CHAPTER 5
DISCUSSION
5.0 DISCUSSION

In the past several decades, and with the advent of more advanced molecular techniques, major advances have been made in understanding the various intricate aspects of mammary gland, particularly in livestock species (Akers, 2006; Hadsell, 2004, Loor & Cohick, 2009). The development of high-throughput tools such as microarray analysis has provided the unique possibility to uncover the molecular networks in mammary tissue during the course of pregnancy, milk synthesis, and involution. Studies conducted to characterize transcriptome changes that occur during mammary gland development have provided insight into key processes involved in signalling and morphogenesis of the mammary gland. Significant proportion of research on mammary development have been conducted on small model animals like mouse which differ appreciably from the farm animals physiologically and also with respect to differences in mammary gland size. Therefore, it may not be possible to translate the rodent data on large milch animals like buffalo. Therefore, the present study was undertaken to elucidate the global gene expression profiling during different physiological stages of buffalo mammary gland with an effort to provide insights into the complex functioning and networks associated with the mammary gland.

In this study, microarray analysis was carried out to profile gene expression changes in the buffalo mammary gland during different physiological stages using the Agilent 60-mer Bovine gene expression Microarray. Unfortunately, many of these bovine transcripts have not been annotated and the majority of the available annotations were based on the sequence similarity to other species to present insights into buffalo mammary transcriptome. In this study, only the annotations based on the strong sequence similarity were used and these annotations should be considered to be reliable.

While delineating the gene expression pattern, a partial list of the 1000 genes belonging to the cluster with the highest expression during buffalo mammary transcriptome was revealed by self-organizing map clustering method as depicted in Table 2 (results). The top of the cluster was found to be predominantly occupied by milk protein genes followed by ribosomal protein genes, genes implicated in transport function, immunological defence and apoptosis, and regulation. To simplify the interpretation of the microarray datasets and associated gene networks in the present work, current findings have been grouped in separate headers relevant to the specific gene expression profile of buffalo mammary gland.
5.1 Expression profiles of the prominent genes in mammary transcriptome

5.1.1 Milk protein genes

The main proteins in milk are caseins and whey proteins (i.e., alpha-lactalbumin, betalactoglobulin, whey acidic protein (WAP), albumin, and immunoglobulin; proteins highly enriched in the milk serum after removal of casein). The milk protein content and composition of the main milk proteins with abundance of each of the caseins and whey proteins in several species has been summarized in Table 1 (review and literature). The clustering of our microarray datasets revealed milk protein genes as the most highly expressed genes during lactation as reported in earlier studies. It has been previously shown in mouse (Rijnkels et al., 1997; Robinson et al., 1995), rat (Rosen et al., 1995), and rabbit (Shuster et al., 1976) that the expression of milk protein genes starts in early to mid-pregnancy, increases throughout the pregnancy and reaches a plateau in late pregnancy and early lactation. This expression pattern may be similar in the buffalo. In addition, lactose synthesis has also shown to start in mid-pregnancy in the rabbit (Mellenberger & Bauman, 1974) and cow (Mellenberger et al., 1973). The temporal patterns of transcription of prominent milk protein genes from pregnancy to lactation in two ruminant species (dairy cow [Bionaz et al., 2012] and dairy goat [Faucon et al., 2009]), two monogastric species (mouse [Rudolph et al., 2007] and pig [Shu et al., 2012]), and kangaroo [Lefevre et al., 2007]) has also been reported and summarized by a recent study. The temporal expression pattern of all the casein genes was reported to be similar, and was consistent for the two ruminant species, with CSN1S2 followed by the CSN1S1 as the most up-regulated casein genes in both species and with an overall greater up-regulation of casein genes in the cow relative to the goat which is consistent with our study. Alpha-lactalbumin was similarly up-regulated in all species except kangaroo. The other caseins, despite being significantly affected by time, were not highly upregulated by lactation in those species as also indicated in buffalo. In the kangaroo, all transcripts coding for caseins were found to be upregulated compared with LALBA which had slight increase in expression during lactation. The low importance of alpha-lactalbumin in this species has been previously established and its presence in milk does not change throughout lactation (Messer et al., 1987). Lactoferrin (LTF) showed expression pattern on expected lines. Significant (p<0.05) higher expression of this gene was observed during involution stage of buffalo mammary gland in comparison to lactating mammary tissues. LTF is known to preferentially synthesize in involuting mammary tissue compared with lactating tissue and its known to be regulated differently than the other milk proteins (caseins, LALBA
etc.) expression (Cheng et al., 2008; Piccinini et al., 2007). Therefore, it is evident from above data that the gene expression profile of milk protein genes followed the expected trend in the present study during lactation stage of buffalo.

5.1.2 Genes implicated in transport of amino acids, glucose, fatty acids

The two major types of transporters that have been mostly involved in active transport of milk constituents are the members of ATP-binding cassette (ABC) transporters superfamily and glucose transporters (GLUT). The ABC transporters, one of the largest families of transporters is known to play an important role in regulating cellular cholesterol homeostasis and transfer of wide variety of substrate across cellular membrane (Higgins, 1992; Dean & Allikmets, 1995). Some members of ABC transporters viz. ABCA1, ABCG1, ABCA7 have been identified specifically to mediate the cellular lipid transport during lactation and dry period of mammary gland and are the active transporters regulating secretion of milk fat from mammary epithelium. Another class of transporters that are involved in glucose uptake is known as glucose transporters (GLUT). At the time of onset of lactation, glucose uptake in the mammary gland increases dramatically and expression of these transporters has been correlated with the milk synthesis (Threadgold & Kuhn, 1984; Zhao & Keating, 2007). During lactation, glucose is predominantly transported across plasma membrane of mammary epithelial cells by passive transport due to establishment of glucose concentration gradient (Faulkner et al., 1981). This facilitative diffusion of glucose across plasma membrane is mediated by a family of glucose transporters. A number of glucose transporter genes (GLUT1, GLUT3, GLUT4, GLUT5, GLUT8, and GLUT12) have been reported, but GLUT1 is the predominant glucose transporter in lactating bovine mammary gland (Zhao & Keating, 2007). Amongst the ABC transporters, expression of ABCA1, and ABCG1 was significantly (p<0.05) high in heifer followed by involution and lactation stages of buffalo mammary gland (Fig.1). Like ABCA1 and ABCG1 genes, ABCA7 gene was more expressed in heifer, however, its expression was higher in lactating as compared to involuting stage of buffalo mammary gland. The expression pattern for these transporters genes, more or less corroborated with the bovine mammary tissue expression data (Farke et al., 2008; Mani et al., 2009). These two studies have compared lactation and non-lactation stages of bovine mammary gland, and similar to our findings, they reported higher expression of ABCA1 gene in non-lactating (involuting) mammary gland in comparison to lactating mammary gland. However, both these studies showed somewhat different profile for ABCA7 gene. The higher expression of ABCA7 in lactating buffalo mammary gland observed in our study matches
with other study (Farke et al., 2008). However, its higher expression was reported in non-lactating mammary tissue (Mani et al., 2010). The differential expression of ABCA1, ABCA7 and ABCG1 transporters in buffalo mammary tissues suggested their distinct physiological roles they play depending on the functional stage of the mammary gland. The relatively higher expression of these genes in heifer mammary tissue could be suggestive of the fact that these genes have important role to play during the developmental process as well. The localization of ABCA1, and ABCG1 in mammary epithelium of human, murine and bovine mammary epithelium (Mani et al., 2010) and their expression in buffalo mammary tissue, clearly supported the involvement of these transporters in regulating lipid transport activity of mammary gland in different species. Their role in conjunction with ABCG in mediating lipid efflux has already been reported in other tissues (Oram & Heinecke, 2005; Cavelier et al., 2006). The role of ABCA1 in removing excess cellular cholesterol from peripheral cells onto apolipoproteins has also been reported. Similar role for these transporters could also be explained in buffalo mammary gland as these transporters might be associated in regulating cholesterol homeostatis and prevention of cholesterol accumulation in lactating mammary epithelial cells. In past studies have shown that ABCA7 has a role in effluxing only phospholipids in peripheral tissues, suggesting its role in phospholipid metabolism. Though, ABCA7 is highly homologous to ABCA1 their differential expression pattern makes both these as important genes whose physiological roles in buffalo mammary gland needs to be investigated further. Additionally, we demonstrated significant (p<0.05) induction of ABCG2 gene in lactating mammary tissue of buffaloes in comparison to involution and heifer stages. Our data set indicated lowest expression of this gene during heifer stage. The expression of this particular lipid transporter has also been shown to be greatly induced during lactation and repressed during involution in mammary gland of mouse, cows and human (Jonker et al., 2005; Cohen-Zinder et al., 2005). Establishing its functional role (Herwaarden et al., 2007), it has been demonstrated that ABCG2 not only secrete drugs but also riboflavin (vitamin b2) in milk secretion. This gene has also been associated as candidate gene with important functional role in regulating milk yield, fat and protein concentration in bovines (Cohen-Zinder et al., 2005). Similar to ABCA1 and ABCG1, the expression of ABCG5 was higher in involution stage in comparison to lactation stage, though the difference in expression was statistically nonsignificant. The expression pattern for ABCG5 observed for buffalo gland was different than reported by Farke et al. (2008) for bovine mammary gland, wherein they have found lower expression of this gene during the dry stages. However, similar to Farke et al. (2008), our data set also
showed much lower expression of this gene during all stages of buffalo mammary gland (high Ct values ranging from 34 to 38). Hence it was quite difficult to relate the expression data and the physiological role that this gene could play in buffalo mammary gland. Amongst the glucose transporters investigated, GLUT1 mRNA showed significantly (p<0.05) higher expression during lactation in comparison to involution and heifer stages (Fig.1). GLUT1 is known as the major glucose transporter in lactating cows and change in its expression has been associated with insulin independent glucose uptake in mammary gland. Studies have shown that there is a linear relationship between the rate of glucose transport and milk yield in cows (Kronfeld, 1982). In the present study, the higher expression of GLUT1 gene in lactating stage suggested its role as predominant glucose transporter in buffalo mammary gland as well. The similar trend was observed for GLUT8 gene, however the differences in expression across three stages were non-significant. The GLUT4 mRNA expression was similar during heifer and lactating stages while lower in involuting samples. The GLUT12 gene expression was significantly (p<0.05) higher in heifer mammary gland in comparison to involution state, though this increase was not significant in comparison to lactating tissue.

5.1.3 Regulator Genes
Expression of various transporter genes is known to be under transcriptional regulation and nuclear orphan receptors. The expression of LXR-α and SREBF1 which is known to be a key regulator of ABCA1 and ABCA7 genes respectively were also analyzed. The mRNA expression of LXR-α gene was more in involuting mammary gland as compared to lactating tissues which could reflect the fact that the expression of ABCA1 and other ABC cholesterol transporters are regulated to a certain extent by expression of LXR-α gene. Similar to our findings, Farke et al. (2008) demonstrated higher expression of LXR-α gene within the second week of dry period. In contrast, Mani et al., (2010) reported slightly lower expression of LXR-α gene in non-lactating in comparison to lactating stages of bovine mammary gland. In their study, the expression of this gene was shown to increase transiently after parturition and decreased again at the end of lactation. The difference with our data set might be due to the fact that Mani et al. (2010) utilized samples during different intervals of lactation whereas we have included samples from peak lactating and involuting stage samples. On the other hand, SREBF1 expression pattern was similar to ABCA7, as its expression was more in lactating tissue as compared to involuting stage, indicating its role in regulating ABCA7 gene. The expression of PPAR-α, which is a ligand activated transcription factor and member of the nuclear hormone receptor superfamily expression was significantly (p<0.05) more in
lactating tissue in comparison to heifer and non-significantly in comparison to involution stage.

5.2 Identification of gene pathways during different physiological stages of buffalo mammary gland

The gene ontology and KEGG analysis linked many up-regulated genes to metabolism and transport processes which is consistent with the observations in mouse mammary gland (Rudolph et al., 2003; Rudolph et al., 2007). During lactation, the mammary gland increased overall metabolism, e.g. large increase in carbohydrate, lipid, and metabolism of other secondary molecules (e.g., glycans). Overall visualization of data suggested a channelling of glucose towards increasing the synthesis of lactose and decreasing use of energy. Further, a reduction of fatty acid metabolism was observed together with an increase in synthesis of triacylglycerol indicated a preferential channeling of fatty acids taken up by the mammary tissue (Miller et al., 1991) towards synthesis of milk fat, including the components of cellular membranes. Those events were coupled with a large increase in membrane transport and signaling and a strong participation of the immune and endocrine system. During early lactation, mammary requirements of amino acids, glucose, and other nutrients for milk synthesis to surge dramatically. It has been estimated that mammary uptakes of glucose, amino acids, and fatty acids in Holstein cows are more than 1.8, 1.4, and 1.2 kg per day at 4 days postpartum, respectively (Bell, 1995). Thus, it is not surprising to see that more than 15% of up-regulated transcripts are associated with the transport processes of amino acids, glucose, fatty acids, and ions. As it was revealed from KEGG pathway analysis that ‘ABC transporters’ and ‘Glycosylphosphatidylinositol (GPI)-anchor biosynthesis’ were among the top impacted pathways. The importance of the GPI-anchored proteins has not been evaluated in buffalo mammary gland. In addition, although the concentration of GPI anchored proteins is high in human and pig milk, it is apparently undetectable in bovine (Kunz et al., 1998) milk. GPI-anchors are pivotal for cell survival and affect many functions such as protein sorting in ER-Golgi trafficking, targeting of GPI-anchored proteins in polarized epithelial cells for apical export, cell-to-cell adhesion, signal transduction associated with cholesterol and sphingolipids in membrane rafts, and sorting in endocytic pathways (Chatterjee & Mayor, 2001). Except for transport of folic acid through the internalization of its receptor, which is a known GPI-anchored protein, to date there are no known roles of GPI-anchored proteins in milk synthesis (Chatterjee & Mayor, 2001). Thus, this finding is novel and more research should be directed towards GPI-anchored proteins. Another category was ‘ABC
transporters’ which was induced overall during lactation. Several ABC transporters seem to be involved in cholesterol transport and some are down-regulated during lactation in buffalo mammary tissue. Complete role and expression pattern for these transporters has already been discussed in this study.

“Cell signal transduction” function was found to be highly impacted and activated during lactation. It can be suggested that the Jak-STAT signaling is essential for the induction of milk protein expression in mammary tissue of non-ruminants, but in ruminants and particularly in the bovine, expression of milk proteins appears to require a basic activity of this pathway but is not directly modulated by the change in expression of Jak and/or Stat genes, rather, it seems to be modulated by down-stream effectors of the pathway (Bionaz & Loor, 2011).

Genes involved in cell division, cell cycle and their related processes, such as microtubule-based process, DNA replication, chromosome organization, and biogenesis were found to be considerably downregulated. During pregnancy, the mammary gland undergoes major structural development and functional differentiation to produce milk. This is accomplished by early-stage ductal morphogenesis and late proliferative phase of alveolar morphogenesis (Hovey et al., 2002; Neville et al., 2002). Another group of genes, which were downregulated at early lactation, was proteasomal genes and other related genes involved in proteolysis. This group of genes were also shown to have a decreased expression throughout pregnancy and lactation and an increased expression during involution in mouse mammary gland (Rudolph et al., 2003). It was speculated that the decline in protein synthesis machinery is a functional adaptation of the mammary gland to conserve biosynthetic processes activated during lactation and/or to require less mRNA to replenish degraded protein (Rudolph et al., 2003). Further a large inhibition of the expression of major histocompatibility complex components suggesting that the mammary gland appeared to have placed substantial effort in preparing the immune system but also simultaneously prevented oversensitivity to pathogen invasion.

5.3 Validation of microarray datasets

5.3.1 Evaluation of suitable housekeeping gene (HKG) for buffalo mammary gland

Expression stability of 16 candidate genes from different functional classes was evaluated to select appropriate HKGs for buffalo mammary tissues. To the best of our knowledge, no concrete information is available about the appropriate HKGs for riverine buffalo mammary gland expression data analysis. Genes from different functional classes representing different pathways were chosen to avoid co-regulation. It is a well-accepted fact that lack of
appropriate HKGs could lead to erroneous interpretation of any qPCR-based expression experiment. Several tools have been developed to test the expression stability of candidate HKGs. We used three freely available softwares: (GENORM, Normfinder and BestKeeper). From the combined analysis, it appeared that most of the genes analysed in this study exhibited sufficient stability and could be used for normalization. Still, we tried to utilize the three algorithms systematically to identify best possible gene combinations for normalization of gene expression data in buffalo mammary gland. RPS15A and EEF1A1 were the most stable genes as per GENORM analysis. The same set of genes was found to be most stable with BestKeeper analysis. The correlation between EEF1A1 and RPS15A pair was sufficiently high (r = 0.775), indicating their suitability as HKGs. As per Normfinder analysis, UXT was found to be most stably expressed followed by EEF1A1 and RPS15A. This variation could be due to the fact that for Normfinder and GENORM analysis, the underlying algorithms are quite different. Overall analysis revealed that the expression of EEF1A1, RPS15A, RPL4 and B2M was most consistent across different physiological stages of buffalo mammary gland, therefore, geometric mean of these genes was used to normalize the quantitative real time PCR data.

5.3.2 Expression profiles of selected genes for validation of microarray data
Quantitative real-time PCR (qPCR) is a commonly used validation tool for confirming gene expression results obtained from microarray analysis. We selected 11 genes viz. Cholesterol transporters viz., ABAC1, ABCG1, ABCA7, ABCG5, ABCG2; their regulators viz., SREBF1, LXR-α and glucose transporters viz., GLUT 1, GLUT8, GLU4 and GLUT12 exhibiting differential level of expression among different physiological stages of buffalo mammary gland. For normalization of our qPCR data, the geometric mean of the Ct values of EEF1A1, RPS15A, RPL4 and B2M genes, the four best reference genes identified in our studies for buffalo mammary gland was used. The expression profile of all of these genes have been discussed previously in this section. A high correlation between the signal log ratios of the real time PCR and the microarray (p<0.0001) was observed confirming the results obtained by the microarray analysis.

5.4 Sequence characterization of selected transcripts in buffalo mammary gland
An attempt was made to sequence characterize some of the major mammary gland derived genes of buffalo. For this purpose, single-pass sequencing of 1031 randomly selected cDNA clones from pre-constructed mammary gland specific cDNA libraries of riverine buffalo
Bubalus bubalis was undertaken. Significant homology analysis of buffalo mammary gland derived ESTs with sequences from different species showed results on expected lines. Almost all the sequences that matched with Bubalus bubalis genes were identical matches (97-100%), with only one lower score of 84% which could be due to sequence ambiguity. The percent identity of ESTs that matched bovine sequences ranged between 76 to 98%, however, majority showed an identity level of 95%. The relatively lower identity percentage observed with other mammalian sequences indicated its evolutionary divergence status.

Of the 416 and 313 informative ESTs generated in lactating and non-lactating libraries respectively, 38 (9.13%) and 117 (37.3%) EST sequences were singletons and represented only once. However, remaining large number of clones represented multiple times in both the libraries, especially the clones representing milk protein genes. This was expected considering the fact that both the libraries were non-normalized and unsubtracted. Multiple clones representing for different genes ranged from 2->50 in lactating and 2->40 in non-lactating library.

Sizeable percentage of annotated proteins was involved in protein biosynthesis, transporter, metabolism and signal transduction. Proteins related to transcription/translation regulators, apoptosis, structural constituents of ribosomes were also expressed. Overall, the analysis of functional distribution of known ESTs in the studied libraries is consistent with the established functional role of mammary tissue.