino acid uptake by the placental tissues. During postpartum period there is marked increase in mammary demands of glucose, amino acids and fatty acid. Therefore, rates of hepatic gluconeogenesis and adipose tissues are greatly increased. Bovine liver has limited capacity to metabolise NEFA into triacylglycerol (TAG) which accumulates in the liver as threshold

Table 5. Oxidative biomarker and antioxidants 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/h)	6.09 ^a ±0.10	7.01 ^b ±0.08	6.31 ^a ±0.13
CAT (µmoles of H ₂ O 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

MDA, malondialdehyde; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase.

^{a-c} Means with different superscripts between rows differs significantly ($p < 0.05$).

crosses the limit. Thereafter, acetyl CoA resulting from oxidation of fatty acid are not utilised by the tri-carboxylic acid (TCA) cycle and later converts into acetone, acetoacetate and BHBA which appears in milk, urine and blood. The free fatty acid and BHBA are considered as indicators of negative energy balance and peak concentrations are seen in the early postpartum period as reported by Bryers [30]. In the present study, the values of BHBA increased from pre-partum to post-partum period but under the normal range of sub-clinical ketosis (<1,200 nmol/mL) in all the three groups. Low prevalence of metabolic diseases such as ketosis can be partially explained with ability of buffering and breakdown of carbohydrates (non-structural), low thyroid activity and retention of minerals [31]. In the present research study, transient decrease in blood glucose concentration from the far-off dry period up to the early lactation was observed. Our results are in accordance with Ambrosio et al [32] suggesting severe negative energy balance characterised by reduced blood glucose and insulin levels.

In-silico analysis of selected potential markers of ketosis/negative energy balance viz; ASTML (upregulated) and GUCA1B (downregulated) validated with the qRT-PCR analysis provides a similar trend in fold expression of ASTML showing upregulation throughout the transition period and GUCA1B giving a downregulated trend. As pathways involved in the upregulation and downregulation of selected markers are cytokine-cytokine interaction, oxidative stress pathways and Glycosphingolipid biosynthesis, respectively. These results suggest that negative energy balance reduces the expression of genes involved in fat biosynthesis and metabolism with upregulation in pro-inflammatory cytokine interaction and free radical stress at molecular level. However, further research is needed to verify whether these genes influence the total amount of fat secretion in the milk or instead influence the fat percentage in the milk.

Similarly, the fold change in expression level of CPT1A in our study showed significant upregulation ($p < 0.05$) as buffaloes progress from dry period to early lactation (3 weeks pre-partum to 3 weeks postpartum). Our results were in congruence with the study of Singh et al [33]. Involvement of CPT1A in stimulation of hepatic fatty acid β -oxidation to maintain energy demands in the hepatic tissues especially for production of glucose from reserve sources can partially explain its upregulation during this phase [34]. Expression fold of IGF-1 in our study has showed a trend of downregulation from pre-calving to immediate calving period and thereafter, a gradual increase was observed during early postpartum period. The down regulation of IGF-1 indicates a relationship of insulin with GHR 1A in liver as GHR 1A is controlled by insulin and growth hormone (GH) in turn controls IGF-I synthesis and secretion. Therefore, reduced negative feedback on GH by IGF-1 promotes lipolysis, which

releases NEFA into blood. The gradual increase in the expression fold of IGF-1 during post-partum period could be an attribute of recovered growth hormone resistance by the hepatic tissue that occurs during this period [35]. However, in some studies suppressed IGF-1 synthesis has been observed in animals having evident negative energy balance [36]. Our study model focuses on transition period as majority of metabolic diseases occur around this time and provides scope for nutritional interventions that can have profound effect on prevention of clinical and subclinical forms of production diseases.

Oxidative stress occurs during the period of high metabolic demands which leads to an imbalance of anti-oxidant defense markers and reactive oxygen species. The reactive oxygen species progressively increased from late lactation to post parturient period, even higher in peak lactation [37]. In our study we found significant increase ($p < 0.05$) in level of LPO during calving and postpartum period and decreased level of SOD, GPx, and catalase. The adaptation to negative energy balance during transition period in various tissues, primarily NEFA leads to oxidation in an intensified manner which eventually results in the formation of reactive oxygen species [38]. The intermediate product of lipid peroxidation, MDA level is increased during this period suggesting a higher level of lipid peroxidation. Superoxide dismutase is considered as the first line of defense against oxidative stress radicals, by converting superoxide radical to hydrogen peroxide and is considered as important antioxidant marker. A significant decrease in SOD is evident of higher oxidative stress during periparturient period in dairy cattle [39]. Glutathione peroxidase plays an important role in antioxidant defense mechanism by reducing the hydrogen peroxide into water and alcohol. Glutathione is used as a reducing agent in the reaction [40]. From the level of GPx during calving and immediate postpartum time, one can say that animals are having a marked reduction in antioxidant markers and an increase in reactive oxygen species during transition period. Also, In-silico analysis revealed pathways regulated by upregulated genes are cytokine interactions causing inflammatory response along with oxidative stress related to significant increase in free radical concentrations at molecular level. This enables us to consider these putative biomarkers for prompt detection of occurrence of metabolic imbalances during transition period in buffaloes. It ultimately provides scope of nutritional or therapeutic interventions to prevent their occurrence.

Physiological conditions associated with insufficient energy supply predispose dairy animals to metabolic and microbial diseases. The role of inflammatory mediators in the decline in fertility/production is not known, given the range of various physiological processes involved. An improved understanding at the molecular level plays an important role in normal immune function, metabolism, and reproduction. This may

improve our ability to predict and prevent transition disorders. Therefore, a scientific approach for the early diagnosis of the metabolic and production disease in dairy animals is required. We have integrated the evidence from in-silico analysis, qRT-PCR validations of potential markers along with putative oxidative stress indicators of ketosis/negative energy balance in buffaloes that can be used for earliest diagnoses as they explain the complex physiological processes in the liver and adipose tissue that are elicited by the plane of nutrition during the periparturient period.

CONCLUSION

In conclusion, bioinformatics analysis of high-throughput gene expression revealed the involvement of multiple pathways, including the inflammatory pathway, fatty acid pathway and cytokine interaction pathway during negative energy balance in transition period. The experimental analysis reveals that molecular biomarkers and oxidative stress indicators may act as potential biomarkers for the early detection of negative energy balance. Furthermore, we should consider other clinical diseases or signs to correlate to improve the accuracy of diagnoses.

AUTHOR CONTRIBUTIONS

Conceptualization, methodology, formal analysis, supervision, S.K., N.S., Resources, investigation, data curation, project administration, N.S., P.K.G., M.K.G; Writing, review, and editing: S.K., N.S., G.B., N.M., Y.O.S.; Statistical data analysis, and editing: M.V.S., A.K.P. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

FUNDING

This work was supported by Department of Biotechnology (DBT), (Grant No. BT/PR26321/SPD/9/1307/2017) Government of India, and National Research Foundation of Korea (Grant No.: 2020R1A2C2004128), and Basic Science Research Program, NRF (Grant No.: 2019R1A6A1A10072987), Ministry of Education, Government of South Korea.

REFERENCES

- Arfuso F, Fazio F, Levanti M, et al. Lipid and lipoprotein profile changes in dairy cows in response to late pregnancy and the early postpartum period. *Arch Anim Breed* 2016; 59:429-34. <https://doi.org/10.5194/aab-59-429-2016>
- Ashmawy NA. Blood metabolic profile and certain hormones concentrations in Egyptian buffalo during different physiological states. *Asian J Anim Vet Adv* 2015;10:271-80.
- Sharma N, Singh NK, Singh OP, Pandey V, Verma PK. Oxidative stress and antioxidant status during transition period in dairy cows. *Asian-Australas J Anim Sci* 2011;24: 479-84. <https://doi.org/10.5713/AJAS.2011.10220>
- Dimri U, Ranjan R, Sharma MC, Varshney VP. 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. *Trop Anim Health Prod* 2010;42:405-10. <https://doi.org/10.1007/s11250-009-9434-4>
- Spears JW, Weiss WP. Role of antioxidants and trace elements in health and immunity of transition dairy cows. *Vet J* 2008; 176:70-6. <https://doi.org/10.1016/j.tvjl.2007.12.015>
- Miltenburg C. Management of peripartum dairy cows for metabolic health and immune function [Doctoral dissertation]. Ontario, Canada: University of Guelph; 2015.
- Ingvartsen KL, Dewhurst RJ, Friggens NC. On the relationship between lactational performance and health: is it yield or metabolic imbalance that cause production diseases in dairy cattle? A position paper. *Livest Prod Sci* 2003;83:277-308. [https://doi.org/10.1016/S0301-6226\(03\)00110-6](https://doi.org/10.1016/S0301-6226(03)00110-6)
- Vanholder T, Papen J, Bemers R, Vertenten G, Berge ACB. Risk factors for subclinical and clinical ketosis and association with production parameters in dairy cows in the Netherlands. *J Dairy Sci* 2015;98:880-8. <https://doi.org/10.3168/jds.2014-8362>
- Holcomb CS, Van Horn HH, Head HH, Hall MB, Wilcox CJ. Effects of prepartum dry matter intake and forage percentage on postpartum performance of lactating dairy cows. *J Dairy Sci* 2001;84:2051-8. [https://doi.org/10.3168/jds.S0022-0302\(01\)74649-8](https://doi.org/10.3168/jds.S0022-0302(01)74649-8)
- Weber C, Hametner C, Tuchscherer A, et al. 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. *J Dairy Sci* 2013;96:5670-81. <https://doi.org/10.3168/jds.2012-6277>
- Fiore E, Arfuso F, Ganesella M, et al. Metabolic and hormonal adaptation in *Bubalus bubalis* around calving and early lactation. *PLoS One* 2018;13:e0193803. <https://doi.org/10.1371/journal.pone.0193803>
- Serrapica F, Masucci F, Romano R, et al. Effects of chickpea in substitution of soybean meal on milk production, blood profile and reproductive response of primiparous buffaloes in early lactation. *Animals* 2020;10:515. <https://doi.org/10.3390/ani10030515>
- Purohit GN, Ruhil S, Daga M, Gaur M, Bihani DK, Ahuja A.

- Parturition related metabolic disorder in buffaloes: a 10 year case analysis. *Rumin Sci* 2014;3:123-6.
14. Ospina PA, Nydam DV, Stokol T, Overton TR. Associations of elevated nonesterified fatty acids and β hydroxybutyrate concentrations with early lactation reproductive performance and milk production in transition dairy cattle in the north-eastern United States. *J Dairy Sci* 2010;93:1596-603. <https://doi.org/10.3168/jds.2009-2852>
 15. Oetzel GR. Herd-level ketosis—diagnosis and risk factors. In: Preconference Seminar 7C: Dairy Herd Problem Investigation Strategies, Transition Cow Trouble shooting American association of bovine practitioners 40th Annual Conference; 2007 September 19, Vancouver, Canada.
 16. Madreseh-Ghahfarokhi S, Dehghani-Samani A. Ketosis (acetoaemia) in dairy cattle farms: practical guide based on importance, diagnosis, prevention and treatments. *J Dairy Vet Anim Res* 2018;7:299-302.
 17. Lockstone HE. Exon array data analysis using Affymetrix power tools and R statistical software. *Brief Bioinform* 2011; 12:634-44. <https://doi.org/10.1093/bib/bbq086>
 18. Zambon AC, Gaj S, Ho I, et al. GO-Elite: a flexible solution for pathway and ontology over-representation. *Bioinformatics* 2012;28:2209-10. <https://doi.org/10.1093/bioinformatics/bts366>
 19. Emig D, Salomonis N, Baumbach J, Lengauer T, Conklin BR, Albrecht M. AltAnalyze and DomainGraph: analyzing and visualizing exon expression data. *Nucleic Acids Res* 2017;38:W755-62. <https://doi.org/10.1093/nar/gkq405>
 20. Fendri K, Patten SA, Kaufman GN, et al. Microarray expression profiling identifies genes with altered expression in Adolescent Idiopathic Scoliosis. *Eur Spine J* 2013;22:1300-11. <https://doi.org/10.1007/s00586-013-2728-2>
 21. Hulin A, Hortells L, Gomez-Stallons MV, et al. Maturation of heart valve cell populations during postnatal remodeling. *Development* 2012;146:173047.
 22. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 2008;3:1101-8. <https://doi.org/10.1038/nprot.2008.73>
 23. Rehman SU. Lead induced regional lipid peroxidation in brain. *Toxicol Lett* 1984;21:333-7.
 24. Aebi H. Catalase in vitro. *Methods Enzymol* 1984;105:121-6. [https://doi.org/10.1016/S0076-6879\(84\)05016-3](https://doi.org/10.1016/S0076-6879(84)05016-3)
 25. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem* 1974; 47:469-74.
 26. Hafeman DG, Sunde RA, Hoekstra WG. Effect of dietary selenium on erythrocyte and liver glutathione peroxidase in the rat. *J Nutr* 1974;104:580-7. <https://doi.org/10.1093/jn/104.5.580>
 27. Loor JJ. Genomics of metabolic adaptations in the periparturient cow. *Animal* 2010;4:1110-39. <https://doi.org/10.1017/S1751731110000960>
 28. Lucy MC, Jiang H, Kobayashi Y. Changes in the somatotrophic axis associated with the initiation of lactation. *J Dairy Sci* 2001;84:E113-9. [https://doi.org/10.3168/jds.S0022-0302\(01\)70205-6](https://doi.org/10.3168/jds.S0022-0302(01)70205-6)
 29. Bell AW. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. *J Anim Sci* 1995;73:2804-19. <https://doi.org/10.2527/1995.7392804x>
 30. Bryers DI. Controlling metabolic diseases. In: Paper presented at Tri-state dairy nutrition conference, held at Michigan State University, Grand Wayne Center Fort Wayne; 1999, April 20-21, IN, USA.
 31. Accorsi PA, Govoni N, Gaiani R, Pezzi C, Seren E, Tamanini C. Leptin, GH, PRL, insulin and metabolic parameters throughout the dry period and lactation in dairy cows. *Reprod Domest Anim* 2005;40:217-23. <https://doi.org/10.1111/j.1439-0531.2005.00581.x>
 32. Ambrosio R, Sannino ML, Cortese L, Nappi C, Ara D, Cioffi M. Validation and application of an immunofluorimetric assay for detection of serum free triiodothyronine and free thyroxine concentrations in buffalo (*Bubalus Bubalis*) under various physiological conditions. *J Vet Diagn Invest* 2009;21: 668-73. <https://doi.org/10.1177/104063870902100511>
 33. Singh R, Randhawa SNS, Randhawa CS. Oxidative stress, Hemato-biochemical and plasma mineral profile in transition buffaloes. *Proc Natl Acad Sci* 2017;87:1091-9. <https://doi.org/10.1007/s40011-015-0684-9>
 34. Huang Y, Zhao C, Kong Y, et al. Elucidation of the mechanism of NEFA-induced PERK-eIF2 α signaling pathway regulation of lipid metabolism in bovine hepatocytes. *J Steroid Biochem Mol Biol* 2021;211:105893. <https://doi.org/10.1016/j.jsbmb.2021.105893>
 35. Rhoads ML, Meyer JP, Kolath SJ, Lamberson WR, Lucy MC. Growth hormone receptor, insulin-like growth factor (IGF)-1, and IGF-binding protein-2 expression in the reproductive tissues of early postpartum dairy cows. *J Dairy Sci* 2008;91: 1802-13. <https://doi.org/10.3168/jds.2007-0664>
 36. Fenwick MA, Fitzpatrick R, Kenny DA, et al. Interrelationships between negative energy balance (NEB) and IGF regulation in liver of lactating dairy cows. *Domest Anim Endocrinol* 2008;34:31-44. <https://doi.org/10.1016/j.domaniend.2006.10.002>
 37. Sordillo LM, Raphael W. Significance of metabolic stress, lipid mobilization, and inflammation on transition cow disorders. *Vet Clin North Am Food Anim Pract* 2013;29:267-78. <https://doi.org/10.1016/j.cvfa.2013.03.002>
 38. Abuelo A, Hernández J, Benedito JL, Castillo C. Oxidative stress index (OSi) as a new tool to assess redox status in dairy cattle during the transition period. *Animal* 2013;7:1374-8. <https://doi.org/10.1017/S1751731113000396>
 39. Herdt TH. Ruminant adaptation to negative energy balance: influences on the etiology of ketosis and fatty liver. *Vet Clin*

North Am Food Anim Pract 2000;16:215-30. [https://doi.org/10.1016/S0749-0720\(15\)30102-X](https://doi.org/10.1016/S0749-0720(15)30102-X)
40. Castillo C, Hernandez J, Valverde I, et al. Plasma malonaldehyde

(MDA) and total antioxidant status (TAS) during lactation in dairy cows. Res Vet Sci 2006;80:133-9. <https://doi.org/10.1016/j.rvsc.2005.06.003>