ORIGINAL ARTICLE

Buffalo liver transcriptome analysis suggests immune tolerance as its key adaptive mechanism during early postpartum negative energy balance

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Abstract

Females undergo negative energy balance (NEB) during the early postpartum period to meet the lactation demands. The liver, being the key metabolic organ, plays a major role in handling NEB. Dairy animals handling high lactation demands are better models to understand the liver adaptive mechanisms during this phase. Therefore, we analyzed the liver transcriptome of dairy buffaloes during early postpartum. Liver biopsies were performed on three lactating buffaloes on the 15th and the 30th days of early postpartum and three heifers (controls) at the diestrous stage. Paired-end Next Generation Sequencing (NGS) identified 509 significantly differentially expressed genes (SDE) in the liver among the three groups. The SDE with log2 fold change > 3 and the unique SDE revealed the promotion of immune suppression (e.g., TCR), apoptosis (e.g., *CCDC103*), PGF2α synthesis, fat accumulation (e.g., *BGLAP*) and liver regeneration (e.g., *FGF10*) pathways, and the downregulation of antigen presentation (e.g., *BOLA-DQA*) on the 15th day of lactation. Consistently upregulated genes on the 15th and 30th days of early postpartum indicated the promotion of immune tolerance (e.g., *IFITM3*), medium and long-chain fatty acids' oxidation (e.g., *ACSM3*), and lipid accumulation (e.g., *INSIG1*). However, consistently downregulated genes during early postpartum showed immunosuppression, the downregulation of gluconeogenesis from amino acids (e.g., *DDO*), and the biosynthesis of taurine (e.g., *CSAD*) and unsaturated fatty acids (e.g., *SCD*). Functional annotation and network analyses also indicated the promotion of immune tolerance, fat accumulation and decreased gluconeogenesis from amino acids, and estrogen metabolism on the 15th day of lactation. Overall, the liver showed immune tolerance as an adaptive mechanism during early postpartum of buffaloes.

Keywords Early lactation · Liver transcriptome · Functional annotation · Network analysis · Hub genes

Abbreviations

NEB	Negative energy balance
NGS	Next generation sequencing
BOLA-DQA	Major histocompatibility complex, class II, DQ alpha, type 1
CCDC103	Coiled-coil domain-containing 103
PGF2α	Prostaglandin F2alpha
BGLAP	Bone gamma-carboxyglutamate
FGF10	Fibroblast growth factor 10
IFITM3	Interferon-induced transmembrane protein member 3
INSIG1	Insulin-induced gene 1
ACSM3	Acyl-CoA synthetase medium-chain family member 3
DDO	D-aspartate oxidase
CSAD	Cysteine sulfinic acid decarboxylase
SCD	Stearoyl-CoA desaturase
ANGPTL4	Angiopoietin like 4
NEFA	Non-esterified free fatty acids
FFA	Free fatty acids
MAMPS	Microbial associated molecular patterns
KEGG	Kyoto encyclopaedia of gene and genome
GO	Gene ontology
NCBI	National Center for Biotechnology Information

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STRING	Search Tool for the Retrieval of Interacting Genes
PPI	Protein- Protein interaction
FPKM	Fragments per kilo-base of exon per million fragments mapped
GIMAP4	GTPase IMAP family member 4
BOLA-DQA5	Major histocompatibility complex, class II, DQ alpha, type 5
BOLA-DQB	Major histocompatibility complex, class II, DQ alpha beta
USH1C	USH1 Protein network component harmonin
M-SAA3.2	Mammary serum amyloid A3.2
SAA3	Serum amyloid A3
SLC22A16	Solute carrier family 16
GSTA1	Glutathione S-transferase A1
ECEL1	Endothelin-converting enzyme-like 1
PPAR	Peroxisome proliferator-activated receptors
DQA2	Major histocompatibility complex, class II, DQ alpha 2
APOA1	Apolipoprotein A1
APOA5	Apolipoprotein A5
UBC	Ubiquitin C
SECTM1A	Secreted and transmembrane protein 1A
SH2D1A	SH2 domain-containing 1A
LOC789452	T cell receptor alpha chain V region 2b4-like
PTGES	Prostaglandin E synthase
LOC617406	Serpin peptidase inhibitor, clade B (ovalbumin), member 6-like
ncRNA	Noncoding RNA
LOC100296286	T cell receptor alpha chain V region RL-5-like
LOC100300282	T cell receptor beta chain V region CTL-L17-like
IFI27L2	Interferon, alpha-inducible protein 27-like 2
HRAS	HRas proto-oncogene, GTPase
GEFR	Guanine nucleotide exchange factor
P2Y	Purinergic receptor 2Y
LOC100848101	Kinesin-like protein KIF13A
TRNAR-CCG	Transfer RNA arginine (anticodon CCG)
LOC615055	Ef-hand calcium-binding domain-containing protein 4A
LOC788915	Caspase-14
LOC107131362	UDP-Glucuronosyltransferase 2B33-like
SERPINE2	Serpin family E member 2
TPGS1	Tubulin polyglutamylase complex subunit 1
ASIP	Agouti signaling protein
LOC101902932	Uncharacterized LOC101902932

C8G	Complement component 8, gamma protein
ASPG	Asparaginase
SPON2	Spondin 2
LOC785540	Cytochrome P450 2C31
LOC107133155	Apolipoprotein C-III-like
C4A	Complement component 4A
LOC100337213	Adhesion G protein-coupled receptor E2
MT2A	Metallothionein 2A
MT1E	Metallothionein 1E
APOL3	Apolipoprotein L, 3
ABCA9	ATP binding cassette subfamily A member 9
GDPD1	Glycerophosphodiester phosphodiesterase domain-containing 1
DEPDC5	DEP domain-containing 5
MINOS1	Mitochondrial inner membrane organizing system 1
S1PR1	Sphingosine-1-phosphate receptor 1
PIK3CD	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta
IGFBP1	Insulin-like growth factor binding protein 1
LOC511161	Nicotinamide N-methyltransferase
NKIRAS2	NF-KB inhibitor interacting Ras like2
LURAP1L	Leucine rich adaptor protein 1 like
SPTCL3	Serine palmitoyltransferase long-chain base subunit 3
KLF10	Kruppel like factor 10
LOC536097	Tyrosine-protein phosphatase non-receptor type substrate 1
ACTA2	Actin, alpha 2, smooth muscle, aorta
MTHFD1L	Methylenetetrahydrofolate dehydrogenase 1-like
OAS1	2′,5′-oligoadenylate synthetase 1
OAS2	2′,5′-oligoadenylate synthetase 2

Introduction

Early postpartum is a critical transition period from pregnancy to lactation in females' life. During this period, females undergo a state of negative energy balance (NEB) by decreased food intake and increased energy requirements of lactation. Dairy animals handling high lactation demands could be better models to understand the molecular adaptive mechanisms during the early postpartum period, as high milk production creates natural NEB. Some animals coping to this NEB very easily than other animals indicate the variation in the adaptive mechanisms among animals. Hence, exploration of the molecular cues in the adaptive mechanisms would be helpful to understand the early postpartum metabolic problems.

The liver, being the key metabolic organ, plays a major role in handling NEB during early postpartum. Interestingly, the histological structure of the liver appears similar between human and dairy animals (Madan and Raju 2014). In ruminants, the liver is the exclusive organ supplying glucose for milk synthesis (Drackley et al. 2001). Therefore, the metabolic load would be very high on the liver in high-yielding ruminants. Particularly, the liver initiates the lipolysis in adipose tissue through certain hepatokines, like angiopoietin like 4 (*ANGPTL4*). Further, it metabolizes the non-esterified free fatty acids (NEFA or FFA) released by lipolysis for maintaining the energy homeostasis during NEB (Loor et al. 2007; McCarthy et al. 2010). Principally, the liver decides the direction of NEFA either towards oxidation or transport or storage. In addition to the role in lipolysis and the metabolism of NEFA, the liver is the first organ after the intestine to be exposed to the digestive materials, including toxins and microbial-associated molecular patterns (MAMPS). Hence, the liver also needs to play a balancing role between immune tolerance and immune susceptibility by both the immune cells and hepatocytes (Crispe 2014). Therefore, it is very important to understand the molecular pathways, networks, and adaptive mechanisms of the liver during early postpartum.

Earlier liver transcriptome studies during the early postpartum period were conducted on cows (Loor et al. 2007; Andersen et al. 2008; McCarthy et al. 2010; Bionaz et al. 2012; McCabe et al. 2012). However, the buffaloes, the premier dairy animals of developing countries, show a wide spectrum of postpartum problems, including mastitis, endometritis, fatty liver, and postpartum anestrus. Particularly, the postpartum anestrus, a similar condition of postpartum amenorrhea in women (Howie and McNeill 1982; Aryal 2007), is a major postpartum problem in buffaloes (Kumar et al. 2014). Therefore, dairy buffaloes could be better models for understanding the postpartum metabolic problems associated with early lactation stress or NEB. The postpartum anestrus or resumption of reproductive activity in females has been addressed by focusing on the hypothalamus-pituitary-ovarian-uterus axis. Although the liver is the principal organ integrating the metabolism in general and steroid metabolism in particular, this organ was not given much attention towards the postpartum fertility problems. Therefore, the present work targeted to understand the liver adaptive mechanisms at the transcriptome level during early postpartum by considering dairy buffaloes as experimental models.

Materials and methods

Animals

A total of six healthy buffaloes were considered in the present study after approval from the Institutional Animal Ethics Committee of the National Dairy Research Institute, Karnal (Approval no. 95/16). The animals were managed according to the standard managemental conditions of the Livestock Research Center, NDRI, India. Three animals were early lactating buffaloes immediately after parturition, and the remaining three were heifers to represent three biological replicates each for early lactating and control groups, respectively, in this study. We have considered three biological replicates according to the earlier research publications, which utilized three biological replicates for transcriptome analysis (Trapnell et al. 2012; Gong et al. 2014; Sollner et al. 2017; Barta et al. 2018). In addition, the maximum percentage of respondents ($n = 168$) polling on biological replicates in RNA-seq blog voted three biological replicates (https://www.rna-seqblog.com/rna-seq-blog-poll-results-8/).

Milk yield data and serum free fatty acid estimation

The data on milk yield were collected from all three lactating animals every day for 22 weeks. These animals were high yielders with a total milk yield of more than 1500 kg for 22 weeks. Weekly blood samples were collected from these animals by the jugular vein puncture from the day of parturition to 70 days of early postpartum. Similarly, the blood samples were also collected from heifers on the same day as control samples. Serum free fatty acids (FFA) were estimated with the EnzyChrom™ Free Fatty Acid Assay Kit (EFFA-100, BioAssay Systems, Hayward, CA, USA).

Liver biopsy

Liver biopsies were performed on heifers at the diestrous stage (10th day of estrous cycle) and lactating animals on the 15th and 30th day of early postpartum. A well-trained veterinarian performed the biopsies with 14G × 6″ disposable Clear Needle™ biopsy needle (NewTech Medical devices, New Delhi, India) following standard minor surgical and post-surgical procedures (Singh 2017). The detailed liver biopsy procedure is mentioned in the supplementary materials and methods. Four liver slices from each animal were collected into a microcentrifuge tube containing all protectant tissue reagent (Qiagen cat # 76405, Germany). The liver tissue samples in the microcentrifuge tubes were brought to the laboratory in a liquid nitrogen tank and stored at − 80 °C in a freezer.

Total RNA isolation from buffalo liver tissue

To obtain a Next Generation Sequencing (NGS) quality RNA from buffalo liver tissue samples, a three-step protocol developed in our laboratory was used (Sharma et al. 2019). The quality of all the RNA samples ($n = 9$), representing three

biological replicates for control, 15th, and 30th days of lactation, were assessed by RIN values.

NGS and differential gene expression

Total RNA samples were custom sequenced by NGS at NXGenBio catalyzing innovations, New Delhi, India. For all nine RNA samples, cDNA libraries were prepared by the Illumina® TruSeq® RNA Sample Preparation Kit v2 protocol (Supplementary Fig. 1), and sequenced by the classical Illumina Hi-Seq-2000 platform. The NGS data were analyzed by the Tuxedo protocol (Fig. 1a). Briefly, the Tophat software with the bowtie aligner was used to align the high-quality (QC score > 20) raw sequence reads to the *Bos taurus* (UMD 3.1) reference genome. The BAM (Binary version of SAM file) and SAM (Sequence alignment mapping) files of the alignment were later used for an abundance estimation by the cufflinks software. The cufflinks output (*.gtf) files of individual RNA samples were merged in Cuffmerge to produce the final transcriptome assembly of the entire data. Later, the expression of the aligned reads was quantified by the Cuffquant tool, which uses both the BAM files of individual samples and the final transcriptome assembly resulted from the cuffmerge tool. The output of the Cuffquant tool (*.cxb file) was further used in Cuffdiff to obtain the expression values of each annotated gene for individual animals and to perform groupwise differential expression analysis. Finally, the statistical measures and differential expression data were visualized by the CummeRbund R package. Five randomly selected genes were validated by standard qPCR (Supplementary Table 5) using Ribosomal Protein Lateral Stalk Subunit P1 (RPLP) as a normalizer. The results were expressed as a fold change calculated by the standard $2^{-(\Delta\Delta Ct)}$ method.

Bioinformatics analysis on differentially expressed genes

The genes with a fold change (FC) > 2 or log2FC > 1 and a FDR *P* value < 0.05 were considered as significantly differentially expressed (SDE) genes among control (heifer), 15th, and 30th days of early lactation. A battery of bioinformatics approaches such as functional annotation, pathway, network, and hub gene analyses were implemented on the SDE genes. Specifically, all the SDE genes together, the SDE genes unique to each group, common among the groups, and common between the 15th and the 30th days of lactation were analyzed separately.

Functional annotation and pathway analyses (Fig. 1b) were performed by online softwares, the GATHER (Gene Annotation Tool to Help Explain Relationships, http://changlab.uth.tmc.edu/gather/gather.py), DAVID (The
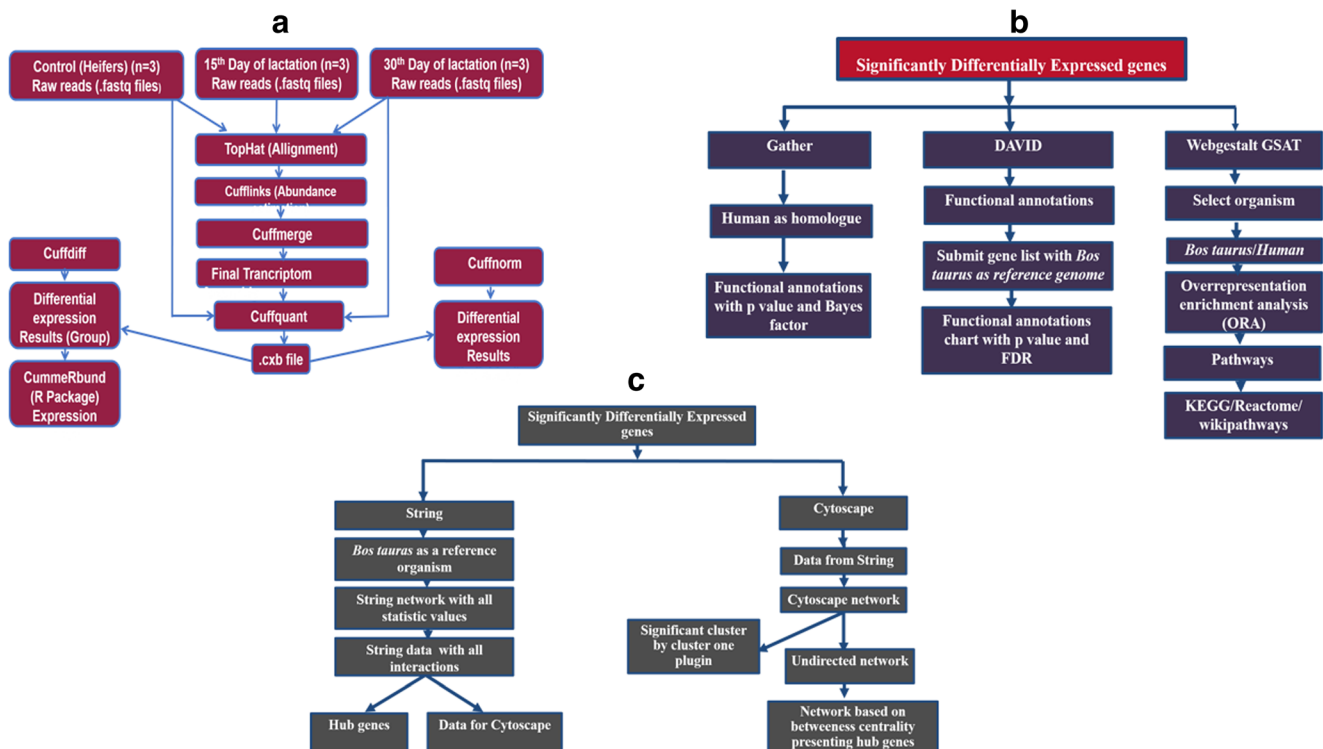

Fig. 1 Workflow of RNA-seq data analysis. Identification of the significantly differentially expressed (SDE) genes from raw reads (**a**); functional annotation of SDE genes with the GATHER, DAVID, and Webgestalt GSAT software (**b**); and the network analysis of SDE genes through STRING and Cytoscape software (**c**)

Database for Annotation, Visualization and Integrated Discovery; https://david.ncifcrf.gov/), and WEB-based GEne SeT AnaLysis Toolkit (Webgestalt, (http://webgestalt.org/option.php). For functional annotation of SDE genes, the *B. taurus* and *Homo sapiens* reference genomes were used on the DAVID and the Webgestalt software, and the *H. sapiens* genome was used on the GATHER software. Pathway analyses were performed by using the KEGG (http://www.genome.jp/kegg/), Reactome (http://www.reactome.org/), Wikipathways (http://www.wikipathways.org/index.php/WikiPathways), and GO annotation (http://www.geneontology.org/page/go-database) databases available on the Webgestalt software. A few number of genes were also annotated by the gene cards, uniprot KB and NCBI literature.

Network analysis was initially performed with the default parameters of the STRING (Search Tool for the Retrieval of Interacting Genes, https://www.string-db.org/) software by considering all 509 SDE genes (Fig. 1c). A significant (*P* value = 8.43E-07) PPI network comprising of 380 SDE genes was created. The STRING-generated output files were later input to ClusterOne plugin of the Cytoscape software (version 3_5_0_64bit; http://www.cytoscape.org/php) to obtain the significant (*P* < 0.05) network clusters/subnetworks. The nodes of the significant clusters were colored as green and red for the up- and downregulated SDE genes, respectively, in the buffalo liver on the 15th day of early postpartum.

In addition to the networks, the hub genes were identified within the networks according to the graph theory for an undirected PPI network. Particularly, the gene or node was considered as a hub when it showed at least the degree of centrality as 3. The degree of centrality $Cd(i) = deg(i) = |N(i)|$, where $deg(i)$ is the degree of node (i), and $|N(i)|$ is the number of neighbor nodes interacted with the node (i) (Pavlopoulos et al.

2011). The hub genes were visualized by using the betweenness centrality parameter in the cytoscape software.

Results

Serum free fatty acid levels and milk yield

Serum FFA levels were significantly (*P* < 0.05) higher (> 100 μM) in early lactating buffaloes than heifers until day 42 of the postpartum (Fig. 2). They reached a peak of 262.49 ± 46.16 μM in the second week of early postpartum. The higher serum FFA could be due to the higher milk yield in early lactating buffaloes. An average weekly milk yield was 55.33 ± 7.75 kg to 80 ± 3.05 kg per animal from weeks 2 to 10 of the lactation. A total average milk yield was 1257 ± 102.52 kg /animal in 22 weeks of the lactation (Supplementary Fig. 2).

RNA and NGS data quality

The isolated RNA with a minimum RIN (RNA integrity number) value of 6.5 (Supplementary Table 1) produced a good quality NGS data with the FastQC quality score of 30 (Supplementary Fig. 3). A minimum of 54 million reads with a read length of 101 bases and a GC content of 47% were obtained from each liver sample (Supplementary Table 2). More than 84% of the reads were aligned to the *B. taurus* genome with a minimum of 77% concordant pair alignment. In addition, more than 50% correlation was observed between the qPCR and NGS data for the expression dynamics of five randomly selected genes (Supplementary Tables 3 and 4, Supplementary Fig. 4). Overall, the assembly statistics and

Fig. 2 Serum free fatty acids (FFA) acid levels in heifers and during early postpartum of buffaloes. The x-axis indicates postpartum days and y-axis shows FFA levels in micromolars (uM). The FFA levels of lactating buffaloes are significantly higher than heifers until 47–49 days, indicating the restriction of negative energy balance to the first 50 days of postpartum in buffaloes. No significant difference in FFA levels between heifers and lactating buffaloes during 19th–21st days was due to an outlier heifer animal affected by social dominance for feed intake in a group housing system during that period
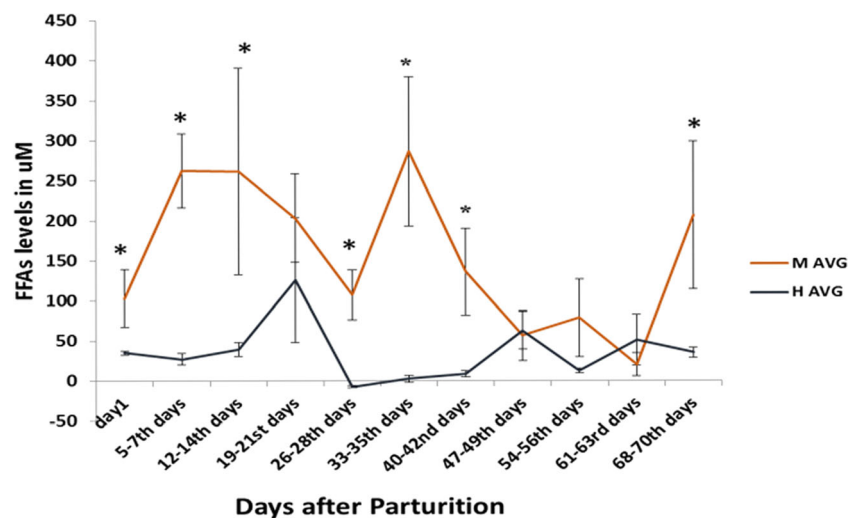
correlation with qPCR indicated that the RNA-seq data was of the best quality in this study.

Differential expression analysis of NGS data

The NGS data of control, 15th, and 30th days of lactation were comparable due to their similar distribution and dispersion of FPKM values (Supplementary Figs. 5, 6, and 7). Among a total of 509 SDE genes, 10, 33, and 22 were expressed uniquely in control, the 15th, and the 30th days of lactation, respectively; 149 were common among the three groups; 129 were common between control and the 15th day of lactation; 77 were common between control and the 30th day of lactation; and 89 were common between 15th and 30th days of lactation (Fig. 3).

Expression dynamics of SDE genes with a log2FC ≥ 3

Expression dynamics of SDE genes with a log2FC ≥ 3 revealed that the liver becomes immune tolerant with less regenerative capability on the 15th day of early postpartum in buffaloes. Among all SDE genes, 16 were highly differentially expressed with log2FC of ≥ 3 on the 15th than the 30th day of lactation and control (Fig. 4). Eight among 16 genes, majorly involving in antigen presentation (e.g., *LOC768255* or *GIMAP4-like*, *BOLA-DQA5, BOLA-DQB* and *USH1C*), acute

phase response (e.g., *M-SAA3.2* and *SAA3*), organic substance transport (e.g., SLC22A16), and detoxification (LOC100138641 or GSTA1), were downregulated on the 15th day of lactation. On the contrary, the *ECEL1* gene associated with lower regenerative capacity of the liver was upregulated on the 15th day of lactation.

Functional annotation and pathway analyses of all SDE genes

Functional annotation revealed the majority of the SDE genes were involved in biological processes, like biological regulation and metabolism, through molecular functions, like the binding of proteins, ions, and nucleic acids on the cellular membrane, nucleus, and vesicles (Supplementary Figs. 8 and 9). Specifically, 491 genes were annotated and 306 of them were uncharacterized using the bovine genome. Similarly, 335 genes were annotated and 42 of them were uncharacterized with the human genome.

Pathway analyses identified that the annotated SDE genes in the top 10 pathways belonged to immunity, steroid biosynthesis, fat, and vitamin metabolism (Figs. 5 and 6). Among these major pathways, highly significantly (FDR *P* value < 0.05) annotated pathways were hematopoietic cell lineage, *Staphylococcus aureus* infection, and
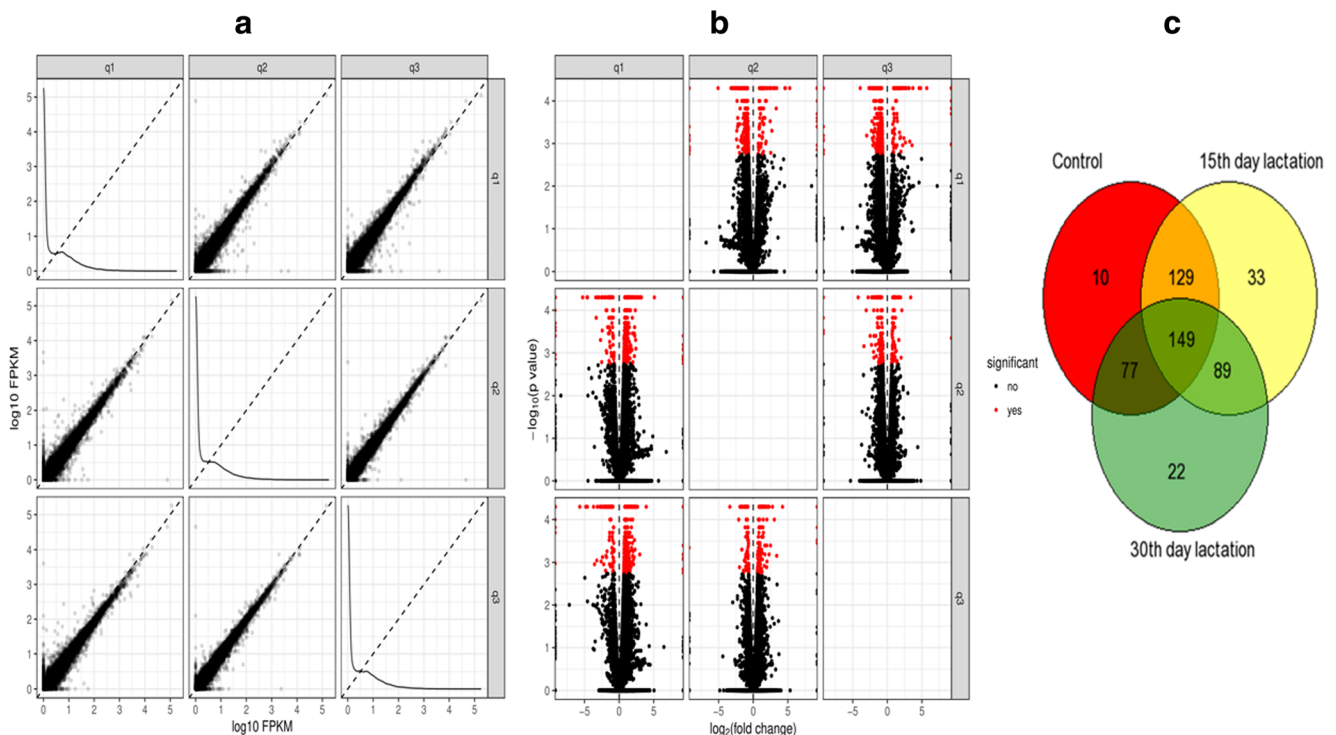

Fig. 3 Differential expression of genes in the liver samples among heifers or control (q1), 15th (q2), and 30th days of lactation (q3). **a** Scatter matrix dots above and below the diagonal represent significantly differentially expressed genes. **b** Volcano plots represent significantly differentially genes on the basis of *P* values. The red dots represent significantly differentially expressed genes. **c** Venn diagram representing

significantly differentially expressed (SDE) genes with fold change > 2 and *q* value < 0.05. A total of 10, 33, and 22 SDE genes are uniquely expressed in control, 15th, and 30th days of lactation, respectively; 149 SDE genes are common among all the groups; 129, 77, and 89 SDE genes are common between the control and 15th day, control and 30th, and 15th and 30th day lactations, respectively
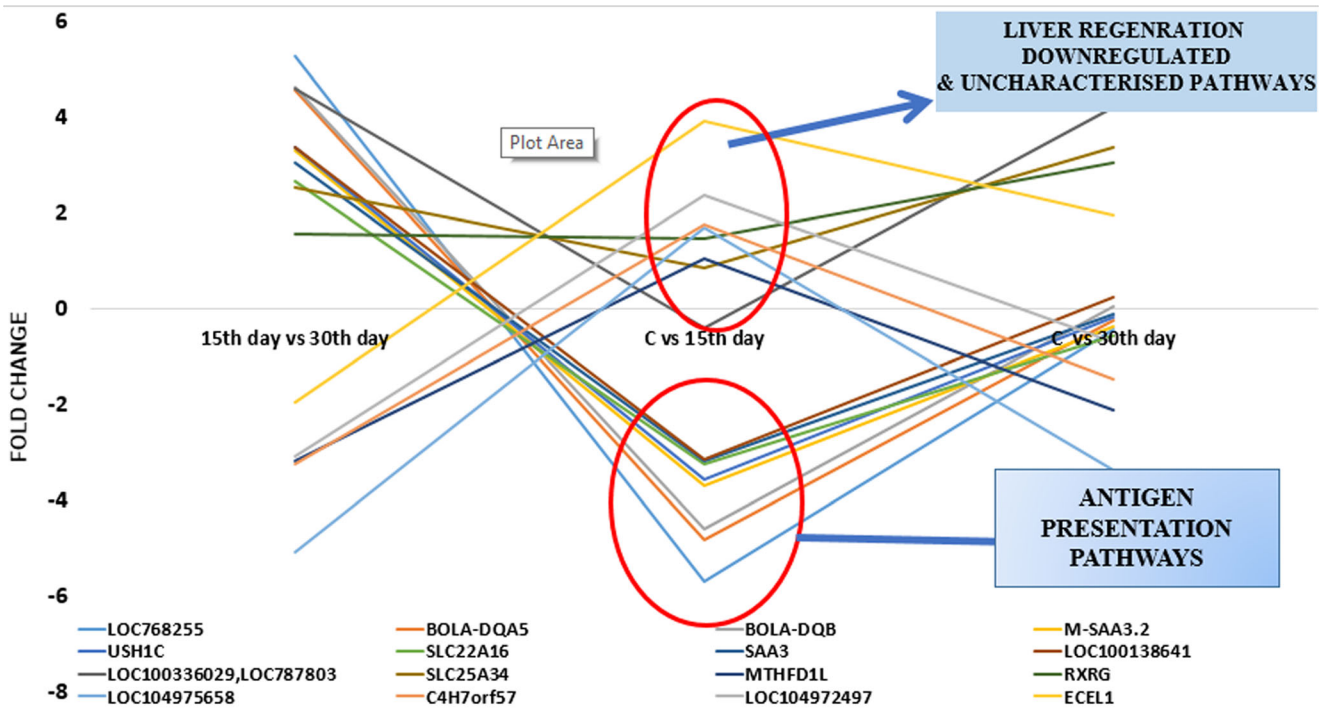
Fig. 4 Expression dynamics of highly significantly differentially expressed genes (log2_foldchange > 3). The genes involved in antigen presentation pathways (e.g., BOLA-DQA5) were downregulated, and the pathways for lower liver regeneration were upregulated on the 15th day of lactation

PPAR-Υ signaling. The SDE genes involved in the hematopoietic cell lineage and *S. aureus* infection pathways were antigen-presenting genes (e.g., *BOLA-DQA2*, *BOLA-DQA5*, and *BOLA-DQB*) which showed a downregulation on the 15th day of lactation (Supplementary Figs. 10 and 11). However, the SDE genes involved in the PPAR-Υ signaling pathway (e.g., *ANGPTL4*, *APOA1*, *APOA5*, *SCD*, and *UBC*)
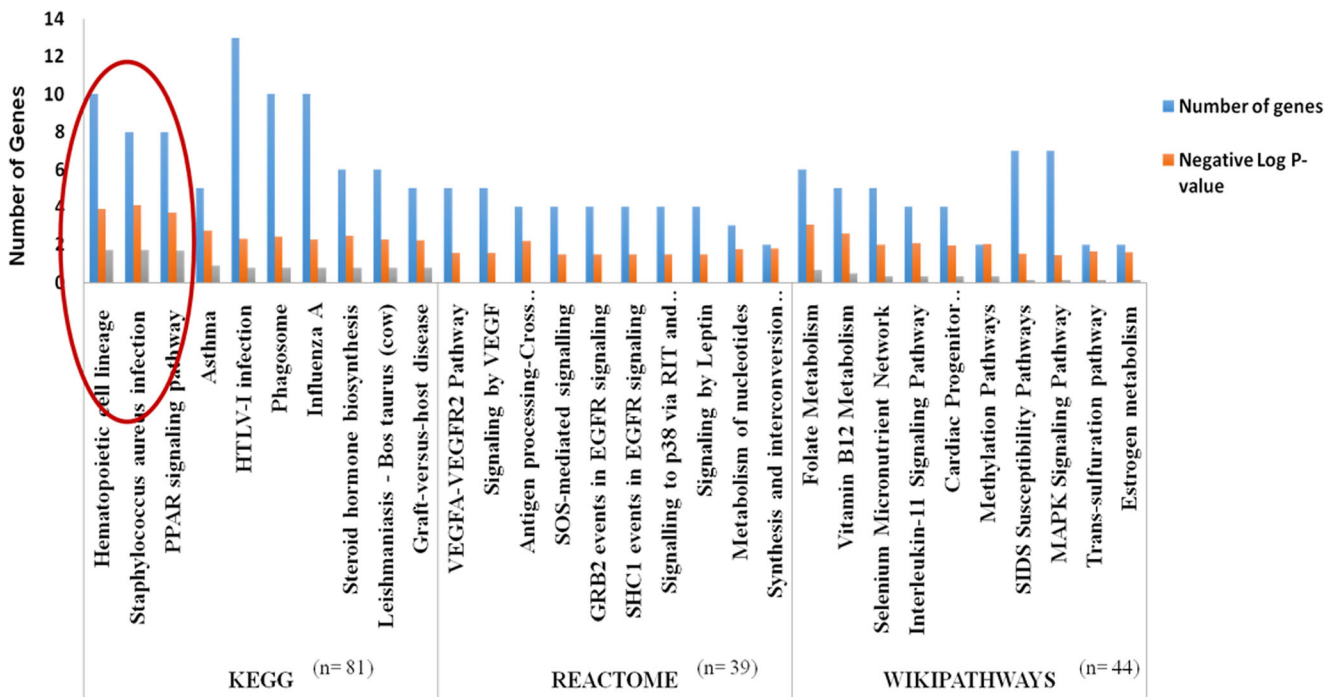

Fig. 5 Pathway analysis of all SDE genes. The top 30 selected pathways from KEGG, Reactome, and Wikipathways are presented. Significant pathways with FDR < 0.05 were encircled
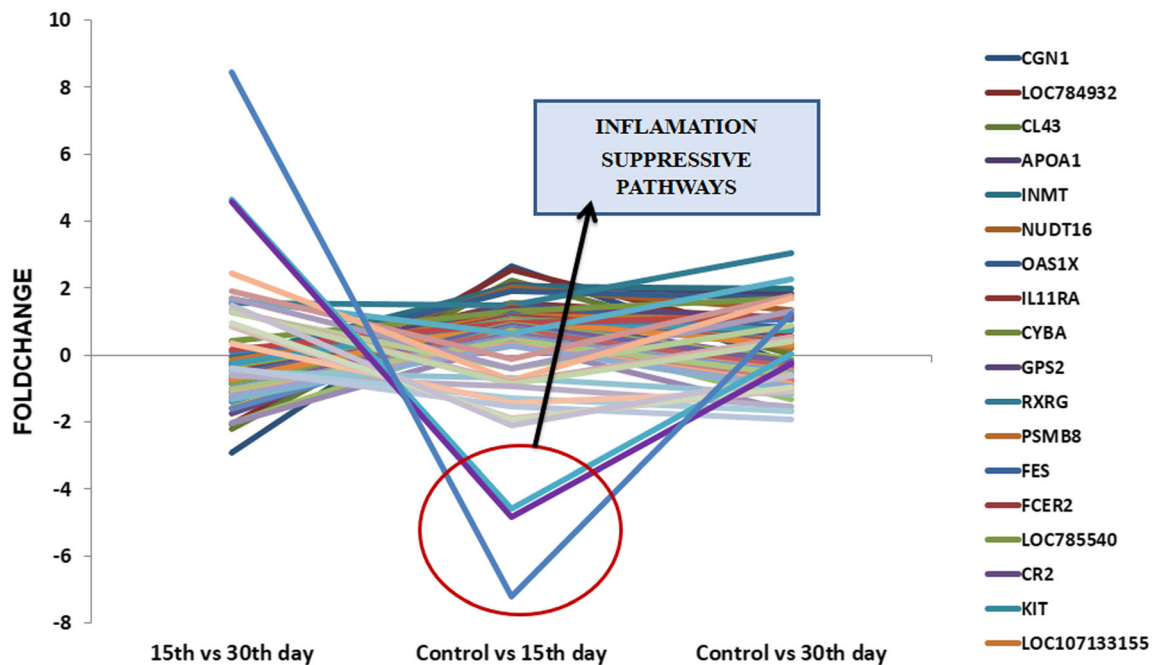
Fig. 6 Expression dynamics of the genes annotated by pathway analyses with KEGG, Reactome, and Wikipathways. The genes involved in inflammation suppressive pathways are active or the genes involved in antigen presentation were downregulated on the 15th day of lactation

(Supplementary Fig. 12) showed a similar expression pattern on the 15th and the 30th days of lactation.

Functional annotation of unique SDE genes

Among 509 SDE genes, 10 were uniquely expressed in the control, indicating their downregulation during early postpartum. Functional annotation of these genes (Supplementary Table 6) showed their involvement in normal immune or inflammatory functions, like cytokine activity (e.g., SECTM1A), natural killer cell-mediated cytotoxicity (e.g., SH2D1A), T cell receptor proteins (e.g., LOC789452), and other inflammation-promoting genes like prostaglandins (e.g., PTGES and LOC617406). Therefore, downregulation of these genes indicated the liver immune tolerance to self-antigens during early lactation of buffaloes.

Functional annotation of the unique genes expressed in the liver on the 15th day of lactation also supported the liver immune tolerance mechanisms against fat-induced inflammation and accumulation. A total of 33 SDE genes were uniquely expressed in the buffalo liver on the 15th day of lactation (Supplementary Table 7). Interestingly, 14 of them were non-coding RNA (ncRNA) emphasizing their role in liver immune tolerance during early postpartum. Among the remaining 19, 14 were annotated to be involved in immune response (e.g., T cell receptor (TCR) subunits like *LOC100296286*, *LOC100300282*, etc.), natural defense mechanisms (e.g., defensins and *IFI27L2*), mild apoptosis (e.g., *CCDC103*), and the regenerative capability of the liver against inflammatory damage (e.g., FGF10) and fatty liver (e.g., BGLAP).

Functional annotation of the 18 among 22 uniquely expressed SDE genes in the liver on the 30th day of lactation (Supplementary Table 8) signified the increased regeneration capacity (e.g., *HRAS*, *GEFR*), immune function (e.g., *P2Y*, *LOC100848101*, *TRNAR-CCG*, *LOC615055*, and *LOC788915*) and the detoxification potential (e.g., *LOC107131362*) of the buffalo liver. Further, the annotation of these uniquely expressed SDE genes emphasized the synthesis of collagen (e.g., *SERPINE2*), phospholipid, and cardiolipin (e.g., *TPGS1*) and the production of hepatokines (e.g., *ASIP* and *LOC101902932*) that are involved in decreasing the lipolysis in adipose tissue.

Functional annotation of common SDE genes among control and early lactation days

Functional annotation of the common SDE genes among control, 15th, and 30th days of lactation further strengthened the immune tolerance, fat uptake and controlled fat accumulation, lower gluconeogenesis from amino acids, and lower taurine synthesis in the liver during early postpartum of buffaloes. A total of 149 SDE genes were common among control, 15th, and 30th days of lactation. Among them, 17 and 11 SDE genes were consistently upregulated and downregulated, respectively, in the buffalo liver on the 15th and 30th days of lactation than the control (Fig. 7, Supplementary Tables 9 and 10). The upregulated genes (Supplementary Table 9) represented the immune tolerance (e.g., C8G and IFITM3), fat uptake (e.g., APOA1), lipid accumulation regulation (e.g., ASPG, INSIG1, SPON2), medium-chain fatty acid oxidation
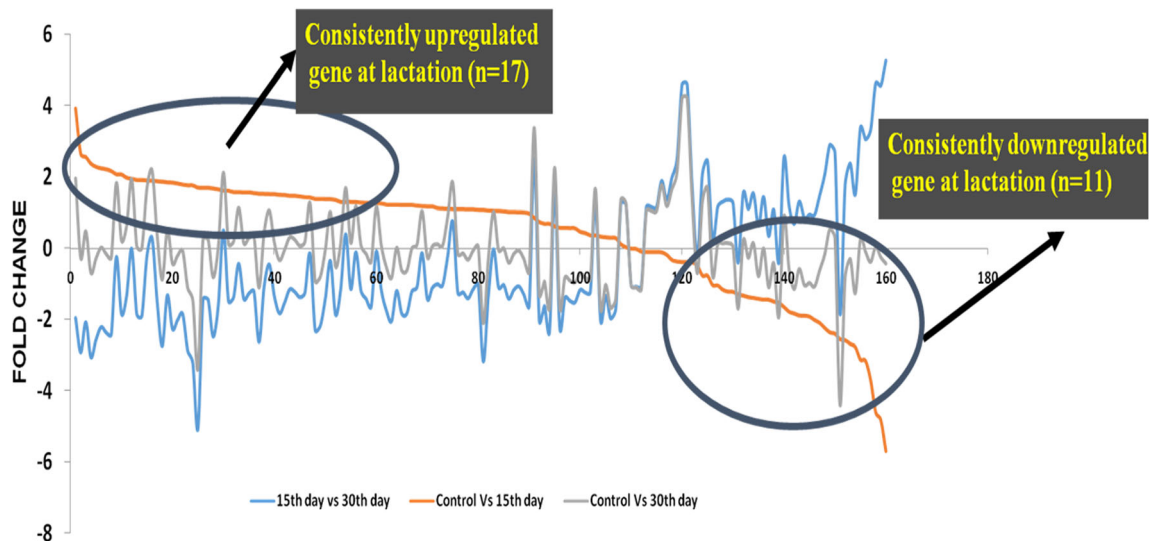
Fig. 7 Expression dynamics of common genes among control, 15th, and 30th days of lactation. A total of 17 genes were consistently upregulated on 15th and 30th days of lactation and a total of 11 genes were consistently downregulated on 15th and 30th days of lactation

(e.g., *ACSM3*), microsomal fatty acid metabolism (e.g., *LOC785540*), hypertriglyceridemia (e.g., *LOC107133155*), and lower regeneration capacity (e.g., *ECEL1* mainly on 15th day of lactation). Consistently downregulated genes on the 15th and 30th days of lactations (Supplementary Table 10) showed immune suppression (*C4A*, *SAA4*, and *LOC100337213*), decreased unsaturated fatty acid biosynthesis (e.g., *SCD*), gluconeogenesis from amino acids (e.g., *DDO*), taurine biosynthesis (e.g., *CSAD*), and the promotion of steatohepatitis (e.g., *MT2A* and *MT1E*).

Functional annotation and pathway analysis of common SDE genes between the 15th and 30th days of early postpartum

A total of 89 SDE genes were common between 15th and 30th days of early postpartum in buffaloes. Similar to the functional annotation results of total SDE genes, these genes are also involved in the biological regulation and metabolism through protein binding, ion binding, and nucleic acid binding on the cellular membrane, nucleus, and vesicles (Supplementary Figs. 13 and 14). Specifically, 87 were functionally annotated with the bovine genome background and 48 of them were uncharacterized. Similarly, 79 of the genes were functionally annotated with the human genome background and 9 of them were uncharacterized. Among 89 SDE genes, 72 were upregulated (Supplementary Table 11) and 12 were downregulated (Supplementary Table 12) with FC > 2 on the 15th than the 30th day of early postpartum. The remaining 5 genes had a FC < 2 between the 15th and 30th day, but with a FC > 2 than control.

Pathway analyses using KEGG, REACTOME, and WIKIPATHWAYS databases identified that the 72 upregulated SDE genes on the 15th than the 30th day of lactation were majorly involved in lipid transport (e.g., *APOL3, ABCA9*),

butyrate metabolism (e.g., ACSM5), hydrolysis of deacylated phospholipids (e.g., *GDPD1*), m-TOR signaling pathway (e.g., *DEPDC5*), mitochondrial inner membrane complex maintenance (e.g., *MINOS1*), spingolipid signaling pathway (e.g., *S1PR1, PIK3CD*), IGF-1 transport (e.g., *IGFBP1*), insulin resistance (e.g., *LOC511161*), and regulation of NF-KB signaling (e.g., *NKIRAS2, LURAP1*L) (Supplementary Table 11). Complimentarily, the 12 downregulated SDE genes on the 15th than 30th day of lactation are also involved in spingolipid metabolism (e.g., SPTCL3), TGF beta signaling (e.g., *KLF10*), negative regulation of phagocytosis, mast cell and dendritic cell activation (e.g., *LOC536097*), mitochondrial DNA replication, and ROS production regulation (e.g., *SLC25A33*) (Supplementary Table 12).

Network analysis of total SDE genes

Network analysis also showed that buffalo liver become immune tolerant with a fatty liver condition during early postpartum, especially on the 15th day of lactation. Specifically, the STRING software with the *B. taurus* genome background produced a very highly significant ($P < 8.43E-07$) network with 380 nodes and 361 edges with an average node degree of 1.9 and a local clustering coefficient as 0.304 (Supplementary Fig. 15). Further analysis of the STRING output by ClusterOne plugin visualized 10 significant ($P < 0.05$) clusters or subnetworks (Fig. 8). The differential expression of the nodes or genes on the 15th day of lactation in each subnetwork depicted the downregulation of antigen presentation pathways (subnetwork 1), acute phase response (subnetwork 4), helper T cell differentiation (subnetwork 7), and neutrophil activity (subnetwork 8), as well as the upregulation of interferon-induced immune response and apoptosis (subnetwork 3), and lysosome trafficking and autophagosome pathways (subnetwork 5). This
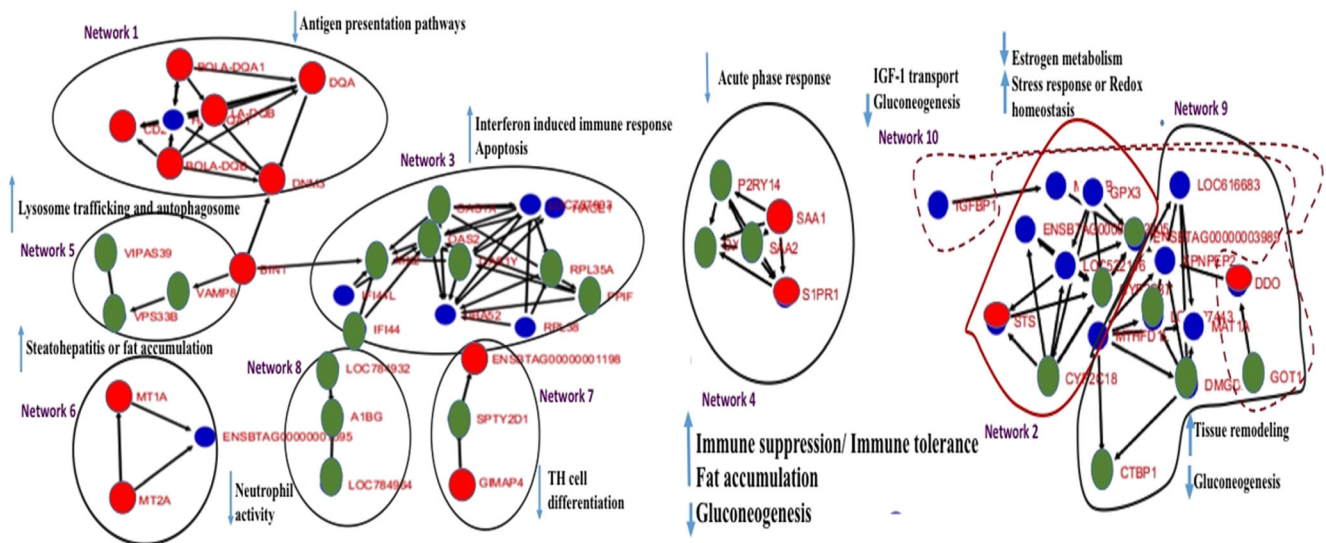
Fig. 8 Significant (P < 0.05) clusters or subnetworks obtained from ClusterOne plugin in the Cytoscape tool. Each node indicates a gene. The green color indicates upregulation and the red color indicates downregulation of the genes on the 15th day of lactation. All the 10

networks together indicate immune tolerance, fat accumulation, and decreased gluconeogenesis from amino acids in buffalo liver during early postpartum

observation indicated the liver immune tolerance on the 15th day of early postpartum. In addition, differential expression of the nodes or genes on the 15th day of lactation showed an increased stress response and redox homeostasis (subnetwork 2), fat accumulation or steatohepatitis pathways (subnetwork 6), and tissue remodeling (subnetwork 9), as well as decreased gluconeogenesis from amino acids (subnetworks 9 and 10). These observations revealed a fatty liver condition with lower gluconeogenesis from amino acids on the 15th day of lactation in the buffalo liver.

Hub gene analysis also emphasized the immune tolerance mechanism as a key hub in liver metabolism during early postpartum on the 15th day of lactation in buffaloes. A total of 41 hub genes interacting at least 3 other nodes were identified in the PPI networks (Fig. 8, Supplementary Figs. 16 and 17, Supplementary Table 13). The expression dynamics of all hub genes further confirmed the downregulation of antigen presentation and acute phase response (Supplementary Fig. 18). In addition, the hub genes with more than 10 interacting partners mainly involved in the regeneration of liver (e.g., *HRAS* and *ACTA2*), one carbon metabolism (e.g., *MTHFD1L*), and innate immune response (e.g., *OAS1* and *OAS2, PIK3CD*) (Supplementary Table 13).

Discussion

The present work targeted to understand the liver adaptive mechanisms at a transcriptome level during early postpartum by considering dairy buffaloes as experimental models. As females show NEB during this period, serum FFA were considered as NEB biochemical indicators. The peak serum FFA

levels (262.49 ± 46.16 μM) in the second week of early postpartum of buffaloes (Fig. 2) were lower than earlier reported (0.48 mmol/l) (Bertoni et al. 1997) due to the use of a colorimetric method than a sensitive RIA (Bertoni et al. 1997). The serum FFA levels were generally less than 50 μM in heifers depending on the feeding status. The higher serum FFA during early lactation could be due to fat mobilization from adipose tissue to meet the demands of higher milk yield (1257 ± 102.52 kg for 22 weeks, Fig. 6) (Accorsi et al. 2005; Koltes and Spurlock 2011). However, similar serum FFA levels between lactating buffaloes and heifers by the 47th–49th days of postpartum indicated that early lactating buffaloes recovered from NEB by certain physiological adaptations.

Liver immune tolerance is one of the adaptation mechanisms to suppress the inflammation and sustain the integrated metabolism for meeting the energy demands of early postpartum. The current transcriptome data, especially the dynamics of SDE with a log 2 FC > 3, clearly demonstrated the liver immune tolerance mechanism on the 15th day of early postpartum. Particularly, the downregulation of antigen presentation (e.g., *BOLA-DQA5, BOLA-DQB,* and *USH1C*), acute phase response (e.g., *M-SAA3.2* and *SAA3*), organic substance transport (e.g., SLC22A16), and detoxification (LOC100138641 or GSTA1) processes on the 15th day of lactation (Fig. 4) represented the liver immune tolerance mechanism (Crispe 2014) for self-antigens, like higher serum FFA. On the contrary, upregulation of the *ECEL1* gene in the liver on the 15th day of lactation indicated the c-jun suppression (Fernandes et al. 2013), an indication of an impaired liver regeneration (Behrens et al. 2002).

Functional annotation and pathway analyses of all SDE genes also emphasized the immune tolerance with a mild fatty

liver condition as an adaptive mechanism in early postpartum. The highly significantly (FDR *P* value < 0.05) annotated pathways were hematopoietic cell lineage, *S. aureus* infection, and PPAR-Υ signaling (Figs. 5 and 6). The downregulation of antigen-presenting genes (e.g., *BOLA-DQA2*, *BOLA-DQA5* and *BOLA-DQB*) of the hematopoietic cell lineage and *S. aureus* infection pathways on the 15th day of lactation (Supplementary Figs. 10 and 11) supported the liver immune tolerance. However, a similar expression pattern of the SDE genes involved in the PPAR-Υ signaling pathway on the 15th and 30th days of lactation indicated a mild fatty liver condition (Supplementary Fig. 12). Particularly, the upregulation of liver *ANGPTL4* might have promoted the lipolysis in adipose tissue during early postpartum of buffaloes akin to Holstein cows (Loor et al. 2007). Similarly, the upregulation of *APOA1* and *APOA5* might have enhanced the HDL production and maturation for increasing the cholesterol uptake from peripheral tissues (Qu et al. 2007) http://www.genecards.org/cgi-bin/carddisp.pl?gene=APOA1). However, decreased *SCD* and *UBC* indicated lower synthesis of unsaturated fatty acids and cellular turnover in the liver (Loor et al. 2007).

The unique expression of the T cell receptor (TCR) subunits (e.g., *LOC100296286*, *LOC100300282*, etc.) on the 15th day of lactation provided a clue for an increased activity of the regulatory T cells to reduce an inflammation. Further, the liver might also have a mild apoptosis on the 15th day of lactation due to the unique expression of *CCDC103*, a part of the NF-KB/TNFα signaling (Wu et al. 2017). In addition, the defensin and *IFI27L2*, which mediate natural defense mechanisms against irritants (http://www.ihopnet.org/UniPub/iHOP/pm/12755248.html?nr=6&pmid=18242016), were uniquely expressed on the 15th day of lactation. The defensin expression inhibits the Δ6- and Δ5-desaturases and alters the membrane phospholipid composition by increasing the monoenoic (mainly C18: 1ω9) and/or dienoic (C18: 2ω6) fatty acids and decreasing the polyenoic fatty acids (Ivanova et al. 2016). This was supported by the downregulation of the SCD gene on the 15th and 30th days of lactation in this study. Although higher expression of PGF synthase like 1 indicates an oxidative stress and inflammation (Marone et al. 2016), its expression in the liver during NEB along with higher levels of serum FFA could be a protective effect against the uptake or promoting the efflux of excess cholesterol through liver X receptor alpha (Seto and Bogan 2015). Additionally, unique expression of PGF synthase like 1, thus higher production of PGF2-α, on the 15th day of early postpartum may be responsible for luteolysis, a probable reason for short luteal cycles in buffaloes during early postpartum. The unique expression of *BGLAP* on the 15th day of lactation further reinforces the liver tolerance mechanism against fatty liver condition by decreasing the oxidative stress and JNK pathway (Bao et al. 2016). Another liver tolerance mechanism on the 15th day of lactation was the upregulation of FGF10, which helps the liver to regenerate from any inflammatory damage (Steiling et al. 2003). Altogether, the liver tried to tolerate fat-induced inflammation and accumulation by promoting specific defense mechanisms on the 15th day of lactation.

Uniquely expressed SDE genes in the liver on the 30th day of lactation showed its regenerative potential during early postpartum of buffaloes. Among 22 uniquely expressed genes in the liver on the 30th day of lactation (Supplementary Table 8), *HRAS*, *GEFR*, and *SERPINE2* might be involved in liver regeneration by enhancing hepatocyte proliferation (Holczbauer et al. 2013; Sibilia et al. 2007) and collagen deposition (Li et al. 2016). The *P2Y*, *LOC100848101*, *TRNAR-CCG*, *LOC615055*, and *LOC788915* genes might be involved in the regeneration of immune function. Particularly, the P2Y signaling promotes the cell cycle and inflammatory response homeostasis, glycogenolysis, and glucose release from hepatocytes (Fausther et al. 2012). *LOC100848101* promotes the mannose-6-phosphate receptor translocation, a required step for lectin binding and lysosomal trafficking in the immune system (http://www.uniprot.org/uniprot/Q9H1H9). The *TRNAR-CCG* gene encodes arginine t-RNA and helps promote arginine incorporation into proteins for boosting the immune system (Popovic et al. 2007). The *ASIP* and *LOC101902932* genes are involved in decreasing lipolysis from adipose tissue (Xue and Zemel 2000). The *ESR1*, *TPGS1*, and *LOC107131362* genes are involved in lipoprotein and cholesterol efflux from liver (Della Torre et al. 2016), phospholipid, and cardiolipin synthesis (Osman et al. 2010), and enhancing the liver detoxification potential (Rowland et al. 2013), respectively.

Among the consistently upregulated common SDE genes (Supplementary Table 9) on the 15th and 30th days of early lactation in the buffalo liver, the upregulation of *C8G*, a component of the complement system, indicated the clearance of apoptotic bodies produced by mild apoptosis (upregulation of *CCDC103*), thereby enhancing the immune tolerance mechanism (Belot and Cimaz 2012). Similarly, *IFITM3* limits the host damage and provides resistance to viral infections (Iwasaki and Pillai 2014). As a part of a tolerance mechanism against lipid accumulation, the buffalo liver enhanced the upregulation of the *ASPG*, *INSIG1*, and *SPON2* genes. *ASPG* maintains hepatic triglycerides through triggering amino acid response by activating the *GCN2* gene (Wilson et al. 2015). Similarly, *INSIG1* regulates lipid accumulation by restricting the SREBP within the endoplasmic reticulum, prevents its movement to the Golgi complex for further proteolytic activation, and inhibits the expression of cholesterol and triacylglycerol biosynthesis enzymes (Ng et al. 2014). Likewise, *SPON2* controls hepatic steatosis by interacting with PPAR-α (Zhu et al. 2014). Consistent downregulation of the *CSAD*, an indicator of the lower taurine synthesis (Supplementary Table 10), may influence the reproductive potential of buffaloes during early postpartum. Since taurine

is a natural antioxidant produced by the liver, its levels are generally found high in thecal cells and oocytes of dominant ovarian follicles (Orsi et al. 2005).

Functional annotation and pathway analyses of the SDE genes common between the 15th and 30th days of early postpartum in buffaloes lead to interpret an increased fat accumulation by lipid uptake and metabolism, and mild controlled inflammation of the liver on the 15th day of lactation. Interpretation towards the mild inflammation of the liver was due to a balance between the promotion and inhibition of NF-KB signaling by the upregulation of *LURAP1L* and *NKIRAS2*, respectively; the downregulation of *KLF10*, thus TGF beta signaling; and the downregulation of *LOC536097*, a key negative regulator of phagocytosis and the activation of mast cells and dendritic cells. However, downregulation of the *BOLA* genes with FC > 6 strongly supports towards the immune tolerance than inflammation in the buffalo liver on the 15th day of early postpartum. Supporting the lipid accumulation and increased serum free fatty acids, the buffalo liver showed essential insulin resistance (e.g., upregulation of *LOC511161*) on the 15th day of lactation. It is well known that fat accumulation in the liver is always associated with

hepatic and systemic insulin resistance as observed in human nonalcoholic fatty liver disease (Utzschneider and Kahn 2006) and early lactating dairy cattle (Chalmeh et al. 2015). As explained above, the liver is the exclusive organ supplying glucose for milk synthesis in ruminants (Drackley et al. 2001). Therefore, insulin resistance in the ruminant liver is a kind of physiological mechanism to promote extensive gluconeogenesis in the liver and lipolysis in the adipose tissue during early lactation. Interestingly, downregulation of one of the gluconeogenesis genes involved in aspartate metabolism (e.g., DDO) on the 15th day of lactation indicated that the buffalo liver majorly depends on fatty acid metabolites, like propionic acid, for gluconeogenesis.

Network analyses also support the presentation of tolerance mechanisms in the liver during early postpartum. Particularly, the differential expression of the nodes/genes in the PPI subnetworks 1, 3, 4, 5, 7, and 8 indicated the liver immune tolerance on the 15th day of early postpartum. Further, the differential expression of the nodes/genes on the PPI networks 2, 6, 9, and 10 revealed the mild fatty liver condition with lower gluconeogenesis from amino acids on the 15th day of lactation in the buffalo liver. These findings are corroborating with the
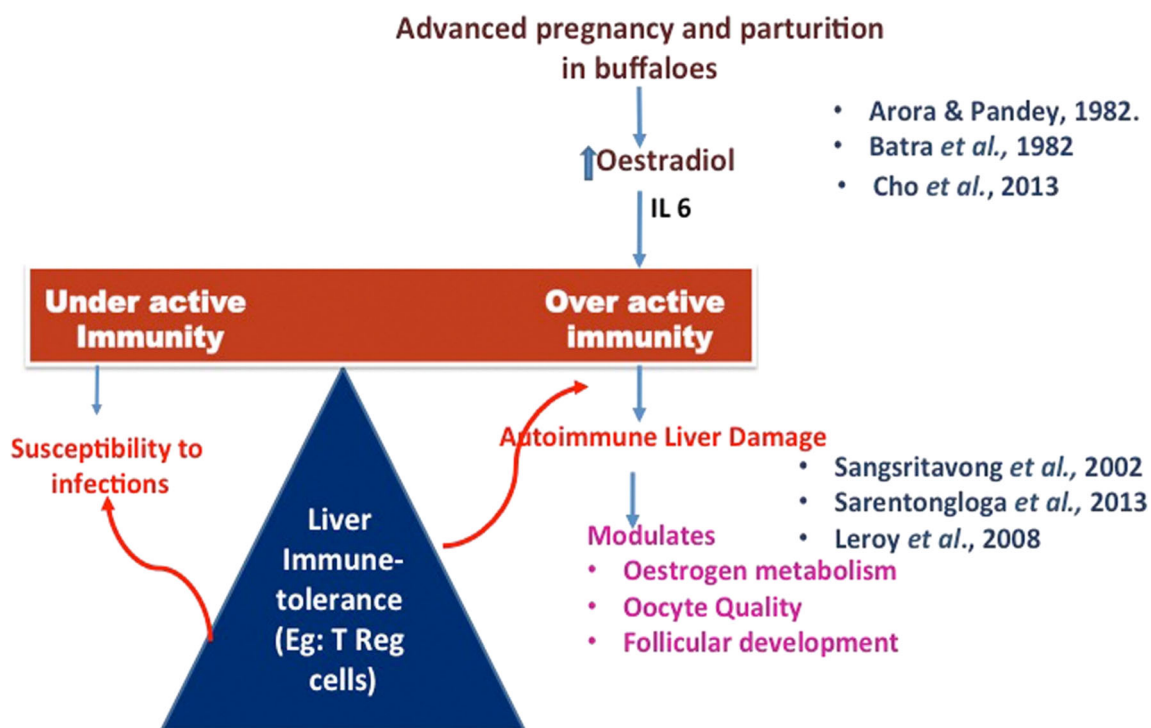

Fig. 9 Proposed hypothesis on liver immune tolerance and postpartum reproductive function in buffaloes. Liver immune tolerance is a balancing mechanism between over- and underactive immunity. As the liver is the key filter organ between the intestine and the rest of the body for several xenobiotics, including microbial antigens, immune tolerance mechanism should be stronger in this organ to maintain animal health. Underactive immunity leads to susceptibility to infections, and overactive immunity leads to autoimmune liver damage. Higher estradiol levels during advanced pregnancy and parturition would increase IL6, which can cause overactive immunity leading to autoimmune liver damage. Such liver damage can compromise female reproduction through the modulation of estrogen metabolism, oocyte quality, and follicular development. Hence, the animals that can maintain liver immune tolerance during early postpartum can resume reproductive function efficiently as in the present study. On the contrary, variations in the liver immune tolerance may affect the postpartum reproductive function in buffaloes. Further studies are required to prove this hypothesis

liver gene networks reported for Holstein cows during early postpartum (Loor et al. 2007). This network analysis leads to a conclusion that the buffalo liver during early postpartum, especially on the 15th day of lactation, becomes immune tolerant with a mild fatty liver condition.

The expression dynamics of all hub genes further confirmed the downregulation of antigen presentation and acute phase response (Supplementary Fig. 18). This emphasizes the immune tolerance mechanism as a key hub for liver metabolism during early postpartum on the 15th day of lactation in buffaloes. As the hub genes with more than 10 interacting partners are mainly involved in the regeneration of the liver (e.g., *HRAS* and *ACTA2*), one carbon metabolism (e.g., *MTHFD1L*), and innate immune response (e.g., *OAS1* and *OAS2*, *PIK3CD*) (Supplementary Table 13), it can be emphasized that the immune tolerance mechanisms might be the key players during early postpartum in buffalo liver.

Liver immune tolerance is an important mechanism to maintain even normal animal reproduction during early postpartum. It is well known that estradiol levels would be higher at the time of advanced pregnancy and parturition in buffaloes (Arora and Pandey 1982; Batra et al. 1982). Higher estradiol levels were found to enhance IL6 and reduce the T-reg immune cells (Cho et al. 2013), resulting in either autoimmune liver injury (Longhi et al. 2004, 2005) or fatty liver (Ma et al. 2007). It was observed in earlier studies that liver injury modulates estrogen metabolism (Sangsritavong et al. 2002), oocyte quality (Sarentonglaga et al. 2013), and follicular development (Leroy et al. 2008). Hence, the buffaloes that can maintain the liver immune tolerance, for example promoting T-reg cell function, during early postpartum can balance the under- and overactive immunity and prevent much liver injury to resume the reproductive function more efficiently. The lactating animals in the present study resumed their estrous cycles within 50 days of postpartum. Hence, the animals having variation in liver immune tolerance mechanisms may be affected by compromised postpartum reproductive function. Future studies are required to prove this hypothesis proposed from this study (Fig. 9).

Conclusion

Buffalo liver transcriptome analysis during early postpartum showed that the immune tolerance could be the major adaptive mechanism in the liver during early postpartum in buffaloes. Liver immune tolerance is a required process not only for efficient function of the liver to meet the demands of early lactation but also to maintain the reproductive function of animals as the liver is the primary site for steroid metabolism. However, more experimental evidence is required to prove this hypothesis in future studies.

Authors' contributions SKO and DS designed and organized the research work. SS, NG, and DaS performed experiments. SS and SKO analyzed the data. SS, DS, and SKO wrote the manuscript.

Compliance with ethical standards

A total of six healthy buffaloes considered in the present study were approved by the Institutional Animal Ethics Committee of the National Dairy Research Institute, Karnal (Approval no. 95/16).

Conflict of interests The authors declare that they have no conflict of interests.

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