Comparative Proteome Profiling of Saliva Between Estrus and Non-Estrus Stages by Employing Label-Free Quantitation (LFQ) and Tandem Mass Tag (TMT)-LC-MS/MS Analysis: An Approach for Estrus Biomarker Identification in Bubalus bubalis

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Functional annotations of GO terms showed enrichment of glycolysis, pyruvate metabolism, endopeptidase inhibitor activity, salivary secretion, innate immune response, calcium ion binding, oocyte meiosis, and estrogen signaling. Over-expression of SERPINB1, HSPA1A, VMO1, SDF4, LCN1, OBP, and ENO3 proteins during estrus was further confirmed by Western blotting. This is the first comprehensive report on differential proteome analysis of buffalo saliva between estrus and non-estrus stages. This study generated an important panel of candidate proteins that may be considered buffalo estrus biomarkers which can be applied in the development of a diagnostic kit for estrus detection in buffalo.

Keywords: biomarker, estrus, label-free quantitation, LC-MS/MS, tandem mass tag, saliva
1 INTRODUCTION

Saliva is an important diagnostic body fluid since it contains explicit biomolecules such as metabolites, hormones, mRNA, miRNA, DNA, and proteins that may be a good source of biomarkers of health and disease (Levine, 1993). Saliva consists of secretion from serous and mucous acinar cells of salivary glands and a small portion originating from the blood (Edgar, 1992; Pedersen et al., 2002). Dietary and physiological variations bring changes in the salivary composition (Prout and Hoppins, 1970; Mass et al., 2002). Furthermore, its stable nature, high biological half-life, and non-invasive approach make it preferable to other body fluids for biomarker discovery (Li et al., 2004; Archunan et al., 2014; Shashikumar et al., 2017). Proteomics using a mass spectrometer enables the identification of new biomarker proteins in a variety of bodily fluids, including saliva (Almeida et al., 2021). In recent past years, salivary proteomics has led to the discovery of biomarkers for several disease conditions such as oral squamous cell carcinoma (OSCC) (Jou et al., 2014; Wang et al., 2015), autoimmune disease (Castagnola et al., 2017), and genetic diseases such as Down’s syndrome (Cabras et al., 2013) in humans. Unlike human saliva, the characterization of salivary proteins of farm animals has been little studied. A global proteome analysis of bovine saliva was carried out by Ang et al. (2011) using both non-targeted and targeted proteomics techniques to identify only extracellular proteins. They identified 402 proteins and 45 N-linked glycoproteins in bovine saliva with enrichment of low-abundance proteins.

Buffalo (Bubalus bubalis) is a premier livestock species that contributes immensely to milk and meat production (20th Livestock Census, 2019). Nevertheless, specific inherent reproductive problems such as delayed puberty, silent or poor expression of estrus signs, and seasonal anestrus compromise its reproductive efficiency and production potential. Accurate and efficient identification of estrus is essential for successful conception and efficient reproduction management of farm animals. However, estrus detection is difficult in buffaloes because of non-manifestation of overt signs of estrus and higher incidences (29%) of silent estrus, especially during the summer season (Singh et al., 2000; Roy and Prakash, 2009). This leads to difficulty in estrus detection, improper insemination time, and conception failure in buffaloes (Sharma et al., 2008; Das and Khan, 2010). In addition, various estrus detection tools, such as teaser bull parading, tail painting, KaMar heat mounting detector, behavioral monitoring using a closed circuit television, and activity monitor using a pedometer, were used in buffaloes that are not very efficient since their sensitivity and specificity vary widely (Selvam and Archunan 2017). Therefore, discovering estrus biomarkers in easily accessible body fluids such as saliva is of utmost importance for developing an easy, reliable, and accurate estrus detection method for buffaloes.

Saliva possesses biomarker potential to detect fertile periods in mammals. Saibaba et al. (2021) reported mass spectrometric analysis of salivary proteome profiles of women during the fertile phase of the menstruation cycle as characterized by mass spectrometry. They identified 16 unique and differentially expressed proteins during the ovulatory phase; among them, cystatin-S offers a biomarker potential. Similarly, Muthukumar et al. (2014a) reported proteome profiling of buffaloo saliva during the estrous cycle by employing in-gel digestion followed by LC-MS/MS analysis. They identified 179 proteins collectively during proestrus (PE), estrus (E), and diestrus (DE) stages, and 37 proteins are found exclusively during estrus. Among estrus-specific proteins, β-enuel, toll-like receptor (TLR)-4, clusterin, lactoperoxidase, serotransferrin, TGM3, and UBA6 proteins are the most predominant. Our previous study reported global proteome profiling of buffalo saliva during the estrous cycle (Shashikumar et al., 2018) and identified 275, 371, 304, and 565 proteins with ≥2 peptides during PE, E, ME, and DE stages, respectively. Among these, 62 proteins are identified exclusively during the estrus stage, and heat shock 70-kDa protein 1A, 17-beta-hydroxysteroid dehydrogenase type 1, inhibin beta A chain, and testin proteins are found to be the most predominant during estrus. These two studies concluded that salivary secretion contains specific proteins, and their expression varies according to the estrous cycle stages. However, differential proteome analysis using labeled quantitation [tandem mass tag (TMT)] and label-free quantitation (LFQ) coupled to mass spectrometry for estrus biomarker discovery, which is lacking in buffaloes. Therefore, the aim of the present study was to identify candidate estrus biomarkers in saliva of buffaloes. We hypothesized that identifying differentially expressed proteins (DEPs) associated with estrus could lead to the identification of estrus biomarkers in buffaloes. Thus, for the first time, we identified differentially expressed estrus-associated proteins in buffalo saliva by employing both label-free quantitation (LFQ) and labeled quantitation (TMT) coupled to high-resolution mass spectrometry (LC-MS/MS) and their validation using Western blotting.

2 MATERIALS AND METHODS

2.1 Experimental Animals

The study was carried out at the Livestock Research Centre, ICAR-National Dairy Research Institute, Karnal, Haryana. Healthy pluriparous Murrah buffaloes (n = 15) of 2–4 parity maintained under iso-managerial conditions were included for the study. The nutrient requirement of the animals was provided as per National Research Council (NRC) standards. After completing the voluntary waiting period, all animals underwent gynecological examination using per-rectal examination and transrectal ultrasonography (Model UST-5820-5). For estrus induction, buffaloes with functional corpus luteum (CL) on the ovary are administered with PGF2α (Vetmate; 500 µg I/M). Samples were collected from the next spontaneous estrous cycle, and stages of the estrous cycle were categorized as proestrus (PE; day 2 to 1 before the onset of estrus), estrus (E; day 0), metestrus (ME; day +3), and diestrus (DE; day +10). All the sampling was completed during the breeding season, that is, from November to February. All the experiments conducted in this study were approved by the Institute Animal Ethics Committee (42-IAEC-18-9).
2.2 Estrus Detection and Confirmation
Estrus in buffaloes was detected by physical observation of estrus signs. Teaser bull parading was carried out twice a day, that is, daily in the morning 5–6 AM and evening 5–6 PM for exhibition of estrus signs. The following estrus signs were observed: standing to be mounted, sniffing/licking the vulva, chin resting, flehmen’s reaction, mounting on or by other buffaloes, restlessness, bellowing, and frequent micturition. The onset of estrus is marked by expression of a typical estrus sign, that is, standing to be mounted behavior (standing estrus). The onset of estrus was further confirmed by reproductive tract examination (uterine horn tonicity, cervical relaxation, tumefaction of vulva, hyperemia, or reddening of the vulvar mucous membrane), biochemical parameters (cervicovaginal mucus crystallization, fluidity, and spinnbarkeit value (Verma et al., 2014)), and progesterone hormone estimation in blood serum. Transrectal ultrasonography (USG) using a real-time B-mode scanner equipped with a 7.5 MHz rectal probe was used to confirm further presence of dominant follicle (DF; ≥ 10 mm) and absence of CL during the follicular phase (proestrus and estrus) and the onset of ovulation and presence of CL during the luteal phase. Progesterone hormone concentration was estimated in serum samples using an ELISA kit (Cayman Chemical, United States) to confirm the stages of the estrous cycle.

2.3 Sample Collection and Processing
Saliva samples were collected from the lower jaw of the mouth of the animal by the direct aspiration method using a 20-ml syringe without a needle in sterile polypropylene tubes (Thermo Fisher Scientific, NY). All the sampling was performed in the early morning (6–7 AM) before feeding, and samples after collection were immediately transported to the laboratory on ice. Samples were centrifuged at 12,000 rpm at 4°C for 30 min to eliminate any particulate matter or feed debris. Supernatant was collected in another 1.5-ml Eppendorf tube, and protease and phosphatase inhibitor (1 x MS-SAFE; Sigma-Aldrich, United States) was added to prevent protein degradation and stored at −80°C until further analysis. Saliva samples from animals wherein estrus and non-estrus stages were confirmed through various methods were selected and processed further for proteomics analysis.

2.4 Quantification and SDS-PAGE Profiling of Saliva Proteins During Different Stages of the Estrous Cycle
Out of 15 buffaloes, seven animals showing all the cardinal signs of estrus confirmed by gynecological examination, USG, cervicovaginal mucus (CVM) parameters, and progesterone estimation were selected for further proteomics study. The protein concentration of neat saliva samples from PE (n = 7), E (n = 7), ME (n = 7), and DE (n = 7) stages were determined with a Bradford assay. Then, salivary proteins, that is, 25 µg from all stages of the estrous cycle, were taken, and initial protein profiling was carried using SDS-PAGE. Initially, proteins were resolved on a 4% stacking and 12% separating acrylamide–bis-acrylamide gel at 15 mA for 3 h using the mini-protein vertical electrophoresis system (Bio-Rad, United States). The gels were stained with Coomassie brilliant blue G (CBB) at room temperature for 1 h, de-stained for 2 h, and scanned using an Epson scanner (GE, Healthcare, United States). The initial protein profiles were identified by running the saliva proteins with a reference protein marker (RTU-BLUeye prestained protein ladder). Based on the intactness and uniformity of the bands, saliva samples from PE, E, ME, and DE stages of five animals were selected for further in-solution digestion and label-free quantification (LFQ) and labeled quantitation using TMT labels and LC-MS/MS analysis.

2.5 Label-Free Quantitation
2.5.1 Trypsin In-Solution Digestion of Proteins for LC-MS/MS Analysis
Neat saliva protein of 50 µg from PE, E, ME, and DE stages were taken from five animals, and all samples were processed separately for in-sol digestion. In brief, samples were dissolved in dissolution buffer (50 mM ammonium bicarbonate) and reduced with 50 mM dithiothreitol (Sigma-Aldrich, United States) at 50°C for 45 min. Further alkylation was carried out using 100 mM IAA at room temperature in the dark for 15 min. Trypsin digestion (Promega, Madison, WI) at 1:20 (enzyme: protein) of protein samples was carried out at 37°C for overnight, followed by quenching with 10% trifluoroacetic acid. Furthermore, tryptic-digested samples were vacuum-dried (SpeedVac concentrator, Thermo Fisher Scientific, United States) and desalted using Pierce™ C18 Spin Columns (Thermo Fisher Scientific, United States). Desalted peptide samples were vacuum-dried using SpeedVac and reconstituted in 0.1% formic acid. Finally, all 20 samples from five animals of four stages were subjected to LC-MS/MS analysis.

2.6 Labeled Quantitation Using TMT Labels
Saliva proteins (75 µg) from each stages of the estrous cycle, that is, PE, E, ME, and DE from five animals were pooled. Proteins were dissolved in dissolution buffer (50 mM ammonium bicarbonate), followed by reduction (50 mM dithiothreitol) at 50°C for 45 min s. Alkylation of cysteine residues was carried out using 100 mM IAA at room temperature in the dark for 15 min s, followed by trypsin digestion at 1:20 (enzyme: protein) at 37°C for overnight. Peptides of PE, E, ME, and DE stages were labeled with 126, 127, 128, and 129 TMT labels, respectively, using TMT 6-plex protocol. Peptides labeled with TMT tags were incubated for 3 h, quenched (5% hydroxylamine), vacuum-dried, and fractionated into eight fractions using a Pierce™ high pH reversed-phase peptide fractionation kit (Thermo Fisher Scientific, Germany), according to the manufacturer’s protocol. Peptides labeled with TMT tags were analyzed using an EASY-nLC 1,000 mass spectrometer (Thermo Fisher Scientific, United States) coupled with Thermo Fisher Q Exactive equipped with nano-ESI.
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Table 1: Behavioral signs of estrus

| Behavioral signs of estrus                        | % of animals showing estrus signs |
|--------------------------------------------------|----------------------------------|
| Standing to be mounted                           | 83.3                             |
| Cervicovaginal mucus discharge                   | 89.9                             |
| Hyperemia of the vulval mucous membrane          | 99.9                             |
| Flehmen’s response                               | 1.1                              |
| Belowing                                         | 77.7                             |
| Vulva tumefaction                                | 72.2                             |
| Frequent micturition                             | 65.1                             |
| Licking/sniffing of vulva                        | 71.1                             |
| Chin resting                                     | 68.2                             |

Figure 1: Behavioral signs of estrus in buffaloes.

Figure 2: Protein expression analysis.

Figure 3: Protein interaction network.

Table 2: Protein expression levels

| Protein expression level | % of animals showing estrus signs |
|--------------------------|----------------------------------|
| Increased               | 83.3                             |
| Decreased               | 89.9                             |
| Unchanged               | 99.9                             |

Figure 4: Protein expression analysis using Cytoscape.

Figure 5: Protein interaction network using the Search Tool for the Retrieval of Interacting Genes (STRING) database.

2.8 Data Processing

The MS/MS data were searched and analyzed with Proteome Discoverer (v2.2) against the UniProt Bos taurus reference proteome database. In Proteome Discoverer, the Sequest search engine was used; the precursor and fragment mass tolerances were set at 10 ppm and 0.5 Da, respectively. The protease used to generate peptides, that is, enzyme specificity, was set at trypsin/P (cleavage at the C terminus of “K/R”: unless followed by “P”) along with a maximum missed cleavages value of two. The “ion score cutoff” was set to 20, thereby eliminating the lowest quality matches and minimum peptide length as six amino acid residues. Carbamidomethyl on cysteine was set at fixed modification and oxidation of methionine, and N-terminal acetylation was considered variable modifications for database search. The maximal number of modifications per peptide was set at 6. The minimum peptide length parameter was set to 6, and the “peptide” re-quantification function was enabled. Both peptide spectra match, and the protein false discovery rate (FDR) was set to 0.01. The decoy-reversed sequences database was included for the calculation of the FDR.

2.9 Bioinformative Analysis and Network Generation

Protein class analysis of the differentially expressed proteins based on molecular function, biological process, and cellular component was performed using Cytoscape ClueGo plugin (v.3.7.2). A protein–protein interaction (PPI) network was constructed with the Search Tool for the Retrieval of Interacting Genes (STRING) database (v.11.5) and Cytoscape.
A hypergeometric test \( (p < 0.05) \) was used in statistical analysis in the ClueGO tool. Furthermore, the online KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database was used for biological interpretation of higher-level systemic functions.

### 2.10 Western Blot Analysis

A quantity of 40 µg salivary proteins from four stages was loaded in a 12% SDS-PAGE gel and then transferred onto a polyvinylidene difluoride (PVDF) membrane using a semidry Western blot transfer system (Invitrogen, United States). The membrane was incubated with 5% BSA in TBST buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, and 5% BSA, pH 7.60) as blocking solution overnight at 4°C. After blocking, the membrane was incubated separately with seven primary antibodies: SERPINB1 (Aviva Systems Biology, San Diego, United States; 1:5,000), HSPA1A (Sigma-Aldrich, United States; 1:5,000), ENO3 (Sigma-Aldrich, MO, United States; 1:1,250), LCN1 (MyBiosource, CA, United States; 1:1,000), SDF4 (Aviva Systems Biology, San Diego, United States; 1:5,000), VMO1 (Thermo Fischer Scientific, United States; 1:5,000), and beta-actin (Sigma-Aldrich, MO, United States; 1:1,667) at room temperature for 1 h. Subsequently, the membranes were washed three times with TBST followed by incubation with horseradish peroxidase–conjugated secondary antibody (diluted 1:4,000, Sigma-Aldrich, United States) for 1 h at room temperature. The membranes were dried and developed using an X-ray film with Clarity Western ECL substrate (Bio-Rad, United States). The band intensity of the protein expression was normalized with beta-actin as an internal control, and values were analyzed using one way ANOVA with SPSS Inc (2007) after normalization.

### 3 RESULTS

#### 3.1 Estrus Detection and Confirmation

Accurate and efficient identification of estrus is essential for successful conception and efficient breeding management of farm animals including cattle and buffaloes. Hence, the onset of estrus in buffaloes was determined by physical signs and confirmed by gynecological examination of reproductive tract, transrectal USG, biochemical parameters (CVM fern pattern, spinnbarkeit value, and other parameters), and progesterone hormone estimation in blood serum. Behavioral signs of estrus are listed in Table 1; Figure 1. Major estrus signs exhibited by female buffaloes were cervicovaginal mucus discharge (89.9%), standing to be mounted (83.3%), bellowing (77.7%), frequent micturition (65.1%), hyperemia of the vulval mucous membrane (89.9%), and vulva tumefaction (72.2%). Further male behavior toward estrus buffaloes such as flehmen’s reaction (81.1%), sniffing the vulva (71.1%), and chin resting (68.2%) were also considered for estrus identification. Other estrus signs such as uterine horn tonicity, CVM crystallization, and spinnbarkeit value were considered for confirmation of estrus. Tonicity of uterine horns (94%) was mostly intense during the estrus stage (Table 2). The CVM crystallization/fern pattern was typical with scores 3 and 4 in 83.3% during estrus (Figures 2, 3). The spinnbarkeit value of CVM was the highest (21.30 ± 1.56 cm) during estrus (Figure 4). Transrectal USG revealed the presence of dominant follicle (DF) during the follicular phase, and the size of DF was 12.1 ± 0.03 mm and 13.5 ± 0.04 mm during PE and E, respectively (Figure 5). The absence of preovulatory follicle confirmed ovulation during the ME stage and presence of CL during the DE stage (Figure 5). The serum progesterone level was found to be lowest during E (0.35 ± 0.04 ng/ml, \( p < 0.05 \)) and PE (0.54 ± 0.08 ng/ml) than that in ME (1.56 ± 0.14 ng/ml) and DE (3.79 ± 0.09 ng/ml) stages (Figure 6).

#### 3.2 Quantification and SDS-PAGE of Salivary Proteins

The salivary proteins were quantified, and the concentration was in the range of 500–1,000, 400–1,000, 400–900, and 466–800 µg/
ml during PE, E, ME, and DE stage, respectively. Furthermore, salivary proteins were separated using SDS-PAGE. A total of seven major and several minor bands were exhibited in the CBB-stained gel invariably among four stages of the estrous cycle and their molecular mass ranged from 17 to 245 kDa (Figure 7).

3.3 LC-MS/MS Analysis and DEPs Comparisons

The LFQ-LC-MS/MS analysis identified 520 DEPs with 1% protein and peptide FDR cutoff. The detailed information related to accession, description, protein FDR confidence, peptide score, coverage %, peptides, PSM, unique peptides, protein groups, MW, calculated pI, Sequest score, gene name, and abundance of the identified proteins is shown in Supplementary Table S1. The number of upregulated and downregulated proteins was distinctly identified based on the fold-change criteria between E, PE, ME, and DE stages. The fold-change values were set between 0.5 and 1.5; the proteins expressed with values greater than 1.5 were upregulated, and the proteins expressed with values less than 0.5 were downregulated. The list of upregulated and downregulated proteins during E compared to other non-estrus stages (PE, ME, and DE) is listed in Table 3. By considering the fold-change range, a total of 59 proteins and four proteins were found to be upregulated and downregulated during E compared to PE, ME, and DE stages, respectively. Among the DEPs, few proteins with higher abundance at E than all other non-estrus stages and identified in all the biological replicate samples were gastric triacylglycerol lipase (LIPF; E/P: 33-fold, E/M: 38.8-fold, and E/D: 19-fold), peptidylprolyl cis-trans isomerase E (PPIE; E/P: 21-fold, E/M: 15-fold, and E/D: 25-fold), fatty acid-binding protein, (FABP3; E/P: 2.4-fold, E/M: 6.2-fold, and E/D: 10.8-fold), leukocyte elastase inhibitor (SERPINB1; E/P: 3-fold, E/M: 2.3-fold, and E/D: 3.4-fold), apolipoprotein A-I (APOA1; E/P: 1.9-fold, E/M: 9.7-fold, and E/D: 6.8-fold), heat shock 70-kDa protein 1A (HSPA1A; E/P: 1.5-fold, E/M: 2.3-fold, and E/D: 3.6-fold), and pyruvate kinase (PKLR; E/P: 5-fold, E/M: 2-fold, and E/D: 4.7-fold) were highly abundant proteins during E vs. PE, ME, and DE comparisons.

Similarly, TMT-LC-MS/MS analysis identified a total of 369 DEPs; among these 74 and 73 proteins were upregulated and downregulated during E compared to PE, ME, and DE stages, respectively. The detail information of identified proteins is listed in Supplementary Table S2. The list of upregulated and downregulated proteins during E than other non-estrus stages (PE, ME, and DE) is presented in Tables 4, 5, respectively.

Among the upregulated DEPs, odorant-binding protein (OBP; E/P: 2.5-fold, E/M: 2.5-fold, and E/D: 69-fold), lipocalin 1 (LCN1; E/P: 7.3-fold, E/M: 14.3-fold, and E/D: 11.3-fold), odorant-binding protein-like (MGC151921; E/P: 1.8-fold, E/M: 2.8-fold, and E/D: 24-fold), lipocln_cytosolic_Fabd_dom_domain-containing protein (LOC104969973; E/P: 11.3-fold, E/M: 14.6-fold, and E/D: 5.8-fold), heat shock protein beta-1 (HSPB1; E/P: 12.5-fold, E/M: 15.5-fold, and E/D: 7.3-fold), galectin (LGALS7B; E/P: 5.3-fold, E/M: 6.6-fold, and E/D: 4.6-fold), metalloproteinase inhibitor 2 (TIMP2; E/P: 11.2-fold, E/M: 11.9-fold, and E/D: 2.7-fold), 45-kDa calcium-binding protein (SDF4; E/P: 62.6-fold, E/M: 88.6-
fold, and E/D: 19.2-fold), peptidyl-prolyl isomerase (LOC526524; E/P: 13.6-fold, E/M: 17.3-fold, and E/D: 37.9-fold), and vitelline membrane outer layer 1 (VMO1; E/P: 62.6-fold, E/M: 88.6-fold, and E/D: 19.2-fold) were highly abundant proteins during E than other non-estrus stages, and few of these were also discovered in our previous investigation.

Hierarchical clustering (Figure 8) and principal component analysis (PCA) (Figure 9) of proteins identified during four stages depict clear cut differences in the expression of proteins among four stages. A Venn diagram analysis revealed that 214 proteins were commonly identified by both techniques, that is, TMT and LFQ; however, 223 and 78 proteins were specifically identified using LFQ and TMT methods, respectively (Figure 10).

### 3.4 Validation of DEPs Using Western Blotting

A total of six DEPs were selected for validation using Western blot analysis based on their involvement in different signaling pathways and their over-expression during E compared to all other non-estrus stages: SERPINB1, HSPA1A, SDF4, VMO1, ENO3, and LCN1. Interestingly, Western blot analysis confirmed the significantly higher expression of these proteins during E than PE, ME, and DE stages. The intensity of the protein expression was normalized with beta-actin as an internal control (Figure 11).

### 3.5 Gene Ontology Annotation of DEPs

Gene Ontology annotation of DEPs was performed on the Cytoscape ClueGo plug-in tool and presented in Figure 12. Based on the molecular function (Figure 12A, Supplementary Figure S1), the DEPs were found to be mainly enriched in cell adhesion molecule binding, endopeptidase regulator activity, protein heterodimerization activity, cadherin binding, cytoskeletal protein binding, oxidoreductase activity, hydrolase activity, transferase activity, fatty acid-binding, cis–trans isomerase activity, lysozyme activity, calcium ion binding, ion binding, antioxidant activity, and protein folding chaperone. Based on biological process (Figure 12B, Supplementary Figure S2), the DEPs were enriched in response to external biotic stimulus,

![Figure 5](https://example.com/figure5.jpg)

**FIGURE 5** | Ultrasonography of ovarian follicles and corpus luteum (CL) during proestrus (PE), estrus (E), metestrus (ME), and diestrus (DE) stages of the buffalo estrous cycle (DF, dominant follicle; SF, small follicle; POF, preovulatory follicle; CL, corpus luteum).
metabolic process, regulation of endopeptidase activity, innate immune response, cytoskeleton organization, tissue homeostasis, generation of precursor metabolites and energy, ATP metabolic process, glycolytic process, chromatin assembly or disassembly, acute inflammatory response, cell redox homeostasis, and mucosal immune response. Based on the GO terms for cellular component (Figure 12C, Supplementary Figure S3), the DEPs belonged to endoplasmic reticulum lumen, plasma membrane, actin cytoskeleton, endoplasmic reticulum chaperone complex, myelin sheath, nucleosome, extracellular matrix, adherens junction, exosome, organelle, vesicle, and extracellular space.

3.6 Protein–Protein Interaction and Network Visualization

We performed protein–protein interaction (PPI) network analysis for DEPs using the STRING tool (v.11.5) with the Bos taurus database. The highly significant protein–protein interaction network ($p < 1.0E-16$) was created with 378 nodes, 810 edges, 4.29 average node degree, and 0.357 average local clustering coefficient (Figure 13A). A total of 31 clusters were found with the highest degree of connectivity among proteins. Furthermore, to explore the interactions between DEPs, we constructed protein networks using Cytoscape ClueGo plug-in tool (Figure 13B). A total 28 KEGG pathways were significantly enriched ($p < 0.05$), including glycolysis/gluconeogenesis, carbon metabolism, biosynthesis of amino acids, salivary secretion, fluid shear stress and atherosclerosis, glutathione metabolism, pentose phosphate pathway, innate immune response, antigen processing and presentation, oocyte meiosis, cell cycle, and estrogen signaling pathway (Table 6).

4 DISCUSSION

Due to the difficulty of detecting estrus in buffalo, the finding of an estrus biomarker in readily accessible bodily fluids such as
### TABLE 3 | List of differentially expressed proteins (DEPs) during estrus (E) vs. proestrus (PE), metestrus (ME), and diestrus (DE) stages using LFQ-LC-MS/MS analysis.

| Accession | Gene symbol | Description | Peptide Fold change | Fold change | Fold change |
|-----------|-------------|-------------|---------------------|-------------|-------------|
| F1MES3    | LIPF        | Gastric triacylglycerol lipase | 4 | 33.125 | 18.989 |
| Q29458    | LIPF        | Gastric triacylglycerol lipase | 7 | 32.65 | 19.173 |
| A4FV72    | PPIE        | Peptidyl-prolyl cis-trans isomerase E | 1 | 21.071 | 15.047 |
| P00639    | DNASE1      | Deoxyribonuclease-1 | 2 | 7.005 | 4.783 |
| F1N09     | —           | Uncharacterized protein | 1 | 5.447 | 3.44 |
| P0CG63    | UBB         | Polyubiquitin-B | 1 | 2.301 | 1.493 |
| P10700    | FABP3       | Fatty acid-binding protein, heart | 1 | 2.455 | 1.617 |
| P02869    | PM2         | Myelin P2 protein | 1 | 2.455 | 1.617 |
| P15497    | APOA1       | Apolipoprotein A-1 | 6 | 1.942 | 1.973 |
| G3M2U3    | LOC733399   | Uncharacterized protein | 4 | 1.075 | 1.783 |
| Q865V6    | CAPG        | Macrophage-capping protein | 1 | 5.134 | 1.633 |
| Q1JPG7    | PPIE        | Peptidyl-prolyl cis-trans isomerase E | 1 | 3.922 | 1.683 |
| G3M2U3    | LOC733399   | Uncharacterized protein | 4 | 1.075 | 1.783 |
| P0CG63    | UBB         | Polyubiquitin-B | 1 | 2.301 | 1.493 |
| P10700    | FABP3       | Fatty acid-binding protein, heart | 1 | 2.455 | 1.617 |
| P02869    | PM2         | Myelin P2 protein | 1 | 2.455 | 1.617 |
| P15497    | APOA1       | Apolipoprotein A-1 | 6 | 1.942 | 1.973 |

(Continued on following page)
saliva is critical for the development of a simple, reliable estrus detection tool. Earlier investigations have confirmed the salivary proteins as a potential candidate to detect fertile periods in woman (Alagendran et al., 2013; Saibaba et al., 2021) and estrus (Muthukumar et al., 2014b; Shashikumar et al., 2018) in buffaloes. However, estrus biomarker discovery in buffalo saliva by differential proteome analysis using TMT and LFQ coupled to mass spectrometry is lacking. Comparative proteome profiling using LFQ-MS/MS and TMT-MS/MS analysis in buffalo saliva identified a total of 520 and 369 proteins as DEPs, respectively. These findings may be helpful for further understanding of buffalo estrus biology and development of an easy, reliable estrus detection method for buffalo.

### 4.1 Proteins Involved in Regulation of Endopeptidase Activity

Our study found the over-expression of leukocyte elastase inhibitor (SERPINB1) during estrus followed by proestrus and almost negligible expression during metestrus and diestrus stages of the estrous cycle. SERPINB1 and several other DEPs (A2M, A2ML1, C3, CST3, CST6, CSTB, LOC404103, LOC786263, LTF, OVO2, PTI, S100A8, SERPINA3-8, SERPINB1, SERPINB6, SERPINB8, SERPINI2, SPN, SPINK5, THBS1, TIMP2, and WFDC2) in the present study were involved in the regulation of endopeptidase inhibitor activity. SERPINB1 and other protease inhibitors inactivate serine proteases and cysteine proteases and mediate important functions in fibrinolysis, coagulation, inflammation, cell mobility, cellular differentiation, apoptosis, and protein C pathways (Law et al., 2006), thus regulating the innate immune response, inflammation, and cellular homeostasis (Choi et al., 2019). The previous study demonstrated altered expression of three SERPINs, SERPINB2, SERPINE1, and SERPINE2 during follicular development in bovine ovarian follicles (Dow et al., 2002; Bedard et al., 2003). Expression of SERPINE2 gene in preovulatory follicles was markedly upregulated immediately after the LH surge and then decreased to the lowest level toward ovulation (Hasan et al., 2002; Cao et al., 2006). Similarly, over-expression of SERPINE2 in granulosa cells of mature follicles compared to that in small and medium follicles has also been observed (Cao et al., 2004).

Previous studies also demonstrated over-expression of SERPINB6 in healthy follicles compared to that in the atretic ones, suggesting follicular development and atresia in bovine ovarian follicles are mediated through SERPINs (Hayashi et al., 2011). The increased abundance of SERPINB1 during the estrus stage may be a protective mechanism of follicular cells against several proteases and intrinsic for follicular development and final follicular maturation and steroid production by ovarian follicles.

### 4.2 Proteins Involved in Glycolysis and Pyruvate Metabolism

We observed increased expression of beta-enolase (ENO3) during E compared to PE, ME, and DE stages. ENO3 is a glycolytic enzyme and regulates the glycolysis process by catalyzing the reversible conversion of 2-phosphoglyceric acid to phosphoenolpyruvic acid (Giallongo et al., 1993). In addition, we observed 14 other DEPs (ALDOA, ALDOB, ALDOC, ENO1, ENO2, GAPDH5, G3P, LDHA, LDHB, LDHC, PGAM1, PGK1, PKLR, and TPI1) and five DEPs (LDHA, LDHB, LDHC, MDH1, and PKLR) were significantly involved in the glycolysis or gluconeogenesis metabolic pathway and pyruvate metabolism, respectively. It is speculated that these proteins associated with active synthesis of energy may support follicular development, steroidogenesis, final follicular maturation, and ovulation process. In a previous study, pyruvate metabolism has been essential for the completion of oogenesis, serving as a vital source of energy during the meiotic maturation of murine oocytes (Johnson et al., 2007). It has also been observed that the mature cumulus oocyte complex (COC) uses two times more glucose and pyruvate than the immature ones (Sutton et al., 2003), indicating high energy demand supported by upregulation of glycolysis by ovarian follicles during the process of final maturation of oocyte. The previous study has demonstrated that increased enolase expression is responsible for higher expression of the FSH gene in granulosa cells during the follicular phase (Hermann and Heckert, 2007). FSH has a significant role in reproduction by regulating granulosa cell differentiation, proliferation and ovarian steroidogenesis (Richards, 1994), and controlling follicular development and

**TABLE 3** (Continued) List of differentially expressed proteins (DEPs) during estrus (E) vs. proestrus (PE), metestrus (ME), and diestrus (DE) stages using LFQ-LC-MS/MS analysis.

| Accession | Gene symbol | Description | Peptide | Fold change E/PE | Fold change E/ME | Fold change E/DE |
|-----------|-------------|-------------|---------|----------------|----------------|----------------|
| G3MZ19    | LOC100295741; ZG16B | HRPE773-like | 8       | 0.445          | 0.463          | 0.134          |
| A6QLQ8    | ENDOU       | Poly(U)-specific endoribonuclease | 2       | 0.453          | 0.364          | 0.433          |
| A4IF6     | HIST3H2A    | Histone H2A | 3       | 0.43           | 0.448          | 0.539          |
| G5ESV1    |             | Uncharacterized protein | 2       | 0.56           | 0.306          | 0.581          |
# List of upregulated proteins during estrus (E) vs. proestrus (PE), metestrus (ME), and diestrus (DE) stages using TMT-LC-MS/MS analysis.

| Accession | Gene symbol | Description | Peptide | Fold change E/PE | Fold change E/ME | Fold change E/DE |
|-----------|-------------|-------------|---------|------------------|------------------|-----------------|
| F1MK5     | SDF4        | 45-kDa calcium-binding protein | 2       | 71.71            | 100              | 19.73           |
| Q3ZBE1    | SDF4        | 45-kDa calcium-binding protein | 3       | 62.663           | 88.648           | 19.262          |
| G2NK68    | LOC526524   | Peptidyl-prolyl isomerase | 1       | 13.676           | 17.355           | 37.964          |
| E1BkA1    | LOC786350   | Protein S100 | 1       | 14.447           | 19.419           | 1.34            |
| G3M22     | —           | Uncharacterized protein | 1       | 13.057           | 8.159            | 2.728           |
| E1BEL8    | HBE1        | Globin B1    | 6       | 12.524           | 15.547           | 7.289           |
| Q58DP7    | HSPB1       | Heat shock protein beta-1 | 5       | 12.524           | 15.547           | 7.289           |
| G3X7S2    | HSPB1       | Heat shock protein beta-1 | 5       | 12.524           | 15.547           | 7.289           |
| Q5KR47    | TPM3        | Tropomyosin alpha-3 chain | 1       | 11.813           | 13.017           | 1.34            |
| A6QOR5    | LOC533277   | LOC533277 protein | 1       | 11.813           | 13.017           | 1.34            |
| Q5KR48    | TPM2        | Tropomyosin beta chain | 1       | 11.813           | 13.017           | 1.34            |
| Q5KR47-2  | TPM3        | Isoform 2 of Tropomyosin alpha-3 chain | 1       | 11.813           | 13.017           | 1.34            |
| F1947     | TUBA1B; TUBA1A | Tubulin alpha-1B chain | 2       | 11.444           | 12.223           | 5.145           |
| F1MNF8    | LOC100141266 | Tubulin alpha chain | 1       | 11.444           | 12.223           | 5.145           |
| G3X700    | LOC104969973 | Uncharacterized protein | 7       | 11.348           | 14.639           | 5.809           |
| Q58DP0    | —           | Uncharacterized protein | 1       | 11.285           | 11.992           | 2.762           |
| Q86288    | TIMP2       | Similar to metalloproteinase inhibitor | 3      | 11.285           | 11.992           | 2.762           |
| P07435    | OBP         | Odorant-binding protein | 8       | 2.573            | 2.481            | 69.029          |
| Q862Q0    | PGAM1       | Phosphoglycerate mutase | 1       | 3.695            | 5.268            | 48.354          |
| Q712W6    | GAPDH       | Glyceraldehyde 3-phosphate dehydrogenase | 3      | 5.445            | 4.678            | 0.97            |
| G3N3D0    | LGALS7B     | Galectin | 6       | 5.339            | 6.629            | 4.633           |
| G3MX66    | VMO1        | Vitelline membrane outer layer 1 homolog | 5      | 5.3              | 5.5              | 4.1             |
| P81265-2  | PIGR        | Isomeric short of polymeric immunoglobulin receptor | 15     | 7.975            | 8.284            | 5.803           |
| G5E5M1    | PIGR        | Polymeric immunoglobulin receptor | 14     | 7.975            | 8.284            | 5.803           |
| G3N2H5    | S100A12     | Protein S100 | 1       | 7.311            | 3.613            | 2.133           |
| A0A1C9E6X0 | HSPB1      | Heat shock protein family B member 1 variant 2 | 7      | 6.972            | 7.193            | 5.125           |
| F1N650    | ANXA1       | Annexin | 2       | 6.562            | 4.819            | 0.85            |
| Q712W6    | GAPDH       | Glyceraldehyde 3-phosphate dehydrogenase | 3      | 5.445            | 4.678            | 0.97            |
| E1BDE6    | LGALS7B     | Galectin | 6       | 5.339            | 6.629            | 4.633           |
| G3X700    | LGALS7     | Galectin | 6       | 5.339            | 6.629            | 4.633           |
| Q58DP0    | —           | Uncharacterized protein | 1       | 3.793            | 3.702            | 1.023           |
| A6QNW3    | PIGR        | Polymeric immunoglobulin receptor | 15     | 7.975            | 8.284            | 5.803           |
| G5E5C8    | TALDO1      | Transaldolase | 3      | 4.986            | 4.795            | 1.281           |
| A5D7E8    | PDI1        | Protein disulfide-isomerase | 2      | 4.969            | 3.617            | 1.212           |
| E1B970    | GOLGA3      | Golgin A3 | 1       | 4.283            | 5.078            | 6.458           |
| A6QQ07    | BTD         | Biotinidase | 1       | 3.945            | 3.735            | 2.313           |
| Q0P569    | NUCB1       | Nucleobindin-1 | 9      | 3.793            | 3.702            | 1.023           |
| P8B252    | YWHAQ       | 14-3-3 protein gamma | 2      | 3.757            | 4.645            | 9.328           |
| G3X8B4    | LOC786263   | Uncharacterized protein | 5      | 3.71             | 5.005            | 1.387           |
| Q58DP0    | RPPG5s      | Fusion glycoprotein F0 | 1      | 3.705            | 4.751            | 3.904           |
| P0S307    | P4HB        | Protein disulfide-isomerase | 3      | 3.338            | 3.649            | 1.108           |
| G3Z2Z0    | PGAM1       | Phosphoglycerate mutase | 1      | 3.242            | 3.288            | 1.021           |
| G3X79B    | OBP         | Uncharacterized protein | 9      | 3.041            | 2.813            | 2.093           |
| F1N614    | HSPA5       | 78-kDa glucose-regulated protein | 2      | 3.018            | 3.507            | 2.316           |
| Q2KJF1    | A1BG        | Alpha-1B-glycoprotein | 2      | 2.625            | 2.919            | 2.093           |
| Q3T1A5    | MDH1        | Malate dehydrogenase, cytoplasmic | 2      | 2.547            | 2.689            | 1.172           |
| F1NK3A    | THBS1       | Thrombospindlin-1 | 10     | 2.524            | 2.7              | 0.95            |
| F1MKE7    | KRT6C       | Uncharacterized protein | 9      | 2.385            | 3.018            | 4.091           |
| Q3T010    | PEBP4       | Phosphatidylethanolamine-binding protein 4 | 2      | 2.122            | 3.24             | 2.012           |
| F2FB41    | MUC5AC      | Mucin-5AC | 6       | 2.197            | 3.168            | 1.523           |
| G3S2H5    | AGT         | Angiotensinogen | 4      | 1.98             | 1.909            | 1.172           |
| F2Z4B6    | HIST2H2AC   | Histone H2A | 5      | 1.928            | 2.208            | 1.153           |

(Continued on following page)
...fertility in females (Hermann and Heckert, 2007). Specific expression of ENO3 in buffalo saliva, particularly during estrus stage has also been observed by an earlier study (Muthukumar et al. (2014b). The increased expression of ENO3 during E compared to other stages of the estrous cycle suggests it has a defined role in follicular development, final follicular maturation, and steroid production by follicular granulosa cells that leads to the onset of estrus in buffaloes.

### 4.3 Proteins Involved in Estrogen Signaling

This study also found a higher abundance of the heat shock protein family A (HSPA1A/HSP70) during estrus than other stages of the estrous cycle. HSPA1A is a molecular chaperon and plays a role in the estrogen signaling pathway. The present study also found other molecular chaperons such as HSPA1L, HSPA2, and HSPA8 as DEPs. Chaperones are stress-response molecules and involved in housekeeping of the cells and facilitating the transport, folding, unfolding, assembly, and disassembly of multi-structured protein units and degradation of misfolded or aggregated proteins (Sorensen et al., 2003). HSPA1A plays a role in the assembly and trafficking of steroid hormone receptors, acts as a co-activator for the nuclear estrogen receptor-a activity, and regulates steroid hormone synthesis by ovarian follicles (Khanna et al., 1995; Liu and Stocco, 1997; Hurd et al., 2000). HSPA1A also mediates luteal regression in murine corpus luteum (Khanna et al., 1995). A previous study also identified a higher abundance of HSPA1A protein in cervical mucus during E than the DE stage of the buffalo estrous cycle (Muthukumar et al., 2014a). Our previous study also observed estrus-specific expression of HSPA1A in buffalo saliva (Shashikumar et al., 2016). Furthermore, our direct saliva transcript analysis also demonstrated a higher level of HSP70 transcript in saliva during estrus than the diestrus stage, suggesting it as a good indicator of estrus in buffaloes (Onteru et al., 2016). HSPA1A protects cells from the negative effect of stress by promoting the folding of proteins and correcting the misfolding of denatured proteins (Lindquist and Craig 1988). Steroid hormone, particularly estradiol plays a key role in inducing estrus signs in farm animals, and its level increased from 22.4–35 pg/ml (Roy and Prakash 2009; Mondal et al., 2010) just before the onset of estrus in buffaloes. An earlier study demonstrated estradiol treatment-induced stress or injury in rat brain vasculature and estradiol-induced heat shock protein expression occurred as a protective mechanism to prevent cellular damage (Lu et al., 2002; Raval et al., 2013). Suggestive increased abundance of HSPA1A protein during estrus could be a protective mechanism of granulosa cells against estrogen-induced stress and its possible role in follicular development and steroidogenesis process.

### 4.4 Calcium Ion Binding Activity

Increased expression of 45-kDa calcium-binding protein (SDF4) was observed during E compared to PE, ME and DE stages of the estrous cycle. SDF4 is a calcium-binding protein and regulates calcium-dependent activities in the lumen of the endoplasmic reticulum and is involved in calcium-ion regulated exocytosis. Calcium-binding proteins are mainly localized in the cytosol and various parts of the secretory pathway (Honore, 2009). They have diversified functions, including secretory process, chaperone activity, and signal transduction (Honore, 2009). Although its exact role in estrous physiology is unknown, its higher expression during estrus suggests its possible involvement in chaperon-mediated steroidogenesis and cellular protection against estradiol-induced stress.

### 4.5 Proteins Involved in Innate Immune Response

The present study also identified several DEPs (PIGR, S100A12, BPIFA2B, B2M, A2M, BPFB1, BPFA1, CATHL3, CATHL5, FGB, and FGA) involved in innate immune response. The whole estrous cycle is regulated by the differential level of steroid hormones; estradiol is predominant during follicular phase and progesterone during the luteal phase. These steroid hormones modulate the immune system depending on the estrous cycle stage. Binding with specific hormone receptors, steroid hormone induces genomic and non-genomic actions in immune cells (Gilliver, 2010; Kovacs et al., 2010; Bellavance and Rivest, 2014). In general, estradiol is immune-enhancing by stimulating functions of innate immune cells, Th1 responses, and antibody production from B cells. However, progesterone is immune-suppressing by...
# TABLE 5 | List of downregulated proteins during estrus (E) vs. proestrus (PE), metestrus (ME), and diestrus (DE) stages using TMT-LC-MS/MS analysis.

| Accession | Gene symbol | Description | Peptide | Fold change E/PE | Fold change E/ME | Fold change E/DE |
|-----------|-------------|-------------|---------|------------------|------------------|------------------|
| B9TUD2    | CATHL7      | Cathelicidin-7 | 1       | 0.01             | 0.01             | 0.01             |
| G3N1R1    | Uncharacterized protein | | 1 | 0.01 | 0.01 | 0.01 |
| B9TUC3    | CATHL2      | Cathelicidin-2 | 1       | 0.01             | 0.01             | 0.01             |
| Q148B6    | CYPI4B1     | Immunoglobulin light chain, lambda gene cluster | 14 | 0.014 | 0.012 | 0.228 |
| Q1RMN8    | IGL@        | Uncharacterized protein | 2 | 0.019 | 0.095 | 0.045 |
| Q28133    | BDA20       | Allergen Bos d 2 | 9 | 0.021 | 0.023 | 0.112 |
| A5D7Q2    | LOC524810   | Uncharacterized protein | 2 | 0.021 | 0.038 | 0.393 |
| K4JR84    | A2M         | Alpha-2-macroglobulin variant 17 | 1 | 0.022 | 0.028 | 0.275 |
| E1B8L2    | LOC525947   | Uncharacterized protein | 1 | 0.028 | 0.057 | 0.069 |
| G3X6K8    | HP          | Haptoglobin | 3 | 0.032 | 0.038 | 0.021 |
| Q32CL0    | CRISP3      | Cysteine-rich secretory protein 2 | 1 | 0.032 | 0.038 | 0.021 |
| F6R3I6    | CRISP3      | Uncharacterized protein | 4 | 0.039 | 0.049 | 0.586 |
| P02702    | FOLR1; FOLR3 | Folate receptor alpha | 1 | 0.041 | 0.049 | 0.056 |
| E1B8Q6    | LOC524080   | Uncharacterized protein | 1 | 0.044 | 0.045 | 0.01 |
| F1MD73    | Uncharacterized protein | 7 | 0.045 | 0.054 | 0.133 |
| P60986    | PIP; LOC107131147 | Prolactin-inducible protein homolog | 10 | 0.047 | 0.062 | 0.056 |
| F1MCV8    | Uncharacterized protein | 3 | 0.047 | 0.062 | 0.056 |
| F1N6D1    | LOC5247724  | Uncharacterized protein | 13 | 0.047 | 0.069 | 0.016 |
| G3N269    | FABP5       | Fatty acid-binding protein, epidermal | 3 | 0.053 | 0.071 | 0.136 |
| F1MHQ2    | BPIFA2A     | Uncharacterized protein | 21 | 0.056 | 0.047 | 0.048 |
| Q6PV3Y    | MIF         | Macrophage migration inhibitory factor | 1 | 0.057 | 0.066 | 0.067 |
| Q6H32O    | KLK1        | Glandular kallikrein | 6 | 0.058 | 0.071 | 0.148 |
| A2NP2     | SOG3B1D     | Secretoglobulin family 1D member | 1 | 0.07 | 0.117 | 0.552 |
| G3N2D7    | LOC100297192 | Uncharacterized protein | 4 | 0.073 | 0.067 | 0.128 |
| K4JR74    | A2M         | Alpha-2-macroglobulin variant 2 | 4 | 0.076 | 0.132 | 0.206 |
| P17697    | CLU         | Clusterin | 5 | 0.08 | 0.08 | 0.444 |
| E1B8Q4    | Uncharacterized protein | 2 | 0.08 | 0.082 | 0.013 |
| F1MSB7    | PLS3        | Plastin-3 | 9 | 0.086 | 0.091 | 0.036 |
| Q8B2H7    | S100A11     | Protein S100 | 1 | 0.086 | 0.075 | 0.01 |
| O6Z672    | MUC19       | Submaxillary mucin 1 | 9 | 0.087 | 0.084 | 0.428 |
| F1MWN7    | Uncharacterized protein | 9 | 0.087 | 0.084 | 0.431 |
| F1N0W1    | FCHSD1      | Uncharacterized protein | 6 | 0.091 | 0.161 | 0.07 |
| Q5E9B1    | LDHB        | L-Lactate dehydrogenase B chain | 2 | 0.101 | 0.083 | 0.032 |
| G1K1L8    | Uncharacterized protein | 9 | 0.106 | 0.117 | 0.104 |
| A6QOF6    | SBSN        | Suprabasin | 1 | 0.109 | 0.112 | 0.06 |
| A2VE41    | EFEMP1      | EGF-containing fibulin-like extracellular matrix protein 1 | 14 | 0.12 | 0.099 | 0.161 |
| E1BAU5    | LOC512548; SLPI | Uncharacterized protein | 1 | 0.124 | 0.171 | 0.479 |
| A4IF0     | IGLL1       | IGLL1 protein | 9 | 0.132 | 0.115 | 0.289 |
| F1MCF8    | LOC100297192 | Uncharacterized protein | 11 | 0.132 | 0.115 | 0.289 |
| A5PK49    | IGL@        | IGL@ protein | 6 | 0.132 | 0.115 | 0.289 |
| A6H7J7    | LOC100297192 | Uncharacterized protein | 9 | 0.139 | 0.121 | 0.297 |
| P02253    | HIST1HC     | Histone H1.2 | 18 | 0.143 | 0.107 | 0.05 |
| F2FB39    | MUC19       | Mucin-19 | 14 | 0.154 | 0.136 | 0.067 |
| G3X6B0    | Uncharacterized protein | 10 | 0.172 | 0.213 | 0.246 |
| P19838    | LDHA        | L-Lactate dehydrogenase A chain | 21 | 0.172 | 0.134 | 0.032 |
| G3X6F7    | Uncharacterized protein | 2 | 0.176 | 0.157 | 0.213 |
| F1MB45    | Uncharacterized protein | 6 | 0.176 | 0.157 | 0.213 |
| G3N0H7    | Uncharacterized protein | 4 | 0.176 | 0.157 | 0.213 |
| A0A1Y0KDJ6 | Beta-casein | 9 | 0.177 | 0.181 | 0.495 |
| F1MG6     | Uncharacterized protein | 13 | 0.181 | 0.165 | 0.052 |
| G9G9X6    | LALBA       | Alpha-lactalbumin protein variant D | 14 | 0.188 | 0.254 | 0.352 |
| G3SYR8    | IGL; JCHAIN | Immunoglobulin J chain | 10 | 0.19 | 0.166 | 0.191 |
| B2BX99    | A2GP1       | Zn-alpha-2-glycoprotein | 3 | 0.197 | 0.19 | 0.156 |
| B2BX70    | A2GP1       | Zn-alpha-2-glycoprotein | 2 | 0.197 | 0.19 | 0.156 |
| A5D7Y3    | ESM1        | ESM1 protein | 2 | 0.213 | 0.297 | 0.145 |
| G5E513    | IGHM        | Immunoglobulin heavy constant mu | 5 | 0.219 | 0.455 | 0.126 |
| P01305    | CST3        | Cystatin-C | 6 | 0.221 | 0.184 | 0.302 |
| G5E506    | TFF3        | Trefoil factor 3 | 3 | 0.229 | 0.232 | 0.22 |
| Q6LC78    | LT1         | Lactoferrin | 8 | 0.23 | 0.432 | 0.369 |
| G5E515    | Uncharacterized protein | 8 | 0.231 | 0.459 | 0.424 |
| E1B8C2    | LOC104596918 | Uncharacterized protein | 5 | 0.241 | 0.216 | 0.775 |
| Q8M8O0    | LT1         | Lactotransferrin | 8 | 0.244 | 0.388 | 0.394 |
| F1M632    | A2ML1       | Uncharacterized protein | 3 | 0.244 | 0.318 | 0.087 |

(Continued on following page)
down-regulating functions of immune cells such as macrophages (Su et al., 2009), natural killer cells (Arruvito et al., 2008), or dendritic cells (Butts et al., 2007; Hughes et al., 2008). In addition, progesterone antagonizes the immune-enhancing function of estrogens on T-cell cycle progression (McMurray et al., 2001) and proliferation of T cells (Butts et al., 2007). Importance of antimicrobial peptides (S100A12, CATHL3, and CATHL5) (Diamond et al., 2009), BPI fold-containing family proteins (BPIFA2B, BPIFB1, and BPIFA1) (Li et al., 2020), polymeric immunoglobulin receptor (PIGR) (Wei and Wang, 2021), alpha-2-macroglobulin (A2M), and beta-2-macroglobulin (B2M) (Vandooren and Itoh, 2021) in mucosal immunity is well established. Mucosal immunity protects the internal genital tract against pathogens. During the estrus period, physical barriers between external genitalia and uterus breached, making pathogens to enter into the uterus. Hence, the estrogen-dominant estrus phase modulates the function of innate immune cells and

TABLE 5 | (Continued) List of downregulated proteins during estrus (E) vs. proestrus (PE), metestrus (ME), and diestrus (DE) stages using TMT-LC-MS/MS analysis.

| Accession | Gene symbol | Description | Peptide | Fold change E/PE | Fold change E/ME | Fold change E/DE |
|-----------|-------------|-------------|---------|-----------------|-----------------|-----------------|
| Q6LB7N7   | LTF         | Lactoferrin | 18      | 0.254           | 0.424           | 0.295           |
| Q3SZ57    | AFP         | Alpha-fetoprotein | 1 | 0.258           | 0.427           | 0.083           |
| Q19K51    | LTF         | Lactoferrin | 7       | 0.269           | 0.484           | 0.223           |
| Q683R8    | Cath        | Cathelicidin | 3 | 0.316           | 0.384           | 0.079           |
| A6QA9A8   | QSOX1       | Sulfhydryl oxidase | 1 | 0.316           | 0.392           | 0.14            |
| P00432    | CAT         | Catalase    | 1       | 0.33            | 0.385           | 0.023           |
| P33046    | CATHL4; LOC786887 | Cathelicidin-4 | 1 | 0.377           | 0.335           | 0.058           |
| F1MH40    | —           | Uncharacterized protein | 1 | 0.417           | 0.43            | 0.413           |
| X5F5B4    | SERPINB4    | Serpin B4-like protein | 2 | 0.456           | 0.557           | 0.566           |
| A6QP24    | SERPINB4    | SERPINB4 protein | 2 | 0.456           | 0.557           | 0.566           |

FIGURE 8 | Hierarchical clustering analysis shows proteins identified during proestrus, estrus, metestrus, and diestrus stages (red to blue indicates highest to lowest abundance).

FIGURE 9 | Principal component analysis (PCA) attributes the more difference between estrus and other non-estrus stages (proestrus, metestrus, and diestrus).

FIGURE 10 | Venn diagram represents number of proteins identified by tandem mass tag (TMT) and label-free quantitation (LFQ).
FIGURE 11 | Western blot analysis for validation of differentially expressed upregulated proteins. (A) Over-expression of SERPINB1, HSPA1A, SDF4, VMO1, LCN1, and ENO3, during estrus (E) compared to proestrus (PE), metestrus (ME), and diestrus (DE) stages of the buffalo estrous cycle. (B) Band intensity was normalized against beta-actin as an internal control.

FIGURE 12 | Gene Ontology (GO) classification of the differentially expressed proteins based on molecular function (A), biological process (B), and cellular component (C) using Cytoscape software with ClueGO plug-in.
secretes several antimicrobial peptides and proteins that protect the mucosa of the female reproductive tract against external pathogens.

4.6 Proteins Involved in Cytoskeleton Organization

The present study also depicted several DEPs involved in the cytoskeleton organization process (APOA1, ARF1, CAP1, CAPG, CFL1, CFL2, CORO1A, CSN1S1, EZR, FKBP1A, HSPA1A, KRT14, KRT20, KRT71, KRT9, LCP1, PFN1, PLS1, PLS3, RAC1, RAC2, and WDR1). It is during estrus that an oocyte that has been diplotene-restricted restarts meiosis before ovulation. Oocyte meiosis entails a number of intricate processes, including change of the mitotic cell cycle, chromosomal segregation, spindle reorganization, and accomplishment of oocyte asymmetry and polar body extrusion. These activities need reorganization of the cytoskeleton, coordination of cytoplasmic dynamics, and regulation of the cortex’s tension forces (Brunet and Verlhac, 2011; Yi et al., 2011). These findings and our results support the role of cytoskeletal proteins in follicular and oocyte maturation during estrus.

4.7 Proteins With Miscellaneous Functions

Notably, we noticed an elevated expression of vitelline membrane outer layer protein 1 (VMO1) in comparisons between E and PE, ME, and DE. It is the first report of VMO1 protein in saliva during the estrous cycle. Its precise involvement in controlling

FIGURE 13 | (A) Protein–protein interaction (PPI) network constructed by the STRING tool using combined score of 0.9–0.4. A PPI network with high combined score was created (p < 1.0E-16) with 378 nodes, 810 edges, 4.29 average node degree, and 0.357 average local clustering coefficient. (B) Functional annotation of a protein–protein interacting hub using Cytoscape software with ClueGO plug-in.
folliculogenesis and estrous physiology is unknown. VMO1 protein was initially found in chicken as an important component of the egg’s vitelline membrane’s outer layer (Kido et al., 1992) and plays a critical function in oviductal recrudescence in laying hens (Lim and Song, 2015). It is anticipated to be a new estrogen-induced gene in laying hens and a possible diagnostic biomarker for ovarian cancer (Lim and Song, 2015). As a result, it is probable that the estradiol peak that occurs during estrus is the cause for the over-expression of VMO1 and its significant relationship with estrus physiology. Additional investigations in large animals using powerful genetic engineering technologies such as CRISPR-based knockout are required to characterize the mechanism of action (Singh et al., 2000). Another protein, nucleobindin-1 (NUCB1), was shown to be significantly increased during estrus compared to other non-estrus phases. Nucleobindins are multidomain Ca2+ and DNA-binding proteins that have a variety of roles in vertebrates. NUCB2 is claimed to operate as a local regulator in the mouse ovary, controlling steroidogenesis and energy balance through autocrine
and paracrine signaling (Kim and Yang, 2012). Additionally, NUCB2 has been shown to bind predominantly to epithelial cells of uterine glands and neutrophils of the endometrium during the mouse estrous cycle, suggesting that its expression in the uterus may be regulated by estrogen secreted by the ovary but not by gonadotropin released by the pituitary gland (Kim et al., 2011). The precise involvement of NUCB1 in estrous physiology in farm animals is unknown and requires additional research. Additionally, the current research showed that two proteins, lipocalin 1 (LCN1) and odorant-binding protein-2B (OBP2B), were overexpressed during estrus compared to other non-estrus phases. Increased expression of LCN1 and OBP2B during estrus compared to other non-estrus phases implies that they be regulated by estrogen secreted by the ovary but not by gonadotropin released by the pituitary gland (Kim et al., 2011).

TABLE 6 | Involvement of differentially expressed proteins (DEPs) in KEGG pathways.

| S.No. | KEGG pathway                        | Number of genes | Associated gene | p-value |
|-------|-------------------------------------|-----------------|-----------------|---------|
| 1     | Glycolysis/gluconeogenesis          | 15              | ALDOA, ALDOB, ALDOC, EN01, EN02, EN03, GADPH2S, GPI, LDHA, LDHB, LDHC, PGAM1, PGK1, PKLR, and TPH1 | 0.00    |
| 2     | PPAR signaling pathway              | 6               | APOA1, D6I, FABP3, FABP4, FABP5, and UBC | 0.00    |
| 3     | Complement and coagulation cascades | 9               | A2M, C3, C4BPA, CF5, CFI, F5, FGA, FGB, and FGG | 0.00    |
| 4     | IL-17 signaling pathway             | 5               | LCN2, LOC786350, MUC5AC, MUC5B, and S100A8 | 0.03    |
| 5     | Salivary secretion                  | 12              | CAMP, CATHL2, CATHL3, CATHL4, CATHL5, CATHL6, CATH2, CATH2L, CATH2L4, CATH2L5, CATH2L6, CATH3, LOC785803, LPO, LY22, LY23, and MUC5B | 0.00    |
| 6     | Pentose phosphate pathway           | 7               | ALDOA, ALDOB, ALDOC, GPI, PGD, TALDO1, and TKT | 0.00    |
| 7     | Fructose and mannose metabolism     | 4               | ALDOA, ALDOB, ALDOC, and TP1 | 0.00    |
| 8     | Cysteine and methionine metabolism  | 5               | LDHA, LDHB, LDHC, MDH1, and SDS | 0.00    |
| 9     | Pyruvate metabolism                | 5               | LDHA, LDHB, LDHC, MDH1, and PKLR | 0.02    |
| 10    | Propanoate metabolism              | 3               | LDHA, LDHB, and LDHC | 0.03    |
| 11    | Glucagon signaling pathway          | 5               | LDHA, LDHB, LDHC, PGAM1, and PYGL | 0.03    |
| 12    | VEGF signaling pathway              | 3               | RAC1, RAC2, and RAC3 | 0.09    |
| 13    | Adherens junction                   | 5               | CDH1, CTNNB1, RAC1, RAC2, and RAC3 | 0.01    |
| 14    | B-cell receptor signaling pathway   | 4               | LOC100300716, RAC1, RAC2, and RAC3 | 0.05    |
| 15    | Fc epsilon RI signaling pathway     | 4               | LOC100300716, RAC1, RAC2, and RAC3 | 0.04    |
| 16    | Fc gamma R-mediated phagocytosis    | 5               | CFL1, CFL2, LOC100300716, RAC1, and RAC2 | 0.02    |
| 17    | Fluid shear stress and atherosclerosis | 13             | CTNB1, CTSV, GSTA2, GSTA3, GSTA5, GSTM1, GSTM3, GSTD1, GSTD1, GSTD1, GSTD1, and GSTD1 | 0.00    |
| 18    | Glutathione metabolism              | 9               | GSTA2, GSTA3, GSTA5, GSTM1, GSTM3, GSTD1, GSTD1, GSTD1, OPLAH, and PGD | 0.00    |
| 19    | Metabolism of xenobiotics by cytochrome P450 | 7 | GSTA2, GSTA3, GSTA5, GSTD1, GSTD1, GSTD1, and GSTD1 | 0.00    |
| 20    | Chemical carcinogenesis             | 7               | GSTA2, GSTA3, GSTA5, GSTD1, GSTD1, and GSTD1 | 0.00    |
| 21    | Fluid shear stress and atherosclerosis | 13          | CTNB1, CTSV, GSTA2, GSTA3, GSTA5, GSTM1, GSTM3, GSTD1, GSTD1, GSTD1, RAC1, RAC2, RAC3, and TXN | 0.00    |
| 22    | Cell cycle                          | 7               | SFN, YWHA2, YWHA2, YWHA2, YWHA2, YWHA2, and YWHA2 | 0.01    |
| 23    | Oocyte meiosis                      | 6               | YWHA1, YWHA2, YWHA2, YWHA2, YWHA2, and YWHA2 | 0.02    |
| 24    | Protein processing in endoplasmic reticulum | 8 | GANAB, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, and UBB | 0.01    |
| 25    | Endocytosis                         | 11              | ARF1, ARF3, ARF5, FOLR1, FOLR2, FOLR3, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, and UBB | 0.01    |
| 26    | Hippo signaling pathway             | 8               | CTNB1, CTSV, YWHA2, YWHA2, YWHA2, YWHA2, YWHA2, YWHA2, and YWHA2 | 0.01    |
| 27    | Antigen processing and presentation | 8               | B2M, CTSV, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, HSPA1A, and UBB | 0.00    |
| 28    | Estrogen signaling pathway          | 4               | HSPA1A, HSPA1A, HSPA1A, and HSPA8 | 0.1     |
CONCLUSION

This is the first comprehensive report on differential proteome analysis of buffalo saliva between the stages of estrus and non-estrus by employing both label-free and TMT-based labeled quantitation and mass spectrometry analysis. Numerous proteins involved in endopeptidase activity, glycolysis, pyruvate metabolism, calcium ion binding, estrogen signaling, and pheromone signaling were identified as differentially expressed between estrus and non-estrus phases. The extensive saliva proteome data set obtained in this work will provide insights into the cellular processes behind buffalo estrous biology. Taken together, this work identified a critical panel of potential proteins in saliva that may be used as a biomarker for buffalo estrus and paves the way for the development of a diagnostic kit for buffalo estrus detection.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institute Animal Ethics Committee, NDRI, Karnal [42-IAEC-18-9].

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AUTHOR CONTRIBUTIONS

RB, TKM, AKM and AK conceptualized the study and arranged the funds. LKS, SP, and Neeraj carried out the sampling. RB, AKM, and MP designed the experiments. MP and LKS performed all experiments. MP, AF, SP, Neeraj, and LKS performed Western blotting. RB and SA performed bioinformatics and statistical analysis. RB, MP, and LS wrote the manuscript, and all other authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

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