SYSTEMS PHYSIOLOGY AND NUTRITION IN DAIRY CATTLE: APPLICATIONS OF OMICS AND BIOINFORMATICS TO BETTER UNDERSTAND THE HEPATIC METABOLOMICS AND TRANSCRIPTOMICS ADAPTATIONS IN TRANSITION DAIRY COWS

BY

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DISSERTATION

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ABSTRACT

Application of systems concepts to better understand physiological and metabolic changes in dairy cows during the transition into lactation could enhance our understanding about the role of nutrients in helping to meet the animal's requirements for optimal production and health. Four different analyses focused on the liver were conducted to analyze metabolic disorder or thermal stress. The first three analyses dealt with supplementation of methionine to prevent clinical ketosis development in high-genetic merit dairy cows. Four groups of cows were formed retrospectively based on clinical health evaluated at 1 week postpartum: cows that remained healthy (OVE), cows that developed ketosis (K), and healthy cows supplemented with one of two commercial methionine products [Smartamine M (SM), and MetaSmart (MS)]. The liver tissue samples (n = 6/group) were harvested at -10 d before calving, and were used for metabolomics (GC-MS, LC-MS; Metabolon Inc.) and transcriptomics (44K-whole-transcriptome microarray; Agilent) analyses. Therefore, the main goals of the analyses were to 1) uncover metabolome and transcriptome patterns in the prepartum liver that were unique to those cows that became ketotic postpartum, and to 2) uncover unique patterns affected by supplemental methionine. The data were analyzed using the MIXED procedure of SAS. The metabolomics analysis ($p \leq 0.10$) resulted in 13, 16, 26, 36, 13 and 43 biochemical compounds out of 313 identified for the comparisons K vs. OVE, SM vs. OVE, MS vs. OVE, SM vs. MS, K vs. SM and K vs. MS, respectively. The transcriptomics analysis ($p \leq 0.05$ and fold change (FC) $\geq |1.5|$) resulted in 3,065, 710, 786, 601, 1,021 and 771 number of differentially expressed genes (DEG) for the respective comparisons. The functional analysis of the data was performed using dynamic impact approach (DIA). The network reconstruction and data integration was performed with

Ingenuity Pathway Analysis (IPA). In the first analysis of K vs. OVE, the results indicated inhibition of several carbohydrate- and lipid-related metabolic pathways, while activation of 'Selenoamino acid metabolism', 'Ribosome', and 'Replication and repair' was predominant. In the second analysis of SM vs. OVE, 'Nitrogen metabolism', 'Glycosaminoglycan biocynthesis-chondroitin sulfate', 'Synthesis and degradation of ketone bodies' and 'Selenoamino acid metabolism' were induced while the 'Cyanoamino acid metabolism', 'Taurine and hypotaruine metabolism' and 'Inositol phosphate metabolism' were inhibited. The analysis of MS vs. OVE revealed activation of 'Riboflavin metabolism', 'Bile secretion' and 'Vitamin digestion and absorption', while inhibition of 'Base excision repair', 'Cyanoamino acid metabolism', and 'One carbon pool'. The analysis of SM vs. MS indicated activation of 'Intestinal immune network for IgA production', 'Antigen processing and presentation', and 'Riboflavin metabolism', while the inhibition 'Glycosaminoglycan degradation', 'Other glycan degradation' and 'Bile secretion'. In the third analysis of K vs. SM, among the top 10 affected pathways, most were inhibited. Examples include 'Cynoamino acid metabolism', 'Fructose and Mannose metabolism', 'Erb signaling' and 'Pentose phosphate pathway'. In contrast, the analysis of K vs. MS revealed an induction of 'Nitrogen metabolism' among the top 10 pathways, while pathways such as 'Riboflavin metabolism', 'Pentose phosphate pathway' and other carbohydrate and glycan biosynthesis related pathways were inhibited. The fourth analysis dealt with the effect of thermal stress on the liver transcriptome as it is related to health and productivity. During this study, we used gene network analysis on transcriptome data to uncover transcription regulators and their target genes in the liver tissue harvested at -30, +3, and +35 d relative to parturition during spring (SP, n = 6) and summer (SU, n = 6). Statistical analysis (FDR $\leq$ 0.10) of data from SU vs. SP revealed a total of 618, 1,030 and 894 DEG at -30, +3 and +35 d, respectively. IPA was used for

gene network reconstructions. A total of 6, 7 and 7 transcription regulators were identified at -30, +3 and +35 d, respectively during SU vs. SP. The evaluation of these results suggests that calving during SU vs. SP is associated with the molecular phenotypes of the liver.

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ABBREVIATIONS

BHBA	β-hydroxybutyrate
d	day(s)
DEG	differentially expressed genes
DIA	Dynamic Impact Approach
DIM	days in milk
DMI	dry matter intake
FA	fatty acid
IPA	Ingenuity Pathway Analysis
K	Ketosis
KEGG	Kyoto Encyclopedia of Genes and Genomes
MS	MetaSmart
NEB	negative energy balance
NEFA	non-esterified fatty acids
OVE	overfed with moderate energy diet
SM	Smartamine M
TG	triglycerides/triacylglycerides
TR	transcription regulators
VLDL	very low-density lipoproteins

CHAPTER # 1

Literature review

The Transition Period in Dairy Cows

"***May you live in interesting times***".

An ancient Chinese proverb (Drackley, 1999).

The transition period also known as the periparturient period in dairy cows ranges from 3 weeks prepartum to 3 weeks postpartum (Grummer, 1995, Drackley, 1999). During this period, cows undergo important physiological, metabolic and nutritional changes. It is also critical because cows become vulnerable to several metabolic disorders and infectious diseases (Simianer et al., 1991, Drackley and Cardoso, 2014). This period requires more attention than the usual time period to prepare cows for early lactation and to prevent the metabolic disorders, reproductive complicacies and subsequent fertility issues (Drackley and Cardoso, 2014). It has been well recognized that amount of dry matter intake (DMI) decreases as calving approaches leading to physiological variations (Grant and Albright, 1995). The demand for nutrients uptake by fetus, placenta and mammary glands is increased especially during the last wk before calving. This demand is met partly by voluntary feed intake and partly by metabolic adaptations.

These metabolic adaptations include increased fatty acid mobilization, muscle degradation and hepatic gluconeogenesis, and decreased glucose utilization by peripheral tissues (Bell, 1995). The lactation phase starts immediately after parturition, where nutrient demands for milk synthesis exceed the available nutrients resulting in metabolic level changes. These altered

metabolic adaptations lead to negative energy balance (NEB) and related metabolic disorders which as a result affect the overall hepatic gene expression patterns in dairy cows (McCarthy et al., 2010). During this period, the nutritional demands of amino acids for milk protein synthesis and immune functions are also greatly affected (Bell, 1995, Goff, 2008). The following section describes about the energy and protein balance conditions.

Negative energy and protein balance

During the transition period, a marked decrease in DMI usually limits the consumption of dietary energy intakes and in turn affects the energy balance equilibrium (Bertics et al., 1992). During the period of early lactation, an increased amount of nutrient requirements over nutrient availability may lead to several physiological disorders such as NEB, fatty liver and ketosis due to increased lipid infiltration or ketone bodies synthesis. These physiological disorders are associated with the onset of lactation and lead to imbalanced immune system in periparturient dairy cows (Bertics et al., 1992). The NEB usually lasts until the milk yield starts to decline (7-10 wk postpartum) and the energy from the DMI becomes sufficient to meet the nutritional requirements (Roche and Berry, 2006). During first few weeks of lactation, dairy cows rely on fats and amino acids mobilization from adipose tissue and muscles for energy and protein requirements (Drackley, 1999). The amino acid requirements are seen in terms of degradable and bypass proteins to support body functions, stabilizing metabolism of carbohydrate and lipids. These serves as precursors for gluconeogenesis, tissue protein synthesis and other metabolic reactions. The net amount of amino acids requirement is increased during the transition period to overcome the increased demand of gluconeogenesis and energy synthesis leading to negative protein balance (Van Saun and Sniffen, 2014). If cows are not provided with the optimal protein

requirements then the resulting situation may lead to metabolic disorders and NEB. As microbial protein provides very small amount of amino acids supply to the total absorbed proteins. So the gap between supply and demands needs to be bridged through both transition and lactation periods in the form of amino acid supplementations e.g., rumen-protected amino acids (Dalbach et al., 2011, Osorio et al., 2013).

Metabolic disorders

Metabolic disorders that result from energy balance disequilibria reflect failures to adapt the shortage of available nutrients that are vital for maintaining the health of dairy cows. The examples of such metabolic disorders include milk fever, ketosis, lipidosis, metritis, displaced abomasum, poor fertility and poor production. Among these, ketosis jeopardizes the dairy health postpartum due to excessive production of ketone bodies leading to several health and productivity related issues such as decreased milk production, and delayed onset of reproductive cycle (Ospina et al., 2013, Zhang et al., 2015). As our main focus is to uncover the effects of methionine supplementation during the close-up dry period (from -21 d to calving) to prevent ketosis development postpartum in dairy cows, so the following discussion mainly revolves around the causes of ketosis development and its preventive measures.

Ketosis

Ketosis is a major metabolic disorder that can affect the productivity and cost of dairy farms. It is associated with NEB, hepatic lipid accumulation, elevated level of ketone bodies and

lower blood glucose concentration. Dairy cows become highly susceptible to developing ketosis during early lactation (Loor et al., 2007). It is characterized by marked increases in circulating ketone bodies and primarily occurs within the first few weeks of lactation. It may result due to severe NEB, where free fatty acids in the form of non-esterified fatty acids (NEFA) are released from adipose tissue and start entering into the liver to meet up the energy requirements. These NEFA are usually esterified in the liver and are exported as triglycerides within very low density lipoproteins (VLDL) to extra hepatic tissues such as mammary glands. At this point, when the rate of NEFA oxidation is increased as compared to its rate of secretion, then this process leads to higher synthesis of ketone bodies (Morrow, 1976). There are three main types of ketone bodies that are generated during this process. These include acetone, acetoacetate and beta hydroxybutyrate (BHBA). Among these, increased synthesis of BHBA plays a key role in ketosis development, whereas acetoacetate is broken down into acetone, which is a volatile compound and is vaporized (Herdt, 2000). The ketone bodies accumulate in the blood when their concentration exceeds their utilization as an energy source (Adewuyi et al., 2005). However, the presence of ketone bodies is normally expected in fresh cows due to NEFA mobilization postpartum where a portion of NEFA is converted into ketone bodies in normal circumstances (Duffield, 2000). However, an excessive amount of ketone bodies above the threshold levels leads to ketosis development.

Ketosis is also associated with lipidosis or fatty liver. The condition when the hepatic uptake of lipid infiltration exceeds over its oxidation and secretion is called lipidosis (Loor et al., 2007). This may also be caused by a decreased synthesis of apo-lipoproteins at the time of parturition leading to severe inflammation response (Bertoni et al., 2004). Dairy cows become

more susceptible to other pathologies when the amount of hepatic lipid accumulation is increased in the liver. During this time, the recovery period usually becomes prolonged (Herdt, 1988).

Ketosis measurement

Ketosis can be measured by several different methods including blood, urine and milk. Most research studies are based on blood BHBA concentration, which is considered as the "***gold standard***" in ketosis testing due to its stability in blood as compared to acetone and acetoacetate (Oetzel, 2004). Defining an exact level at which ketones are too high has been something of an enigma. Ketosis is a threshold disease, meaning, cows will only be affected after a certain level of threshold has been reached. Most sources use 1000 to 1400 µmol/L of BHBA as a threshold (Duffield, 2000).

General types of Ketosis

Ketosis has been classified into different types based on perceived risk factors, etiology, pathophysiology or clinical symptoms. Currently used classification scheme divides ketosis into two main types (type I and II) based on the physiology and time of occurrence (Holtenius and Holtenius, 1996, Herdt, 2000).

Type I Ketosis

During this type, the gluconeogenesis mechanism is fully involved in utilization of available glucose precursors. However, the required need for glucose synthesis is relatively insufficient due to the lower supply of available glucose precursors in the diet (e.g., propionate,

and amino acids). This type of ketosis is characterized when cows have reached a high level of milk production during 3 to 6 weeks of postpartum and are still in NEB. It is not further associated with fatty liver infiltration. Type I condition can be prevented by feeding cows with starch rich diet. Therefore, it is not as dangerous as the following type II (Goff, 2006).

Type II Ketosis

Type II Ketosis usually occurs during the first few weeks of lactation and is related to fatty liver infiltration or lipidosis (Herdt, 2000). It is similar to type I, but in more aggravated condition. It is characterized by low level of blood glucose, elevated levels of NEFA and ketone bodies concentrations. This type is accompanied with various other risk factors such as retained placenta, metritis, displaced abomasum, adipose sensitivity (increased lipolytic response to a given stimulus) and insulin resistance. The treatment duration is usually prolonged as compared to type I (Herdt, 2000). It has been reported as the most common type in the United States dairy farms (Loor et al., 2006).

Clinical types of Ketosis

Ketosis is also classified based on subclinical and clinical symptoms (Baird, 1982). A brief description about these types is provided in the following sections.

Subclinical Ketosis

Subclinical ketosis is characterized by greater than normal ketone bodies (BHBA) level (1000-1200/1400 µmol/L) in blood or milk circulation with no adverse effects observed during early lactation (Oetzel, 2004, Duffield et al., 2009). It is a threshold disease. The elevated level

of BHBA concentration above 1400 µmol/L (14.4 mg/dL) is used as a cut of point for its diagnosis (Oetzel, 2004). Subclinical ketosis is associated with lower milk production, poor reproductive performance and increased rate of periparturient diseases development (Duffield, 2000, Raboisson et al., 2014). It is a critical disorder and needs to be detected in earlier stages, as if it remains undetected, then it may leads to similar effects as caused by clinical ketosis (Baird, 1982).

Clinical Ketosis

Clinical ketosis is characterized by higher concentration of circulating BHBA (2000-2500 µmol/L) as compared to subclinical ketosis. It is further associated with other physiological symptoms such as inappentence (reduced DMI), lower blood pH, hypoglycemia, hyperketonemia, and reduction in body weight and body condition score (Baird, 1982). Reduced DMI is often accompanied by lower milk production and increased susceptibility to infectious diseases (Gerloff, 2000). It has been estimated that approximately 50% of high producing dairy cows experience a case of subclinical ketosis and nearly 6% of the subclinical cases proceed to clinical during early lactation (Grohn et al., 1989, Geishauser et al., 1998).

Ketosis treatment

Treatment of ketosis involves increased glucose supply to meet energy and lactation demands. The incidence of ketosis can be minimized by supplementing the diet with extra nutritional requirements and proper dairy management according to the National Research Council (NRC) recommended guidelines (Baird, 1982, NRC, 2001b, a). The following literature

describes the dietary supplementation with essential amino acids such as methionine and choline during the close-up dry period (3 wk prepartum).

Amino Acid Supplementation

During the onset of lactation, essential amino acids such as methionine and lysine are required to maintain DMI, milk yield, milk protein concentrations and healthy immune system (Soder and Holden, 1999, St-Pierre and Sylvester, 2005). These amino acids have been considered as limiting factors for lactating dairy cows during early- and mid-lactation (Schwab et al., 1992, Schwab et al., 2003). Methionine supplementation in this regard has been the subject of various studies (Smith et al., 2005, Nikkhah et al., 2013, Osorio et al., 2013). As discussed earlier, the NEB prepartum is associated with extensive mobilization of fatty acids from adipose tissue, causing marked elevation of circulating NEFA in the blood and subsequently triglycerides (TG) accumulation in the liver. The excess amount of TG impairs the liver from its normal functioning leading to lipid infiltration or fatty liver and consequently compromising the immune response. The ultimate goal of this stage is to increase the rate of oxidation and the evacuation of TG from the liver through VLDL secretion process. The VLDL secretion is relatively slow process and requires sufficient amount of additives to work properly. In this regard, methionine and choline have been tested as an effective additive to increase the hepatic VLDL secretion during early lactation (Grummer, 2008). In rats, it has been shown that choline deficiency causes TG accumulation in the liver (Tinoco et al., 1965, Juggi and Prathap, 1979). In several other studies it has been reported that both methionine and choline serve as a methyl donor and enhance the process of hepatic VLDL secretion (Auboiron et al., 1994, Grummer, 2008,

Martinov et al., 2010). Methionine deficiency may also interfere with the process of choline synthesis as demonstrated by Ardalan et al. (2009). If both supplementations, provided in an adequate amount, may help to improve the milk yield and protein contents (Sales et al., 2010).

However, there is a limited amount of data available to support the role of methionine supplementation to increase the milk yield during the transition period. The above mentioned and other related studies were mostly focused on achieving the 3:1 ratio of Lysine to Methionine in metabolizable protein (MP) as estimated by National Research Council (NRC, 2001a). Only fewer related studies have reported the clinical significance of methionine supplementation, for example (Osorio et al., 2013). A recent work by Osorio et al. (2014) further highlighted the role of different genes that were affected by methionine supplementation. Soder and Holden (1999) reported that methionine supplementation may also improve the immune response especially during early lactation to avoid farmer costly diseases such as mastitis. It has been shown that rumen protected methionine supplementation is helpful in heat stressed periparturient dairy cows especially during early lactation to increase the productivity (Nikkhah et al., 2013).

Heat Stress in Dairy Cows

Environmental factors affect animal heath by means of complex interactions between them. These factors include heat stress, ambient temperature, cold stress, solar radiation, wind speed and humidity, and have direct or indirect role on animal's health and productivity (Collier et al., 1982a). Consequently, these factors also impact the production of livestock worldwide at a large scale (Nienaber et al., 1999). Many of these interacting factors are difficult to measure, as

these appear only as a seasonal effect in a disease incidence. We have reviewed the phenomena of heat stress with respect to liver tissue in transition dairy cows.

Heat stress during the transition period adversely affects the health, performance, productivity, and immune response in dairy cows (do Amaral et al., 2009, do Amaral et al., 2011). It alters the overall pattern of hepatic gene expression that further results in several metabolic and physiological disorders (Tao et al., 2012, Shahzad et al., 2015). The increased environmental temperature, humidity index and rectal temperature beyond the critical threshold level lead to reduced DMI (Fuquay, 1981, West, 2003). The notable feature caused by heat stress in transition dairy cows is that despite the reduced DMI, fats are not mobilized from the adipose tissue. Consequently, the NEFA level remains lower during the heat stress (Shwartz et al., 2009). However, the pattern of endocrine hormones is changed (Collier et al., 1982b). As a result, the lower feed intake leads to reduced rumination, decreased nutrient absorption and increased energy level maintenance requirements. This whole process results in the net decrease in energy level of the animal leading to NEB.

The NEB during early lactation is further associated with increased risk of metabolic disorders and health related complications as discussed earlier. It has been reported that heat stress results in an incredible loss of both dairy and beef industries. The example include a loss of around one billion dollars in dairy industry when a heat wave struck in California in 2006 (Collier and Zimbelman, 2007). The proper management strategies in the form of proper temperature maintenance and environmental cooling may help to cope with these circumstances (Collier et al., 1982b). In normal circumstances of heat stress, feed supplementation through rumen protected methionine has also provided a significant measure in improving the milk yield and dairy health (Nikkhah et al., 2013).

Bioinformatics Analysis

"*A mind of the caliber of mine cannot derive its nutriment from cows*".

GEORGE BERNARD SHAW, The Star, Apr. 5, 1890.

Bioinformatics and systems biology are key areas to handle and analyze the vast majority of data that are being generated through 'omics' technologies (Shahzad and Loor, 2012). Bioinformatics provides tools and expertise while systems biology helps in data manipulation and integration. In general, bioinformatics analyses are conducted using newly generated or even previously available datasets and their related information to come up with a proof of established knowledge and novel conclusions. The analyses are usually conducted by developing novel software tools or pipelines. Some of the existing software packages include but are not limited to R Bioconductor packages and SAS for statistical evaluations, and Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City) and Dynamic Impact Approach (Bionaz et al., 2012) for post statistical analysis. Examples of the online available databases containing biological data and analysis pipeline tools include but are not limited to the KEGG database (Kanehisa, 2002) and DAVID bioinformatics resource (Huang et al., 2007).

In the current literature, we have considered the bioinformatics analysis for the following two main areas of research such as transcriptomics and metabolomics for the review purpose. We have used transcriptomics data generated through Agilent bovine microarrays platform and metabolomics data generated through GC-MS and LC-MS techniques.

1. Transcriptomics Research

Today's research is mainly focused on systematic approaches rather than reductionist approaches. Systematic approaches make use of the big datasets generated through high throughput omics techniques to come up with novel observations. We have utilized this approach to analyze the data obtained from dairy cows fed with moderate energy diet and supplemented with commercial products of methionine such as Smartamine M (SM) and MeteSmart (MS). Some of the cows that developed ketosis postpartum were taken into consideration to explore the mechanisms associated with the disease development as compared with the healthy and supplemented group of cows. Literature search shows that relatively fewer studies have been performed underlying the mechanism of ketosis development using omics techniques. Loor et al., (2007) conducted a study using microarrays dataset to identify the etiology of ketosis development in the liver of transition dairy cows under different feeding conditions. The overall objective of the study was to test the hypothesis of ketosis development in transition dairy cows fed with restricted (~80% of energy requirement) and ad libitum (~140% of energy requirement) diets prepartum and consequently to evaluate the role of diets on hepatic metabolism by means of differential gene expression within the two groups. During the study, it was found that feeding different diets resulted in differential gene expression with the two groups. It was identified that genes involved in oxidative phosphorylation, protein ubiquitination, ubiquinone biosynthesis, cholesterol metabolism, growth hormone signaling and proton transport were down regulated. Whereas the genes associated with cytokines signaling, fatty acid uptake/transport and fatty acid oxidation were upregulated. There are numerous other studies dealing with the role ketosis development in the liver of transition dairy cows suggesting the etiology of the disease and its treatment mechanisms (McCarthy et al., 1968, Waterman and Schultz, 1972, Osorio et al., 2013).

2. Metabolomics Research

Metabolomics analyses are conducted to better understand the metabolic adaptations occurring in an organism due to several reasons such as diet, treatment, animal physiology, and environmental factors (Berge et al., 2011, Bujak et al., 2014, Wu et al., 2014). It has been shown that metabolomics is well suited for detection of different response variables and metabolic alterations (Beckonert et al., 2007). These analyses are usually conducted using nuclear magnetic resonance (NMR) and mass spectrometry coupled with liquid or gas chromatography. These types of analyses along with transcriptome profiling are helpful in understating the biological mechanisms under pathophysiological conditions.

NMR spectrometry is a quantitative technique that provides a detailed information on solution-state molecular structures, based on atom-centered nuclear interactions and properties (Liu et al., 1996, Beckonert et al., 2007). It has been extensively used for multivariate metabolic profiling of cells, tissues and biological fluids since 1970s (Brown et al., 1977, Nicholson et al., 1983, Nicholson et al., 1984). Several NMR based studies have been published dealing with the underlying mechanisms involved in ketosis development (Klein et al., 2012, Sun et al., 2014, Tetens et al., 2015).

In mass spectrometry, a neutral protein sample is ionized usually through electron bombarding procedure. The ionized molecules are then separated based on a mass to charge ratio (m/z). Then corresponding results are displayed on a mass spectrum, which represent the characteristics of either the molecular mass of molecules and/or structure of proteins or peptides (Fenn et al., 1989, Horgan and Kenny, 2011). The mass spectrometry is often coupled with other chromatographic techniques such as Gas chromatography (GC-MS) or Liquid chromatography (LC-MS). Both techniques work on a similar principle except the physical state of the matter

(Pitt, 2009). The GC-MS technique involves the separation of metabolites or biochemical compounds using a gas chromatograph. The LC-MS technique involves the separation of metabolites or biochemical compounds in liquid phase (usually mixed with water) by chromatography before they are introduced into the ion source (e.g., electron). The results are then interpreted though analytics and statistical methods.

There are couple of mass spectrometry studies that were performed in transition cows with developed ketosis postpartum (Zhang et al., 2013, Li et al., 2014). Among these, Li et al., (2014) reported plasma metabolic profiling using LC-MS technique. The study highlighted 13 potential metabolic biomarkers that are responsible for plasma ketosis. These include glycocholic acid, tetradecenoic acid, and palmitoleic acid (fatty acid metabolism), arginine, valine, glycine, lysine, and leucine/isoleucine (amino acid metabolism), nicotine, tryptophan, aminobutyric acid, creatinine, and undecanoic acid (other metabolisms). In another study, Zhang et al. (2013) reported novel biomarkers by means of GC-MS technique from two main types of ketosis such as clinical and subclinical. These biomarkers were identified in carbohydrates, fatty acids, amino acids, sitosterol and vitamin E isomers. In our current study, we have obtained the results using both GC/LC-MS technique. Our finding are reported in the following chapters.

Summary

Dairy cows are confronted with a large array of physiological and nutritional challenges during the transition period. Failure to meet these challenges often lead to overall production related complicacies and metabolic disorders such as ketosis, and fatty liver. In addition to these

challenges, environmental factors also influence the productivity of dairy cows during the transition period. The studies about methionine supplementation in periparturient dairy cows show that methionine is not only required at cellular and tissue levels but also for treating the heat stress, metabolic disorders and to cope with optimal levels of protein requirements especially after calving. Diagnosis of metabolic related disorders such as ketosis using omics techniques promises to unravel the complex biological mechanisms associated with the onset of the disease. Integrating the results from more than one omics techniques will also help us to identify the interrelated biological mechanisms and their interactions.

Several informatics methodologies have been adopted to improve the health and productivity of dairy cows during transition and lactation phases. These methodologies rely heavily on statistical and bioinformatics approaches to deal with datasets obtained from various high throughput omics techniques including metabolomics and transcriptomics.

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CHAPTER # 2

Integrating metabolomics and transcriptomics of liver to study susceptibility to ketosis in response to prepartal nutritional management

Abstract

Postpartal ketosis is associated with body fat mobilization postpartum. Sub-clinical and clinical ketosis arise more frequently in cows that are overfed energy during the entire dry period or during the close-up (i.e. last 21 d prior to parturition). Metabolomics (GC-MS, LC-MS; Metabolon Inc.) and transcriptomics (44 K-whole-transcriptome microarray; Agilent) analyses were performed in liver tissue harvested at -10 d relative to parturition from cows that were healthy (H) on 7 d postpartum or were diagnosed with clinical ketosis (K). Cows in K consumed a higher-energy diet (OVE) from -21 d to calving. Cows in H consumed OVE (n = 8) or a high-straw lower-energy diet (CON; n = 8) from -21 d to calving. Out of 313 biochemical compounds identified, statistical analysis ($p \leq 0.10$) of metabolomics data for K vs. CON, OVE vs. CON, and K vs. OVE revealed 34, 33 and 25 affected compounds, respectively. The top-five affected and up-regulated biochemical compounds in K vs. CON were taurocholate, adenine, hypotaurine, gamma-glutamylcysteine, and taurochenodeoxycholate. In OVE vs. CON cysteine, methylphosphate, cysteinylglycine, and taurocholate were up-regulated and gamma-glutamylthreonine was down-regulated. In K vs. OVE the top-five affected compounds were all down-regulated: xylitol, 1-palmitoylglycerophosphoglycerol, leucylaspartate, sphinganine, and glycylvaline. Bioinformatics analysis revealed that primary bile acid production through cysteine and taurine precursors, and oxidative stress-like activities were affected in both K and OVE vs.

CON groups. In contrast, in K vs. OVE ketone body production was up-regulated and cell signaling pathways were inhibited. Bioinformatics analysis of 2,908 differentially expressed genes (DEG, $p \leq 0.05$) using the Dynamic Impact Approach (DIA) revealed that the top-five impacted pathways in K vs. OVE were 'hedgehog signaling', 'glycosphingolipid biosynthesis - globo series', 'renin-angiotensin system', and 'other glycan degradation' all of which were inhibited. The 'circadian rhythm' pathway was among the most-induced pathways. Furthermore, there was marked inhibition in K vs. OVE of pathways associated with cellular growth, communication, signal transduction, fatty acid biosynthesis, and immune related responses. These results suggest that prepartal diet alters the hepatic metabolome and transcriptome. Liver from cows developing ketosis postpartum appears to exhibit unique alterations in the transcriptome and metabolome.

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Introduction

Ketosis is a metabolic disease often seen in high producing dairy cows during early lactation (Baird, 1982). It arises more frequently in cows that are fed high-energy diet during the entire dry period or during the close-up dry period (i.e. last 21 d prior to parturition). It is characterized by partial anorexia, depression and body fat mobilization (Morrow et al., 1979). Nutritional management during this period may affect susceptibility of cows to several metabolic disorders and infectious diseases (Forslund et al., 2010). Dairy cows are highly susceptible to developing metabolic disorders such as ketosis (Loor et al., 2007) and become more susceptible to infectious diseases such as mastitis and metritis (Forslund et al., 2010). Ketosis can affect the productivity and cost of dairy forms in term of decreased milk production and animals curing (Berge and Vertenten, 2014). It is associated with negative energy balance (NEB), hepatic lipid accumulation, and increased blood ketones while blood glucose concentration is decreased (Drackley, 1999). The prepartal plane of nutrition is highly correlated with body fat mobilization around parturition (Khan et al., 2015). High-energy protein diet without or with low protein supplementation may cause fatty liver or other metabolic disorders as compared to high energy high protein or low energy low protein dietary planes of nutrition (Bell et al., 2000). The improper plane of nutrition can lead to animal stress and provoked immune response during early phases of lactation (Ingvartsen, 2006, Shahzad et al., 2014).

Metabolic adaptations during early lactation lead to fatty acid mobilization because of NEB. These fatty acids in the form of non-esterified fatty acids (NEFA) either enter into the liver tissue or directed towards the mammary glands. The higher concentrations of NEFA in the liver give rise to higher synthesis of acetyl-CoA through beta-oxidation or triglycerides (TG) synthesis

through esterification reactions. Due to the decreased amount of available oxaloacetate resulting from lower glucose concentration, the acetyl CoA cannot enter into the TCA cycle and results into marked elevation of ketone bodies such as β-hydroxybutyrate (BHBA), acetyl acetate, and acetone (Grummer and Carroll, 1991, Li et al., 2014a). On the other hand, when the concentration of TG exceeds its extra hepatic export through very low-density lipoprotein (VLDL), lipid infiltration occurs in the liver leading to fatty liver disease (Loor et al., 2006). At this point the ability of liver to oxidize NEFA and export them as TG is reduced due to the severe NEB (Morrow, 1976). During this process, ketone bodies are produced as a result of partial oxidation of NEFA. There are three main types of ketone bodies such as BHBA, acetone, and acetoacetate (Grummer and Carroll, 1991, Li et al., 2014a). Among these, BHBA plays a key role in the development of ketosis, whereas acetoacetate is broken down into acetone, which is a volatile compound (Herdt, 2000). The ketone bodies start accumulating in the blood when their concentration exceeds their utilization (Adewuyi et al., 2005). However, the presence of ketone bodies is normally expected in fresh dairy cows because of NEFA mobilization postpartum where a portion of NEFA is converted into ketone bodies (Duffield, 2000). The excessive amount of ketone bodies production at this stage leads to the metabolic disorder commonly known as ketosis.

Ketosis is also associated with fatty liver or lipidosis. The condition, when the hepatic uptake of NEFA exceeds over oxidation and secretion, is called lipidosis (Loor et al., 2007). It might also be caused by reduced synthesis of apo-lipoproteins during severe inflammation at the time of calving (Bertoni et al., 2004). When the amount of hepatic lipid accumulation is increased, dairy cows become more susceptible to other pathologies and at the same time when the treatment is functional, the recovery period is prolonged (Herdt, 1988).

Transcriptomics and metabolomics have been widely used in dairy research to explore the molecular mechanisms and biological behaviors using computational methods (Loor et al., 2007, Loor et al., 2013, Zhang et al., 2013). Transcriptomic techniques help to unravel differentially expressed genes (DEG), genetic variants, point mutations and noncoding RNAs from a list of whole genome array by means of unbiased statistical applications. On the other hand, metabolomics techniques help to understand the role of different biomarkers involved in particular etiology from a list of identified metabolites (Horgan and Kenny, 2011, Zhao et al., 2014). The methodologies involved in metabolomics research include nuclear magnetic resonance (NMR), and mass spectrometry coupled with liquid chromatography (LC-MS) or gas chromatography (GC-MS) (Li et al., 2014b). These techniques have been employed at different scales in dairy research (Coffey, 2007, Wang et al., 2012, Ferreira et al., 2013), however, the integration techniques have not been reported yet. We have taken this strategy into consideration and used them together to explore the transcriptional and biochemical level alterations to unravel the etiology of ketosis development.

In the current study, we have explored the metabolic pathways involved in ketosis development by means of transcriptomics and metabolomics. We have used the dynamic impact approach (DIA) (Bionaz et al., 2012) and Ingenuity pathway analysis tools (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity) in this regard. The objective of the study was to use both metabolites and DEG in hepatic tissue extracted prepartum at -10 d and retrospectively explore the mechanisms associated with ketosis development postpartum in cows fed moderate energy diet during the close-up dry period. The present work provides necessary information that may help to provide ketosis diagnosis prepartum and improve the health and milk production during early lactation.

Materials and Methods

Experimental design and dietary treatments

The procedure for this protocol (#09214) was approved by The Institutional Animal Care and Use Committee (IACUC) of the University of Illinois (Urbana). The experiment was conducted as a randomized complete block design as explained elsewhere (Osorio et al., 2013). All cows received the same far-off diet (1.24 Mcal/kg of DM; 14.3% CP) from −50 to −22 d before expected calving, a close-up (moderate energy) diet (1.54 Mcal/kg of DM; 15.0% CP) from −21 d to calving, and fresh cow lactation diet from calving (1.75 Mcal/kg of DM; 17.5% CP) through 30 days in milk (DIM). Supplements of methionine were top-dressed from −21 to 30 DIM. The other factors such as animal husbandry, sampling of ingredients, total mixed ration (TMR), body weight (BW), body condition score (BCS), milk weights, sampling for milk composition, and housing of cows pre- and postpartum have been reported elsewhere (Osorio et al., 2013) and are not included in this this study. For the current study, we have selected a subset of 24 cows which were evaluated at +7 d postpartum such as healthy cows fed moderate energy diet (OVE, n=6), OVE cows with developed ketosis (K, n=6), OVE plus supplemented with either Smartamine M (SM, n=6), or MetaSmart (MS, n=6). For this chapter, we have focused on K and OVE groups. The experimental design of the study is shown in the Figure 2.1.

Liver biopsies and RNA extraction

Liver tissue samples were collected via puncture biopsy (Dann et al., 2006) from cows under local anesthesia at approximately 0730 hour once prepartum on d −10 (±3 d), and then

postpartum on +7 and +21 d. The samples were stored in liquid nitrogen immediately and then at -80°C until RNA extraction. We used liver samples from –10 d prepartum for the ketosis etiology. Total RNA was extracted from the samples using established protocol in our laboratory. Briefly, liver tissue sample was weighed (~55 mg on average) and straightway put inside a 2 ml centrifuge tube (Corning Inc. ®, Cat. No. 430052, Corning, NY, USA) with 1 ml of Qiazol reagent to proceed with RNA extraction. This extraction procedure also utilizes chloroform (Ambion® Cat. No. 9720, Austin, TX, USA), which removes DNA. Any residual genomic DNA was removed from RNA with DNase enzyme using miRNeasy Mini Kit columns (Qiagen, Hilden, Germany). The RNA concentration was measured using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The purity of RNA (A260/A280) for all samples was above 2.0. The quality of RNA was evaluated using the Agilent Bioanalyzer system (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). The average RNA integrity number (RIN) for all samples that were used for the subsequent analysis were around 6.9.

Blood profiling

A large metabolic blood profiling including 33 biomarkers was performed in plasma collected at -12, -3 and +3 d relative to parturition. The raw results were recorded and were further used for statistical analysis. For ketosis evaluation, ketosticks were used in the urine to measure BHBA concentrations. The ketotic cows were identified based on the presence of large amounts of blood NEFA and ketone bodies in the urine at a certain time point. The results are included here in the study for further discussion along with transcriptomics and metabolomics datasets.

Metabolomics

Metabolomic analysis was performed by Metabolon Company (Metabolon Inc. NC). The liver tissue samples were prepared according to the sample preparation guidelines. Briefly, the 500 milligram of the liver tissue per sample was weighed, packed in dry ice, and then shipped to the company. The analysis was performed using mass spectrometry coupled with gas chromatography (GC-MS) and liquid chromatography (LC-MS). Total 313 biochemical compounds (metabolites) were identified by the assay.

Transcriptomics

For transcriptomics analysis, we used ~44 K bovine (v2) gene expression Agilent microarray platform. The microarrays experiment was performed according to our laboratory's established protocol and the instructions provided by Agilent technologies. Four groups of cows were used, which are OVE, K, SM and MS. The complete microarray hybridization design is given in the Figure 2.2. The detailed description of the microarrays experiment is provided elsewhere (Shahzad et al., 2015). Briefly, the RNA with 200 nanograms (ng) per sample was used for cDNA synthesis. The cDNA was reverse transcribed to cRNA and was further used for cy3 or cy5 fluorescent dye according to the manufacturer's instructions. Purification of the labeled cRNA product was performed with RNeasy mini spin columns (Qiagen, cat# 74104), and it was subsequently eluted in 30 µL of DNase-RNase-free water. The eluted cRNA concentration was measured using NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA) to confirm the manufacturer's recommended criteria for yield and specific activity of at least 0.825 µg and ≥ 6 respectively. The labelled cRNA was fragmented using 10X blocking Agent and 25X

fragmentation buffer and then the reaction was stopped using 2X GEx hybridization buffer. The samples were loaded onto the Agilent bovine microarray's slides and were hybridized in a rotating hybridization oven at 65°C for 17 hours. After that, slides were washed and scanned using a GenePix 4000B scanner (Axon Instruments Inc., Sunnyvale, CA) and GenePix Pro v.6.1 software. The scanned images were then edited using the bovine gal file. Resulting spots with substandard features were flagged as bad and were excluded from the subsequent analysis. The results were saved in GPR files and were further processed using Perl scripts before statistical analysis.

Statistical analysis

For metabolic profiling parameters, a normal distribution was assessed using the procedure UNIVARIATE of SAS (SAS Institute Inc., Cary, NC). A MIXED model procedure with a spatial power as a covariance structure was used. The model included time, status, and time x status as fixed effects, with cows as random variable. Means between treatments and time point were separated using the PDIFF. Data were deemed to be significant if overall time x status interaction was $p \leq 0.05$. Single point comparisons were determined to be significantly different if $p \leq 0.05$.

For metabolomics analysis, total 313 biochemical compounds were used for statistical analysis. The data was normalized in terms of raw area counts. Each biochemical compound from the raw value was rescaled to set the median value equal to 1. The missing values were imputed with the minimum value. Following the log transformation and imputation of missing values, if any, with the minimum observed value for each compound, we used a mixed procedure

of SAS to identify the biochemical compounds that differed significantly between the experimental groups. Significantly affected biochemical compounds with $p \leq 0.10$ were selected for further analysis.

For microarray's statistical analysis, data from 12 arrays (24 samples) were used. The oligo IDs with bad flags (-100) were removed before normalization. The data was log transformed and then corrected across dye and array effects using loess normalization and array centering method. After normalization, a mixed procedure of SAS was used. The statistical model included dietary treatments as a fixed effect. The raw *p*-values were adjusted for the number of genes tested using Benjamini and Hochberg's false discovery rate (FDR; Benjamini and Hochberg, 1995) to account for multiple comparisons. However, we could not find enough differentially expressed genes (DEG) with corrected *p*-values. So, we used a $p \leq 0.05$ and fold change (FC) $\geq |1.5|$ criteria for evaluation purpose. For the current chapter's discussion, we used K vs. OVE comparison for both metabolome and transcriptome.

Pathways analysis

For metabolomics analysis, the biochemical compounds were annotated with their corresponding sub-pathways. The results were further furnished with FC- and *p*-values resulting from SAS analysis. For functional analysis of the microarray data, a dynamic impact approach (DIA) was used to unravel the Kyoto Encyclopedia of Genes and Genome (KEGG) pathways. A list of DEG along with their Oligo IDs, Entrez gene IDs, *p*-values and FC was used as an input for DIA. We obtained a total of 3,065 DEG (2,091 up and 974 down regulated) with a $p \leq 0.05$ and FC $\geq |1.5|$ for K vs. OVE comparison. A minimum of 30% annotated genes on the

microarray versus the whole genome were selected for the analysis consideration as explained elsewhere (Bionaz et al., 2012). The DIA was run on the selected DEG to obtain the impact and flux values of the KEGG categories, sub-categories and their respective pathways. The impact values reflect the overall perturbation and the flux values reflect the overall direction of a pathway. This strategy allows evaluation of transcriptome profiles in a more holistic fashion across different pairwise comparisons.

Network analysis and data integration

The network analyses for both metabolomics and transcriptomics were performed using IPA software. The Metabolon's biochemical compounds were annotated with PubChem identifiers (https://pubchem.ncbi.nlm.nih.gov/). For metabolomics network constructions, a list of significantly (13, $p \leq 0.10$) affected biochemical compounds was used along with their FC values. For transcriptomic network reconstructions, a list of DEG with a $p \leq 0.05$ and FC $\geq |1.5|$ was uploaded to run the core analysis. From the analysis results, we selected upstream transcription regulators and their downstream target genes for network reconstructions. The data integration was performed with transcription regulators and biochemical compounds.

Results and Discussion

Relatively fewer studies have been conducted underlying the mechanism of ketosis development in dairy cows during transition period by using omics techniques. One of the studies dealing with ketosis development during transition period was conducted by our group

using microarrays dataset to identify the molecular events of ketosis progression in the liver of dairy cows (Loor et al., 2007). During the study, it was identified that the genes involved in oxidative phosphorylation, protein ubiquitination, ubiquinone biosynthesis, cholesterol metabolism, growth hormone signaling and proton transport were down regulated. In contrast, the genes associated with cytokines signaling, fatty acid uptake/transport and fatty acid oxidation were up regulated. Furthermore, this study explains the relationship of metabolic pathways and gene networks under NEB, and unravels the mechanisms of lower protein synthesis and altered immune response as a result of low and high energy feed intakes. The study highlights the mechanism of ketosis development in terms of two types of dietary feeds with different energy sources such as ~80% of energy requirements (restricted energy) and ~140% of energy requirements (ad libitum). There are other studies highlighting the mechanisms of ketosis development in the liver of transition cows, suggesting the significance of nutritional management to prevent ketogenesis. One of important technique is to supplement the diet with limiting amino acids to cope with lower protein synthesis (McCarthy et al., 1968, Waterman and Schultz, 1972, Osorio et al., 2013). It is considered that decreased concentration of plasma amino acids and lower protein synthesis is also one of the main cause behind the ketogenesis. The accelerated demand of limiting amino acids for gluconeogenesis, milk production and energy synthesis is also met through muscle protein degradation (Bell et al., 2000, Kuhla et al., 2016).

Mass spectrometry has been used in several studies to explore the process of ketosis development in transition dairy cows (Zhang et al., 2013, Li et al., 2014a). In one of the study, Zhang et al., (2013) reported a set of novel biomarkers from clinical, subclinical and control groups of cows using plasma metabolomic profiling by GC-MS technique. These biomarkers were from carbohydrates, fatty acids, amino acids, sitosterol, vitamin E isomers, and many

others. The study reveals that these metabolites respond differentially under different forms of ketotic stages. By identifying the metabolic pattern of each stage, it will help to identify and prevent the progression of ketosis development. On the other hand, Li et al., (2014a) reported 13 potential plasma biomarkers by LC-MS technique to identify the progression of ketosis development in dairy cows. In the study, two groups of cows, clinical ketosis and control were chosen based on the plasma glucose and BHBA concentrations. The identified biomarkers were related to Fat metabolism: glycocholic acid, tetradecanoic acid, and palmitoleic acid; Amino acid metabolism: arginine, valine, glycine, lysine, and leucine/isoleucine; and Other metabolisms: nicotine, tryptophan, aminobutyric acid, creatinine, and undecanoic acid. Among these, clinical group showed a significant uprise response towards fat related metabolites such as glycocholic, tetradecanoic acid and palmitoleic acid, and amino acids related metabolites such as valine, and glycine. However, the other metabolites showed a declining response. These results indicated an increased response of fatty acids and amino acids metabolism to promote the gluconeogenesis due to the decreased level of blood glucose. An increased level of bile production was also observed in clinical group of cows.

In our study, we have combined transcriptomic analysis with GC-MS/LC-MS to reveal the changes occurring at genes, metabolic, and pathways level. This integration will help to diagnose the progression of ketosis development before parturition. It will also help ketosis prevention during early stages of lactation in dairy cows. The results from our experiments are discussed in the following sections.

Overall experimental design

The overall experimental design for the groups OVE and K is shown in the Figures 2.1. The figure shows the plane of nutrition from far-off (-50 d) to early lactation (+7 d) along with tissue extraction (-10 d) and calving time points (0 d). However, the Figure 2.2 shows the overall microarrays design with the two (OVE and K) highlighted conditions. The two colored hybridization plan is shown with two different colors red and green.

Expression patterns of transcriptome, metabolome and blood biomarkers

We observed a large number of DEG (3,065) with FC $\geq |1.5|$ and p-value ≤ 0.05 cut off criteria. Among these 2,091 DEG were up regulated and 974 DEG were down regulated. By reducing the $p \leq 0.01$ and doubling the FC $\geq |3|$ criteria, we obtained a total of 121 DEG, of which 22 were up regulated (Table 2.1) and 99 were down regulated (Table 2.2). It was found that the up regulated DEG were mostly linked with innate immune responses, DNA replication, and protein synthesis, whereas the down regulated DEG were linked with the cell signaling, and cell cycle. Overall, a mixed response of both up and down regulated DEG was observed within the metabolism and immune system. For metabolomics results, we used a $p \leq 0.10$ to enlist the significantly affected biochemical compounds along with their sub-pathways as shown in the Table 2.3. The selection criteria resulted into a total of 15 biochemical compounds, of which three were up regulated and the remaining were down regulated. The table shows the FC values to represent the overall trend of the pathways for each of the biochemical compound.

The results from 33 blood biomarkers are shown in the Table 2.4. From these results, we did not find enough significant differences ($p \leq 0.05$) between the two states such as K and OVE

before parturition. However, there were significant differences within the time factor. Noticeably, the NEFA and BHBA values were different at all the three time points. We also did not find enough significant results between status and time point interactions except BHBA and creatinine. The results indicate that both BHBA and creatinine concentration has significant differences at 3 d in K vs. OVE group. Both biomarkers were found increased in the ketotic group. Creatinine has been considered as one of the biomarker along with urea to determine the kidney functions in the ketotic cows (Tyler et al., 1994).

Summary of the KEGG pathways

For the KEGG summary results, we focused on five main categories and their subcategories as shown in the Figure 2.3. The first two categories such as 'Metabolism' and 'Genetic Information Processing' were overall induced, while the remaining three categories such as 'Environmental Information Processing', 'Cellular Processes' and 'Organismal systems' were moderately inhibited. The KEGG pathways linked with these categories and subcategories were further classified into two main groups such as 'metabolic' (Figure 2.4) and non-metabolic (Figure 2.5) pathways.

Networks of transcription regulators and biochemical compounds

The network analysis for both DEG and biochemical compounds was conducted using IPA software. For DEG, we used upstream analysis results to identify the transcription regulators. The transcription regulators are involved in regulation of several other genes by modulating the interaction with nucleosomes, transcription factors and histones. The analysis

identified 8 up regulated (Figure 2.6: A-D) and 7 down regulated (Figure 2.7: A-B) transcription regulators. The up regulated transcription regulators include *HMGB1* (high mobility group box 1), *HOXA13* (homeobox protein Hox-A13), *SREBF2* (sterol regulatory element binding transcription factor 2), *MECOM* (MDS1 and EVI1 complex locus protein EVI1), *NKX2-1* (NK2 homeobox 1), *SATB1* (special AT-rich sequence binding protein 1), *HIF1A* (hypoxia-inducible factor 1-alpha), and *NFE2L2* (nuclear factor erythroid 2-like 2). Among these, HMGB1, STAB1 and NEF2L2 are involved in immune response, injury and inflammation. The HOXA13, and MECOM are involved in gene regulation, cell development, differentiation and proliferation. The HIF-1 plays an important role in cellular response to systemic oxygen levels, glucose and iron metabolism, SREBPF2 controls cholesterol homeostasis by regulating transcription of sterol-regulated genes and NKX2-1 also known as 'thyroid specific enhancer binding protein' is involved in regulation of genes that are involved in thyroid, lung, and diencephalon. The down regulated transcription regulators include *CSHL1* (chorionic somatomammotropin hormone-like 1), *NCOA2* (nuclear receptor coactivator 2), *HDAC5* (histone deacetylase 5), *GLI3* (Zinc finger protein GLI3), *SKIL* (SKI-Like proto-oncogene), *SPIB* (Spi-B transcription factor) and *SNAI1* (Snail family zinc finger 1). These are involved in growth control (CSHL1), transcriptional activity and cell signaling (GLI3, SNAI1), transcriptional regulation, cell cycle progression, growth and differentiation (HDAC5, SKIL, SPIB) and nuclear hormone receptors including steroid, thyroid, retinoid, and vitamin D receptors (NCOA2). The function of these genes were retrieved from the NCBI gene database.

The networks of biochemical compounds are shown in the Figure 2.8. From the 15 biochemical compounds we have shown here the networks of two compounds, glucose-6-phosphate and glycochenodeoxycholate under the carbohydrate metabolism (Figure 2.8A). The

same compounds along with D-erythro-C16-ceramide are shown under the cellular functions (Figure 2.8B). For data integration, we have combined the results from transcriptomics and metabolomics to find out the interconnecting links between the two datasets. The Figure 2.9 shows the data integration using transcription regulators and biochemical compounds. In the figure, the transcription regulators are linked with the other potential downstream target genes and biochemical compounds by direct and indirect interactions. The results from both approaches along with blood profiling are discussed along with their KEGG pathways in the following sections.

Metabolic pathways

The results from our top 20 most impacted metabolic pathways (Figure 2.4) including some of those from the remaining pathways (not shown) are discussed in the following subsections.

Carbohydrate Metabolism. The metabolism of 'Glycolysis / Gluconeogenesis', 'Pentose phosphate pathway', and 'Inositol phosphate metabolism' were inhibited indicating a reduced synthesis and utilization of glucose (Figure 2.4). Similarly, the results from metabolomics study, such as 'xylitol', 'ribulose' and 'glucose-6-phosphate (G6P)' as shown in the Table 2.3 also support the inhibition of these pathways. Among these biochemical compounds, xylitol acts as a precursor for xylulose 5-phosphate, and hence serves as an intermediate in the pentose phosphate pathways (Dupriez and Rousseau, 1997). Interestingly, xylitol can reduce ketone production in dairy cattle potentially though its ability to stimulate energy production via glucose-dependent pathway and its stimulation of insulin release which both act to suppress ketogenesis in the liver

(Sakai et al., 1996, Toyoda et al., 2008). Xylitol enters into the gluconeogenesis system via pentose phosphate pathway to synthesize glucose and as a result it stimulate insulin secretion to provide antiketotic effects (Mäkinen, 2000). The inhibition of xylitol and related pathways may indicate a gradual increase in ketogenesis as indicated by other studies (Zhang et al., 2013, Sun et al., 2014). The other carbohydrate metabolic pathways such as 'Glyoxylate and dicarboxylate metabolism', 'Propanoate metabolism', 'Pyruvate metabolism', 'TCA cycle', and 'Butanoate metabolism' (not shown here) were overall induced prepartum in the ketotic group. These results may indicate a shift of energy source from carbohydrate towards fatty acids and amino acids in the ketotic group as compared to the healthy group.

Lipid Metabolism. Among the lipid metabolic pathways, 'Fatty acid biosynthesis', 'Biosynthesis of unsaturated fatty acids', and 'Fatty acid metabolism' were inhibited. The inhibition of these pathways suggest fatty acid synthesis feedback mechanism in the ketotic group. Surprisingly, the expression of SREBF2 gene transcription regulator was found to be upregulated (Figure 2.6A), which has a major role in regulation of sterol biosynthesis (Piantoni et al., 2010). This gene was earlier found to be down regulated in the ketotic group of cows postpartum (Loor et al., 2007). As a response 'Steroid biosynthesis', 'Steroid hormone biosynthesis' and 'Sphingolipid metabolism' were induced in K vs. OVE group. The induction of 'Synthesis and degradation of ketone bodies' pathway indicates the ketone bodies production in the liver even before parturition due to high energy diet as shown in the earlier study (Wu et al., 2014). The induction of 'Primary bile acid synthesis' was also supported by metabolomics results partially in the form of glycochenodeoxycholate as shown in the Figure 2.8A. The bilirubin was also significantly altered at different time points as shown in the Table 2.4. The elevated level of bilirubin has been reported in the ketotic cows (Steen et al., 1997), which, if not cleared properly, may result in

poor performance in productivity and pronounced immune response (Bertoni et al., 2008). Liver is the main site of bile acid production, which in turn helps to reduce the excessive cholesterol level (Hofmann, 1999). The concentration of bile acid in liver and peripheral blood can be an influenced by many factors such as production activity, transport across membranes, secretion, secondary bile acid production by luminal bacteria, enterohepatic recirculation, liver glycine- or taurine-conjugation, and fecal elimination. It was found that CYP7B1 gene was among the up regulated genes that might be involved in the conversion of cholesterol to bile acid (Chiang, 1998). This gene is under the influence of SREBF2 transcription regulator (Figure 2.6A).

Amino Acid Metabolism. We found amino acid related several metabolic pathways that were induced in the ketotic group. In addition to the pathways that are listed in the Figure 2.4, Other induced pathways include 'Cysteine and methionine metabolism', 'Arginine and proline metabolism', 'Lysine degradation', 'Tryptophan metabolism', and 'Alanine, aspartate and glutamate metabolism' (results not shown). The higher rate of metabolism in these pathways suggest that K vs OVE group has more consumption and utilization of amino acids during transition period. For example, increased metabolic rate of cysteine and methionine indicate a shift of balancing the methionine supply as a limiting amino acid. Methionine plays an important role in fatty acid catabolic reactions to avoid its accumulation in the liver tissue and lowering the cholesterol level in the body (Sun et al., 2016). These results were also supported by metabolomics profiling such as 'butyrylglycine' and '1-methylimidazoleacetate' (Table 2.3). The down regulation of dipeptides such as 'glycylvaline', 'leucylaspartate', 'tyrosylglycine' and 'glycylisoleucine' may indicate an increased degradation of amino acids in a 'Valine, leucine and isoleucine degradation' pathway. These results were in accordance with the metabolic profiling

of plasma in dairy cows where an induction of 'Valine, leucine and isoleucine degradation' was observed (Zhang et al., 2013).

Glycan Biosynthesis and Metabolism. Glycans are carbohydrate molecules that are either linked with lipids to form glycolipids or proteins to form glycoproteins. These are mainly involved in cellular signaling pathways (Shahzad et al., 2014). In the ketotic group, we found that 'Other glycan degradation', 'Glycosylphosphatidylinositol(GPI)-anchor biosynthesis', 'Glycosaminoglycan degradation', and 'O-Glycan biosynthesis' were inhibited, whereas the 'Glycosphingolipid biosynthesis - ganglio series' and 'N-Glycan biosynthesis' were most importantly induced. The intricate roles of the inhibited pathways are associated with cellular signaling and communication, and extracellular matrix (ECM) interactions along with growth factors (Ernst et al., 1995, Wopereis et al., 2006). However, the induced pathways are involved in handling of misfolded proteins due to stress response in the endoplasmic reticulum (ER) and cellular growth (Ruddock and Molinari, 2006, Lingwood, 2011).

Metabolism of Cofactors and Vitamins. The cofactors and vitamins play an important role in different metabolic functions. Except the two pathways 'Riboflavin metabolism', and 'One carbon pool by folate', (Figure 2.4) all of the other cofactors and vitamins pathways were induced in K vs. OVE. Riboflavin is part of Flavin mononucleotide (FMN) and Flavin adenine dinucleotide (FAD) cofactors and plays a critical role in fatty acid metabolism, citrate cycle and electron transport chain (Powers, 2003). One carbon metabolic pathway is centered around folate, which is required for different functions such as *de novo* synthesis of purines and thymidylate and remethylation of homocysteine to methionine (Stover, 2009). The deficiency of these vitamins may lead to reduced or complete shutdown of energy related pathways in the liver and ultimately leading to metabolic disorders such as ketosis.

Non-metabolic pathways

We have categorized non-metabolic pathways as shown in Figure 2.5 separately from the metabolic pathways to uncover the etiology of ketosis development under this category. This will help us to enhance our understanding of the metabolic disorder beyond the well-established role of metabolic pathways.

Protein Synthesis. We discovered 'Ribosome' among the top 20 most impacted KEGG pathways (Figure 2.5). 'Ribosome' along with other pathways such as 'Ribosome biogenesis in eukaryotes', 'RNA transport' and 'mRNA surveillance pathway' is involved in protein synthesis and turn over reactions. Similarly, several pathways related to folding, sorting and degradation were induced. These include 'Protein processing in endoplasmic reticulum', 'RNA degradation', 'Protein export', 'Ubiquitin mediated proteolysis', and 'Proteasome'. The overall greater protein turnover may be involved in acute phase response protein such as haptoglobin and serum amyloid A (SAA) (Osorio et al., 2014). These proteins are characterized by increased inflammatory responses. It has also been shown that genes involved in endoplasmic reticulum (ER) stress induced unfolded protein response are expressed in the liver of cows during transition period (Gessner et al., 2014, Winkler et al., 2015). The expression of these protein responses in prepartum cows may reflect an onset of inflammatory responses before calving. These conditions further give rise to metabolic disorders during early lactation.

DNA Replication. The pathways related to DNA replication such as 'Nucleotide excision repair', 'Base excision repair' and 'DNA replication' were induced (Figure 2.5). The induction of these pathways may suggest liver regeneration and cell proliferation. Similar mechanisms of cellular proliferation were indicated by other studies (Loor et al., 2007, McCarthy et al., 2010). As an example, we found MECOM (MDS1 and EVI1 complex locus, Figure 2.6A) transcription

regulator in our network. The upregulation of MECOM is involved in regulation of cellular proliferation (SKIL) (Kim et al., 2016), metabolism and bile acid synthesis related genes (ACACA, ACAA2) (Nakamura et al., 2014).

***Cellular Functions*.** We found several genes, biochemical compounds and pathways involved in different cellular functions. Most of the signaling pathways such as 'mTOR signaling pathway', 'ErbB signaling pathway', 'VEGF signaling pathway', 'Phosphatidylinositol signaling system', 'MAPK signaling pathway', 'Calcium signaling pathway', 'Hedgehog signaling pathway', 'Notch signaling pathway', 'Wnt signaling pathway', and 'Jak-STAT signaling pathway' were inhibited in K vs. OVE group except 'TGF-beta signaling pathway' which was found as an induced pathway. The inhibition of these pathways may indicate a poor response of cell to cell communication and regulation mechanisms among the hepatic cells. These signaling pathways were more likely involved in regulating cell metabolism (mTOR, Phosphatidylinositol, Calcium, Notch) (Kuhla et al., 2009, Laplante and Sabatini, 2009, Arai et al., 2010), growth, proliferation and survival (mTOR, ErbB, MAP Kinase, Hedgehog, Wnt) (Varjosalo and Taipale, 2008, Hynes and MacDonald, 2009), differentiation, apoptosis, and cell motility (ErbB, MAP Kinase, Phosphatidylinositol, Notch, Wnt) (MacDonald et al., 2009, Guruharsha et al., 2012) , vascular development (VEGF) (Zhang et al., 2014), and immune response (Jak-STAT, MAP Kinase) (Shuai and Liu, 2003, Arthur and Ley, 2013). These pathways are in accordance with network analysis of transcription regulators. TGF-beta signaling along with its downstream genes such as SMAD1, TGIF1, and BAMBI was an indication of cell proliferation and apoptosis regulation (Heldin et al., 1997) in the hepatic cells of cows developing ketosis postpartum.

The other signaling pathways such as 'GnRH signaling pathway', and 'Insulin signaling pathway' from endocrine system were also found to be inhibited indicating mechanisms of

reduced reproductive functionality (Aguilar-Rojas and Huerta-Reyes, 2009) and insulin resistance (Pires and Grummer, 2008) respectively. The Figure 2.8B highlights the role of three important biochemical compounds such as glycochenodeoxycholate, D-erythro-C16 ceramide and glucose-6-phosphate that are involved in cellular functions and their maintenance. The induction of glycochenodeoxycholate shows depolarization of mitochondria leading to hepatocyte apoptosis (Lemasters et al., 1998, Higuchi et al., 2001). This process indicates lower rate of energy synthesis that leads to mitochondrial apoptosis, liver dysfunction and NEB. The downregulation of D-erythro-C16 ceramide indicates reduced functioning of adherens junctions and tight junctions. These junctions play an important role in cell to cell adhesions in epithelial cells (Yap et al., 1997). Ceramides are important cell signaling lipid molecules that serve in different cellular processes such as apoptosis, cell signaling, growth, differentiation and proliferation (Wang et al., 2009, Kjellberg et al., 2015). These results indicate a shutdown of several signaling molecules in the liver of ketotic cows.

Immune System. Understanding the immune system is essential to uncover the metabolic changes occurring inside the liver of dairy cows. The liver plays an essential role during stressful phases of the transition period, where this organ undergoes through metabolic, inflammatory, and immune responses. One of the study published by our group shows several biomarkers including those involved in inflammation such as albumin, ceruloplasmin, SAA, haptoglobin and interleukin 6 (IL-6) during transition period (Osorio et al., 2014). The study compares the OVE group with two other groups supplemented with methionine (Smartamine M and MetaSmart). The OVE group of cows showed a greater response of positive acute phase proteins such as haptoglobin and SAA while lower response of negative acute phase protein such as albumin. These acute phase proteins are mainly synthesized in the liver and provide a measure of

inflammation around calving (Jain et al., 2011). In our results, Table 2.4 shows no significant responses for haptoglobin and albumin ($p > 0.05$). However, the time effect shows a significant response for these biomarkers ($p < 0.05$). From our transcriptome analysis, 'Antigen processing and presentation' and 'Complement and coagulation cascades' (results not shown) were the most induced pathways within immune response in K vs. OVE group. Among the up regulated transcription regulators, we found HIF1A, HMGB1 and NFE2L2 (Figure 2.6: A, C-D) that were linked with several downstream immune related genes. HIF1A (Hypoxia Inducible Factor 1 Alpha) is mainly involved in regulation of cell metabolism, stress and innate immune response (Nizet and Johnson, 2009). The protein expression of HMGB1 (High Mobility Group Box 1) can act as a cytokine to respond against cellular injury, infection and inflammation reactions (Lotze and Tracey, 2005). The third gene NFE2L2 (Nuclear Factor, Erythroid 2 Like 2) is an important regulator of inflammatory responses (Figarska et al., 2014). The blood profiling data (Table 2.4) along with transcription regulators suggest an onset of inflammatory response well ahead of calving. The inflammatory response around this time may also be under the cellular injuries leading to the compromised liver functions, NEB and increased ketone bodies production during early days of lactation. The compromised immune response might be an indication of direct effect of ketone bodies on the immune system (Gregory et al., 1993).

Integration of metabolome and transcriptome profiling

The methodologies involved in metabolomics research include nuclear magnetic resonance (NMR), and mass spectrometry coupled with either liquid chromatography (LC-MS) or gas chromatography (GC-MS) (Li et al., 2014b). These techniques have been employed at different levels in dairy research (Humer et al., 2016, Dervishi et al., 2017), however, the

integration methods have been applied at a very limited scale (Ferreira et al., 2013). For example, a network reconstruction in mammary gland tissue (Wang et al., 2012) is one of the example where metabolic data from publically available databases (NCBI, KEGG and Uniprot) was used for integration purpose. However, the integration of transcriptomics and metabolomics datasets in dairy research has not been reported yet. The integration of data with the help of suitable bioinformatics tools may enhance our understanding at cellular and molecular levels that render cows more susceptible to ketosis development. The figure 2.9 shows an example of data integration using transcription regulators and biochemical compounds. In the figure transcription regulators are linked with the other potential downstream genes by direct and/or indirect means. From the network it can be inferred that the transcription regulators and biochemical compounds are linked with downstream genes and metabolites that are involved in cell growth, proliferation, apoptosis, immune response, insulin signaling and metabolism.

Conclusion

Predisposition to early-postpartal ketosis in cows overfed moderate energy prepartum is associated with alterations in transcriptome- and metabolome-wide metabolic and non-metabolic pathways. Ketosis is a critical metabolic disorder that arises during early days of lactation due to improper nutritional management. By taking necessary precautions during the close up dry period, the rate of this metabolic disorder can be abridged in high producing dairy cows. Our results of transcriptomics, metabolomics, and blood profiling highlight several causative agents at transcriptome, metabolome and blood profiling levels that can potentially be involved in ketosis development postpartum. We have highlighted several metabolic and non-metabolic

pathways that were affected in ketotic group of cows as compared with healthy group of cows. Transcriptome analysis revealed many transcription regulators and their role in regulation of several pathways. On the other hand, metabolomics analysis also provided the information of significantly affected biomarkers that might predict the metabolic level disorders leading to the etiology of ketosis development. Integration analysis of transcription regulators and biochemical compounds summarizes the phenomena of ketosis development along with potential pathways level variations. From this study, we have uncovered the proof of established knowledge along with novel observations. These observations along with novel biomarkers might help to predict ketogenesis during close up dry period, which might help us to diagnose and overcome the metabolic disorders well ahead of time.

Figures and Tables

Table 2.1: A list of differentially expressed gene with $p \leq 0.01$ and fold change (FC) ≥ 3. The table shows the gene symbols, their descriptions and respective FC values.

Symbol	Description	K vs. OVE
CCL2	chemokine (C-C motif) ligand 2	5.12
CLCA2	chloride channel accessory 2	5.09
CACNA1D	calcium channel, voltage-dependent	4.51
ADARB2	adenosine deaminase, RNA-specific	4.51
KRT9	keratin 9	4.45
KIFC2	kinesin family member C2	4.34
MARK1	MAP/microtubule affinity-regulating kinase 1	4.22
POLE2	polymerase (DNA directed), epsilon 2 (p59 subunit)	4.19
SLC22A2	solute carrier family 22 (organic cation transporter), member 2	4.06
GPR63	G protein-coupled receptor 63	3.98
ANO3	anoctamin 3	3.88
XK	X-linked Kx blood group (McLeod syndrome)	3.83
DUSP4	dual specificity phosphatase 4	3.73
SPATA7	spermatogenesis associated 7	3.64
ZC3H4	zinc finger CCCH-type containing 4	3.59
SYCE3	synaptonemal complex central element protein 3	3.57
MYRIP	myosin VIIA and Rab interacting protein	3.40
LOC787081	PREDICTED: UPF0632 protein A	3.36
CNR1	cannabinoid receptor 1 (brain)	3.21
SPATA17	spermatogenesis associated 17	3.20
PCBP3	poly(rC) binding protein 3 (PCBP3)	3.09
ADAM32	ADAM metallopeptidase domain 32	3.08

Table 2.2: A list of differentially expressed genes with $p \leq 0.01$ and fold change ≤ -3.

Symbol	Description	K vs. OVE
FBP2	fructose-1,6-bisphosphatase 2	-8.60
DOCK3	dedicator of cytokinesis 3	-8.12
FAM131A	family with sequence similarity 131, member A	-6.91
LOC528412	multidrug resistance-associated protein 4	-6.80
COBRA1	cofactor of BRCA1	-6.24
CPXM2	carboxypeptidase X (M14 family), member 2	-6.13
UNC13D	unc-13 homolog D (C. elegans)	-5.18
FBP2	fructose-1,6-bisphosphatase 2	-5.14
LOC780781	keratin associated protein	-5.11
BTBD10	BTB (POZ) domain containing 10	-5.09
BAD	BCL2-associated agonist of cell death	-5.08
NEU4	sialidase 4	-5.08
SS18	synovial sarcoma translocation, chromosome 18	-4.82
TAPBPL	TAP binding protein-like	-4.80
KIAA0922	KIAA0922 ortholog	-4.77
APOBR	apolipoprotein B receptor	-4.68
PROK2	prokineticin 2	-4.64
WDR6	WD repeat domain 6	-4.60
EVC2	Ellis van Creveld syndrome 2	-4.58
SYN3	synapsin III	-4.50
GLCCI1	glucocorticoid induced transcript 1	-4.50
DLGAP5	discs, large (Drosophila) homolog-associated protein 5	-4.47
CIB2	calcium and integrin binding family member 2	-4.47
TBC1D19	TBC1 domain family, member 19	-4.41
ARNT	aryl hydrocarbon receptor nuclear translocator	-4.40
ACPT	acid phosphatase, testicular	-4.40
MAPK3	mitogen-activated protein kinase 3	-4.39
TUBG1	tubulin, gamma 1	-4.39
NCOA2	nuclear receptor coactivator 2	-4.29
REN	renin	-4.25
DST	dystonin, transcript variant 1	-4.23
PTK2	PTK2 protein tyrosine kinase 2	-4.21
HCN1	hyperpolarization activated cyclic nucleotide-gated potassium channel 1	-4.20
DEFB1	defensin, beta 1	-4.16
TTF2	transcription termination factor, RNA polymerase II	-4.13
NPB	neuropeptide B	-4.13

Table 2.2 (Cont.)

RAB27A	RAB27A, member RAS oncogene family	-4.09
ZYG11A	zyg-11 homolog A (C. elegans)	-4.08
MAP3K4	mitogen-activated protein kinase kinase kinase 4	-4.06
TPGS1	chromosome 7 open reading frame, human C19orf20	-4.04
C1QTNF3	C1q and tumor necrosis factor related protein 3	-4.00
TOE1	target of EGR1, member 1 (nuclear)	-3.98
ADARB2	adenosine deaminase, RNA-specific, B2	-3.97
TRIM65	tripartite motif containing 65	-3.94
LOC505465	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein	-3.92
SLC25A39	solute carrier family 25, member 39	-3.90
AP2A1	adaptor-related protein complex 2, alpha 1 subunit	-3.86
RP1	retinitis pigmentosa 1 (autosomal dominant)	-3.81
C1QTNF5	C1q and tumor necrosis factor related protein 5	-3.79
PMM1	phosphomannomutase 1	-3.76
MYO6	myosin VI	-3.76
ABCB7	ATP-binding cassette, sub-family B (MDR/TAP), member 7	-3.75
DUSP15	dual specificity phosphatase 15	-3.75
FIGF	c-fos induced growth factor (vascular endothelial growth factor D)	-3.74
NAT1	N-acetyltransferase 1 (arylamine N-acetyltransferase)	-3.72
CLDN8	claudin 8	-3.69
KDM2B	cDNA clone	-3.67
CENPE	centromere protein E	-3.64
TBX5	T-box 5	-3.64
CBX2	chromobox homolog 2	-3.63
LOC788554	olfactory receptor 8B3	-3.62
CA5B	carbonic anhydrase 5B, mitochondrial	-3.61
DCST2	DC-STAMP domain containing 2	-3.57
FGF13	fibroblast growth factor 13	-3.57
PLLP	plasmolipin	-3.57
PIGT	phosphatidylinositol glycan anchor biosynthesis, class T	-3.55
ZBTB32	zinc finger and BTB domain containing 32	-3.54
LOC788703	olfactory receptor, family 52, subfamily J, member 3-like	-3.54
PITPNM1	phosphatidylinositol transfer protein, membrane-associated 1	-3.53
LOC789869	Zinc finger and BTB domain-containing protein 8	-3.50
MAP3K8	mitogen-activated protein kinase kinase kinase 8B-like	-3.48
PLOD3	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	-3.48
HPS3	Hermansky-Pudlak syndrome 3	-3.47
PYGO2	pygopus homolog 2	-3.46
STK11IP	serine/threonine kinase 11 interacting protein	-3.46

Table 2.2 (Cont.)

NCOA3	nuclear receptor coactivator 3	-3.44
ZNF565	zinc finger protein 565	-3.40
ARHGAP22	Rho GTPase activating protein 22	-3.39
ACRV1	acrosomal vesicle protein 1	-3.37
HDAC5	histone deacetylase 5	-3.36
SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1	-3.35
ADORA2B	adenosine A2b receptor	-3.34
FAM214B	KIAA1539 ortholog	-3.33
GTPBP4	GTP binding protein 4	-3.32
EML3	echinoderm microtubule associated protein like 3	-3.31
OR5AS1	olfactory receptor, family 5, subfamily AS, member 1	-3.27
TKTL1	transketolase-like 1	-3.26
NCAN	neurocan	-3.22
PDE4DIP	phosphodiesterase 4D interacting protein	-3.20
DEFB7	defensin beta 7	-3.16
ACP5	acid phosphatase 5, tartrate resistant	-3.13
AGFG2	ArfGAP with FG repeats 2	-3.12
RALGAPA1	ral GTPase-activating protein subunit alpha-1	-3.11
MAP1S	microtubule-associated protein 1S	-3.10
KLHL30	kelch-like family member 30	-3.09
TBC1D5	TBC1 domain family, member 5	-3.08
ABHD16A	abhydrolase domain containing 16A	-3.07
ADM	adrenomedullin	-3.04
RAE1	RAE1 RNA export 1 homolog (S. pombe)	-3.01

Table 2.3: A list of significantly affected biochemical compounds along with their sub-pathways with $p \leq 0.10$ and respective fold change (FC) values. The FC < -1.0 indicates the down regulation, whereas FC ≥ 1.0 indicates up regulation of the biochemical compounds.

Biochemical Name	Sub Pathway	K vs. OVE	
		p -value	FC
glycylvaline	Dipeptide	0.01	-1.41
leucylaspartate	Dipeptide	0.01	-1.46
tyrosylglycine	Dipeptide	0.03	-1.30
glycylisoleucine	Dipeptide	0.03	-1.49
xylitol	Pentose Metabolism	0.04	-1.34
1-palmitoylglycerophosphoglycerol*	Lysolipid	0.06	-1.37
glycochenodeoxycholate	Primary Bile Acid Metabolism	0.06	2.32
glucose-6-phosphate (G6P)	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.07	-1.24
1-methylimidazoleacetate	Histidine Metabolism	0.07	1.62
N-palmitoyl-D-erythro-sphingosine	Sphingolipid Metabolism	0.07	-1.24
1-nonadecanoylglycerophosphocholine(19:0)*	Lysolipid	0.08	-3.79
3-dehydrocarnitine*	Carnitine Metabolism	0.08	-1.15
ribulose	Pentose Metabolism	0.09	-1.29
butyrylglycine	Fatty Acid Metabolism (also BCAA Metabolism)	0.09	1.34
tetradecanedioate	Fatty Acid, Dicarboxylate	0.09	-1.40

Legends

***p* -value**	***p* < 0.05**	**0.05 ≤ *p* < 0.1**
FC	**FC < -1.0**	**FC ≥ 1.0**

Table 2.4: Metabolic profiling of blood biomarkers. The table shows 33 metabolites their estimates, standard error means (SEM) and *p*-values for each status (OVE, and K), time point (-12, -3, and +3) and their interaction (status x time).

						Status X Time									
	Status		Time			OVE			K				*p* -value		
Metabolites	OVE	K	-12	-3	3	-12	-3	3	-12	-3	3	SEM	Status	Time	Status X Time
Glucose	3.69	3.80	4.01[a]	3.66[b]	3.56[b]	3.95	3.55	3.57	4.08	3.76	3.55	0.17	0.49	0.00	0.65
Cholesterol	2.35	2.51	2.85[a]	2.32[b]	2.11[b]	2.84	2.18	2.01	2.85	2.45	2.21	0.21	0.49	0.00	0.55
Urea	4.99	4.71	5.16	4.84	4.55	5.54	5.12	4.30	4.78	4.56	4.79	0.43	0.51	0.15	0.09
Calcium	2.38	2.42	2.51[a]	2.43[a]	2.25[b]	2.52	2.45	2.17	2.51	2.41	2.33	0.08	0.55	0.00	0.22
Phosphorus	2.20	2.06	2.09	2.14	2.16	2.16	2.23	2.21	2.02	2.05	2.11	0.15	0.23	0.87	0.95
Magnesium	0.89	0.87	0.91	0.89	0.85	0.91	0.90	0.87	0.92	0.87	0.83	0.05	0.66	0.30	0.82
Sodium	146.72	146.79	145.35[a]	148.19[b]	146.72[a]	145.40	148.37	146.39	145.31	148.01	147.06	0.94	0.92	0.00	0.75
Potassium	4.66	4.44	4.65[a]	4.69[a]	4.33[b]	4.81	4.76	4.43	4.49	4.62	4.23	0.13	0.07	0.00	0.66
Chloride	105.79	106.56	106.17[a]	107.87[a]	104.5[b]	105.95	107.40	104.03	106.38	108.33	104.98	1.07	0.35	0.00	0.95
Zinc	11.62	10.17	13.25[a]	10.17[b]	9.267[b]	13.46	11.23	10.16	13.03	9.10	8.37	1.27	0.06	0.01	0.77
Ceruloplasmin	2.61	2.48	2.42	2.47	2.75	2.50	2.29	3.05	2.34	2.66	2.45	0.26	0.55	0.25	0.07
Total protein	72.87	72.47	74.34[a]	70.79[b]	72.89[a]	75.76	70.69	72.16	72.92	70.88	73.61	1.74	0.82	0.02	0.22
Albumin	36.08	36	36.95[a]	35.63[b]	35.54[b]	37.17	35.54	35.52	36.73	35.72	35.55	0.52	0.88	0.00	0.70
Globulin	36.64	36.28	37.42[a]	34.63[b]	37.35[a]	38.64	34.65	36.63	36.19	34.60	38.06	1.81	0.86	0.02	0.23
GOT	76.40	74.69	58.81[a]	63.48[a]	104.33[b]	62.08	64.42	102.70	55.55	62.55	105.97	12.55	0.86	0.00	0.89
GGT	21.75	21.31	21.96	20.51	22.13	23.59	20.07	21.59	20.32	20.94	22.67	40.70	0.81	0.34	0.19
Bilirubin	4.25	4.63	1.37[a]	4.34[b]	7.60[c]	1.49	4.17	7.07	1.25	4.51	8.12	1.81	0.82	0.00	0.89
Alkaline phosphatase	26.51	28.86	26.03[a]	26.26[a]	30.77[b]	24.64	24.42	30.47	27.42	28.10	31.06	2.98	0.54	0.01	0.63
Haptoglobin	0.67	0.77	0.44[a]	-	1.00[b]	0.40	-	0.94	0.49	-	1.05	0.18	0.53	0.00	0.94
NEFA	0.72	0.77	0.37[a]	0.78[b]	1.09[c]	0.41	0.77	0.98	0.33	0.79	1.20	0.14	0.69	0.00	0.40
BHBA	0.75	0.91	0.50[a]	0.84[b]	1.16[c]	0.52[a]	0.83[b]	0.91[b]	0.48[a]	0.86[b]	1.41[c]	0.17	0.36	0.00	0.02

Table 2.4 (Cont.)

Creatinine	101.84	105.17	100.76	104.19	105.56	101.13	104.03	100.36	100.39[a]	104.35[a]	110.75[b]	3.05	0.16	0.18	0.03
Paraoxonase	57.03	56.30	68.46[a]	55.77[b]	45.77[c]	64.82	58.80	47.48	72.10	52.75	44.06	7.03	0.91	0.00	0.42
ROMt	12.01	13.10	12.02[a]	11.78[a]	13.87[b]	11.79	11.04	13.22	12.26	12.51	14.52	1.05	0.20	0.04	0.83
SHp	145.79	159.33	138.51	148.09	171.08	116	168.29	153.08	161.02	127.89	189.09	29.82	0.58	0.36	0.13
NOX	41.78	42.45	39.87[a]	51.55[b]	34.94[a]	39.26	52.50	33.59	40.48	50.59	36.28	4.19	0.88	0.00	0.63
NO2	9.60	9.13	11.72[a]	8.53[b]	7.84[b]	11.97	9.36	7.47	11.48	7.71	8.21	1.43	0.74	0.00	0.48
NO3	31.72	32.75	28.07[a]	41.54[b]	27.10[a]	27.14	41.91	26.12	29.00	41.17	28.07	3.81	0.80	0.00	0.81
Myeloperoxidase	471.23	499.45	408.96[a]	484.64[b]	562.44[c]	392.98	484.42	536.30	424.93	484.85	588.58	43.79	0.43	0.00	0.73
ORAC	11438	11737	12006	11267	11490	12033	11219	11063	11980	11315	11916	647.38	0.69	0.16	0.38
Retinol	24.23	20.46	31.42[a]	20.91[b]	14.71[c]	32.72	22.50	17.46	30.11	19.31	11.97	3.92	0.33	0.00	0.84
Tocopherol	2.33	2.53	2.58	2.73	1.97	2.78	2.42	1.78	2.38	3.03	2.16	0.39	0.53	0.06	0.28
Beta-Carotene	0.10	0.10	0.12[a]	0.10[a]	0.08[b]	0.12	0.10	0.08	0.12	0.11	0.08	0.02	0.85	0.01	0.66

[a-c] Values with different superscript letters in rows are different ($p < 0.05$)

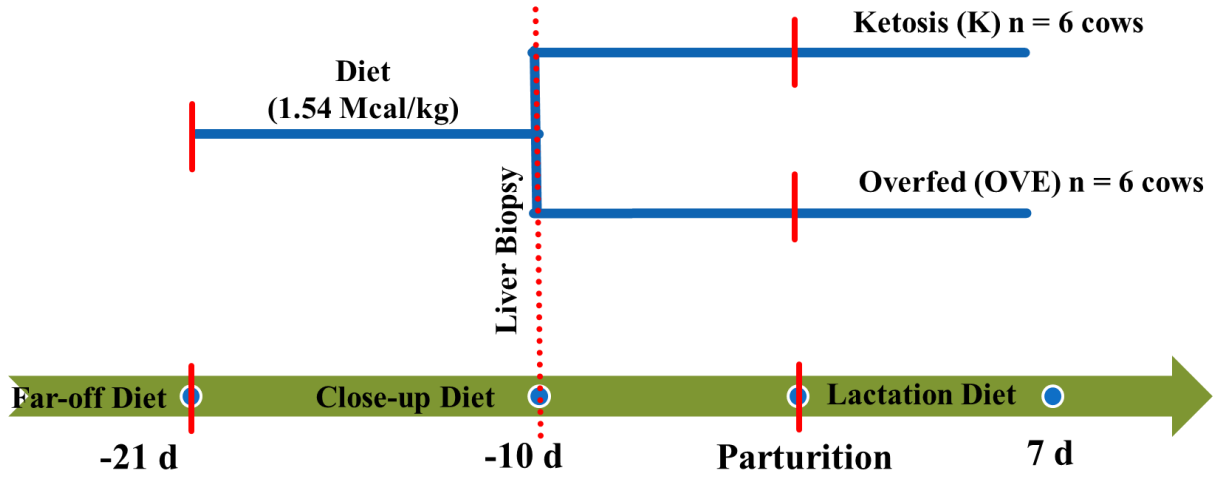


Figure 2.1: Experimental design for the current study: The tissue biopsies were taken at -10 d relative to parturition.

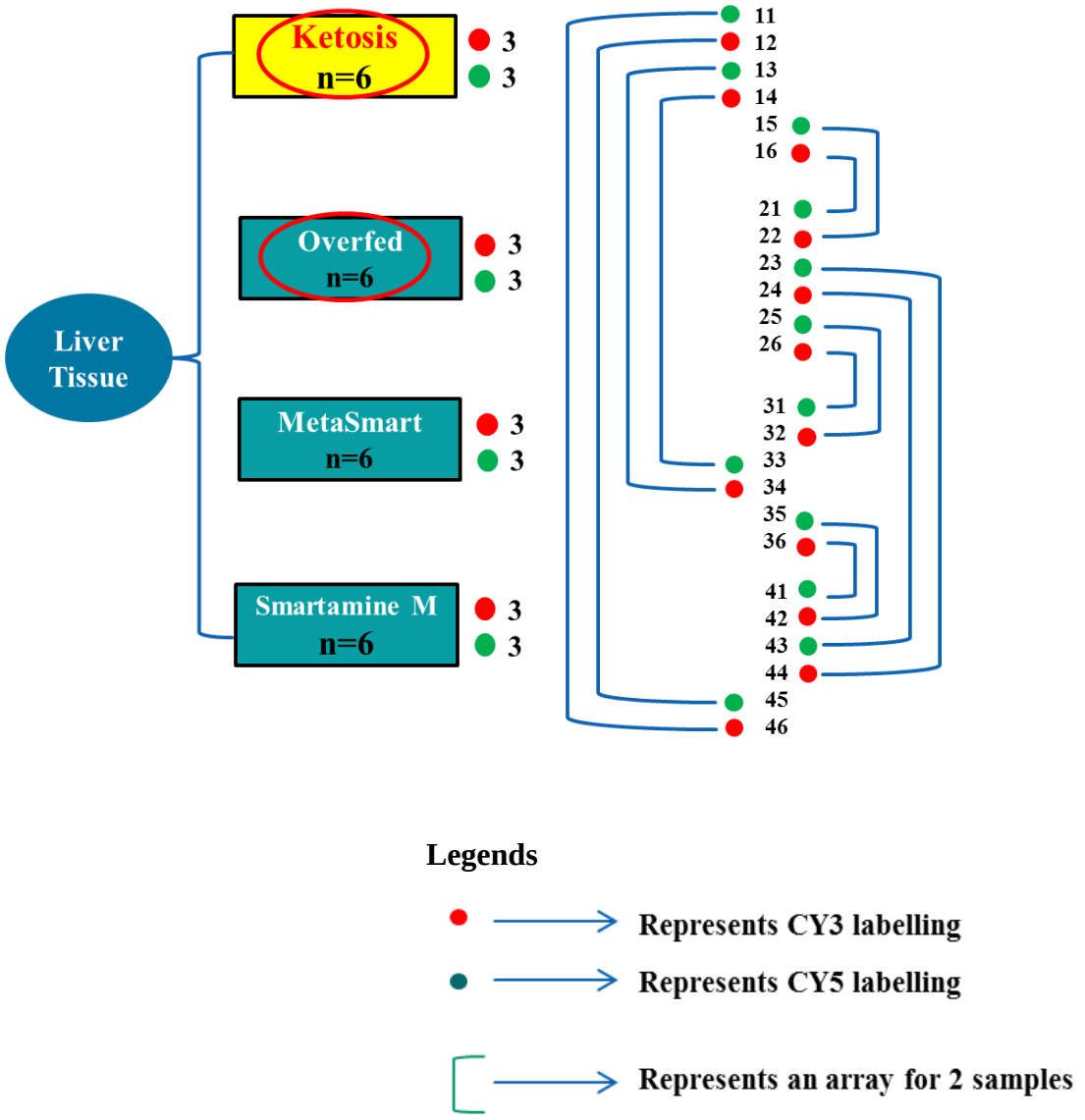


Figure 2.2: The microarray's hybridization design is shown here. A two colored channel (red and green) hybridization plan was used. The cows were fed with moderate energy diet, remain health (OVE), and with developed Ketosis (K) postpartum are encircled with red color.

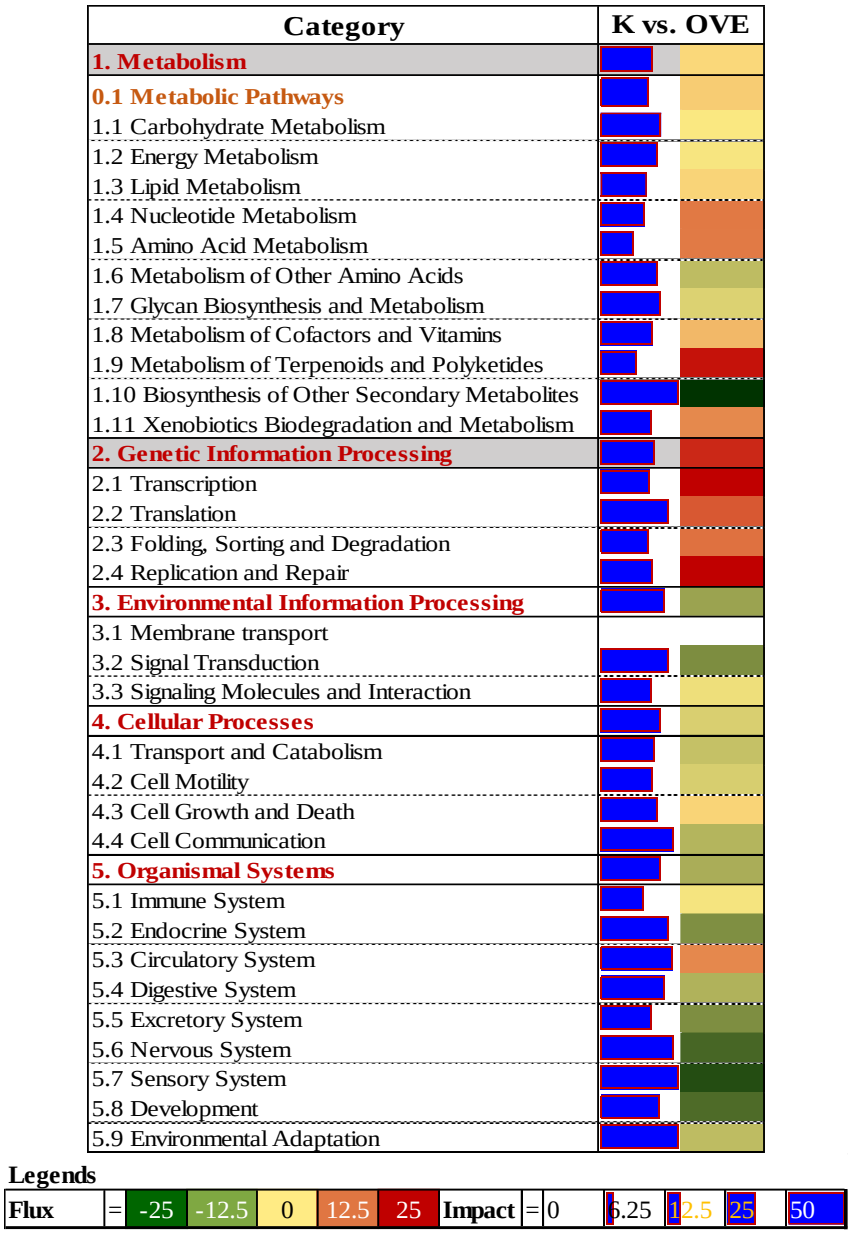


Figure 2.3: Summary of the Kyoto Encyclopedia of Genes and Genomes (KEGG) categories and subcategories resulting from analysis by Dynamic Impact Approach (DIA). The transition dairy cows with developed ketosis and healthy group (K vs. OVE) were fed moderate energy diet (1.54 Mcal/Kg) prepartum (-21 d to parturition). The impact values are represented by blue bars ranging from 0 to 50. The flux values are represented by colors ranging from green (inhibited, -25 to 0) to red (activated, 1 to 25).

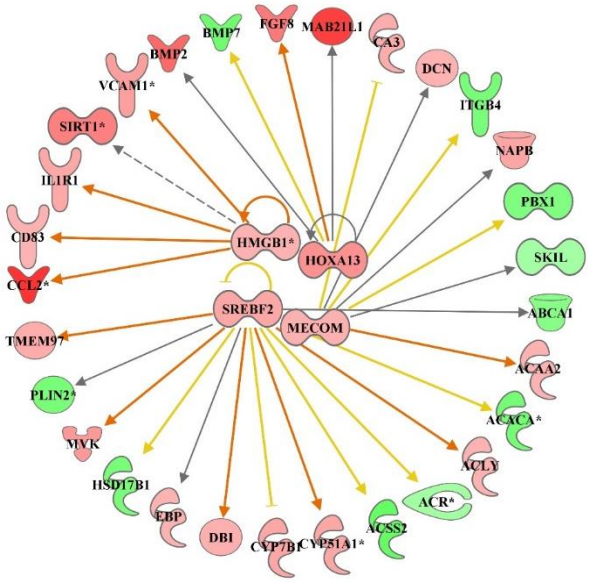
Sub category	Metabolic Pathways	K vs. OVE
1.8 Metabolism of Cofactors and Vitamins	Riboflavin metabolism	
1.10 Biosynthesis of Other Secondary Metabolites	Caffeine metabolism	
1.7 Glycan Biosynthesis and Metabolism	Other glycan degradation	
1.3 Lipid Metabolism	Fatty acid biosynthesis	
1.1 Carbohydrate Metabolism	Pentose phosphate pathway	
1.7 Glycan Biosynthesis and Metabolism	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis	
1.6 Metabolism of Other Amino Acids	Selenoamino acid metabolism	
1.1 Carbohydrate Metabolism	Inositol phosphate metabolism	
1.1 Carbohydrate Metabolism	Glyoxylate and dicarboxylate metabolism	
1.7 Glycan Biosynthesis and Metabolism	Glycosaminoglycan degradation	
1.7 Glycan Biosynthesis and Metabolism	Glycosphingolipid biosynthesis - ganglio series	
1.1 Carbohydrate Metabolism	Fructose and mannose metabolism	
1.3 Lipid Metabolism	Steroid biosynthesis	
1.3 Lipid Metabolism	Sphingolipid metabolism	
1.1 Carbohydrate Metabolism	Propanoate metabolism	
1.1 Carbohydrate Metabolism	Ascorbate and aldarate metabolism	
1.3 Lipid Metabolism	Biosynthesis of unsaturated fatty acids	
1.6 Metabolism of Other Amino Acids	Cyanoamino acid metabolism	
1.8 Metabolism of Cofactors and Vitamins	One carbon pool by folate	
1.2 Energy Metabolism	Nitrogen metabolism	

Figure 2.4: The top 20 most impacted metabolic Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways ranked by overall impact values. The impact and flux columns are shown on the right hand side of the figure. The impact values are represented by blue bars ranging from 0 to 50. The flux values are represented by colors ranging from green (inhibited, -25 to 0) to red (activated, 1 to 25).

Sub category	Non-metabolic Pathways	K vs. OVE
5.7 Sensory System	Taste transduction	
5.6 Nervous System	Long-term potentiation	
5.2 Endocrine System	Renin-angiotensin system	
5.2 Endocrine System	GnRH signaling pathway	
2.2 Translation	Ribosome	
3.2 Signal Transduction	mTOR signaling pathway	
5.4 Digestive System	Salivary secretion	
5.4 Digestive System	Bile secretion	
5.7 Sensory System	Phototransduction	
3.2 Signal Transduction	ErbB signaling pathway	
5.9 Environmental Adaptation	Circadian rhythm - mammal	
5.3 Circulatory System	Vascular smooth muscle contraction	
2.4 Replication and Repair	Nucleotide excision repair	
5.4 Digestive System	Gastric acid secretion	
5.2 Endocrine System	Insulin signaling pathway	
4.4 Cell Communication	Gap junction	
5.8 Development	Dorso-ventral axis formation	
4.4 Cell Communication	Adherens junction	
4.3 Cell Growth and Death	Oocyte meiosis	
5.6 Nervous System	Glutamatergic synapse	

Figure 2.5: The top 20 most impacted non-metabolic Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways ranked by overall impact values in K vs. OVE. The impact and flux columns are shown on the right hand side of the figure. The impact values are represented by blue bars ranging from 0 to 50. The flux values are represented by colors ranging from green (inhibited, -25 to 0) to red (activated, 1 to 25).

A



B

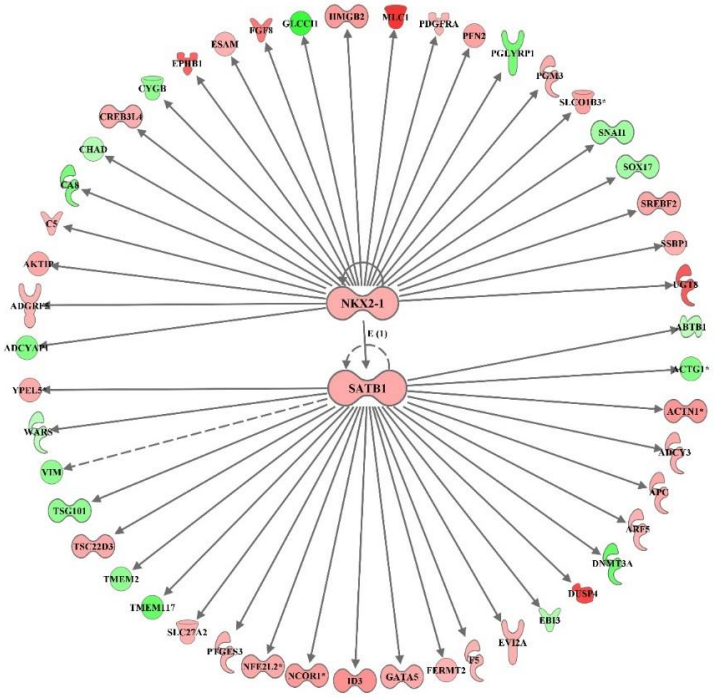
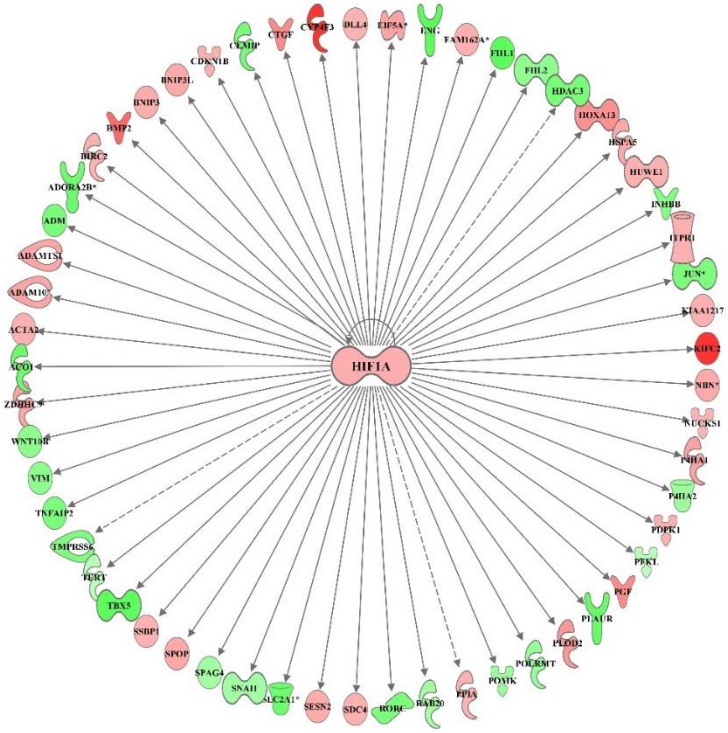


Figure 2.6: Representation of upstream analysis (A-D). The eight (8) molecules in the center are shown as up regulated transcription regulators (red), while the molecules in the periphery are the target genes from our differentially expressed gene (DEG) list.

C



D

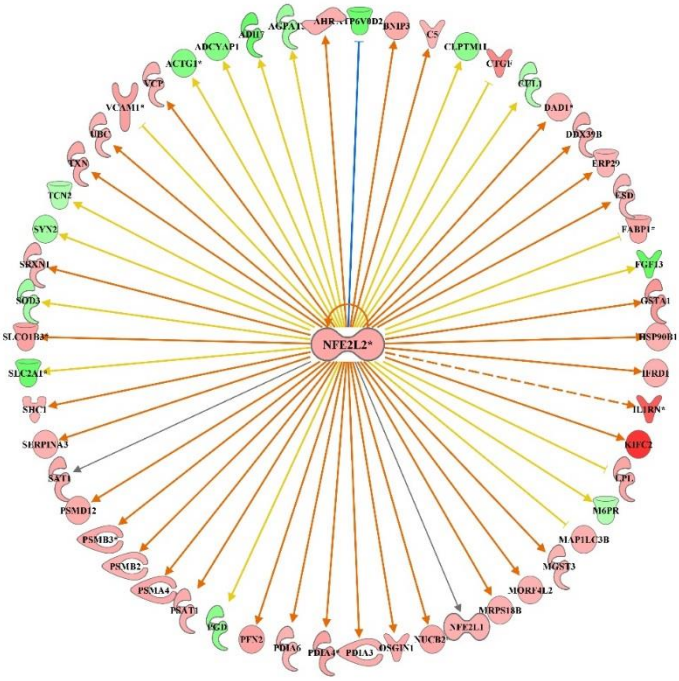


Figure 2.6 (Cont.): Representation of upstream analysis (A-D). The eight (8) molecules in the center are shown as up regulated transcription regulators (red), while the molecules in the periphery are the target genes from our differentially expressed gene (DEG) list.

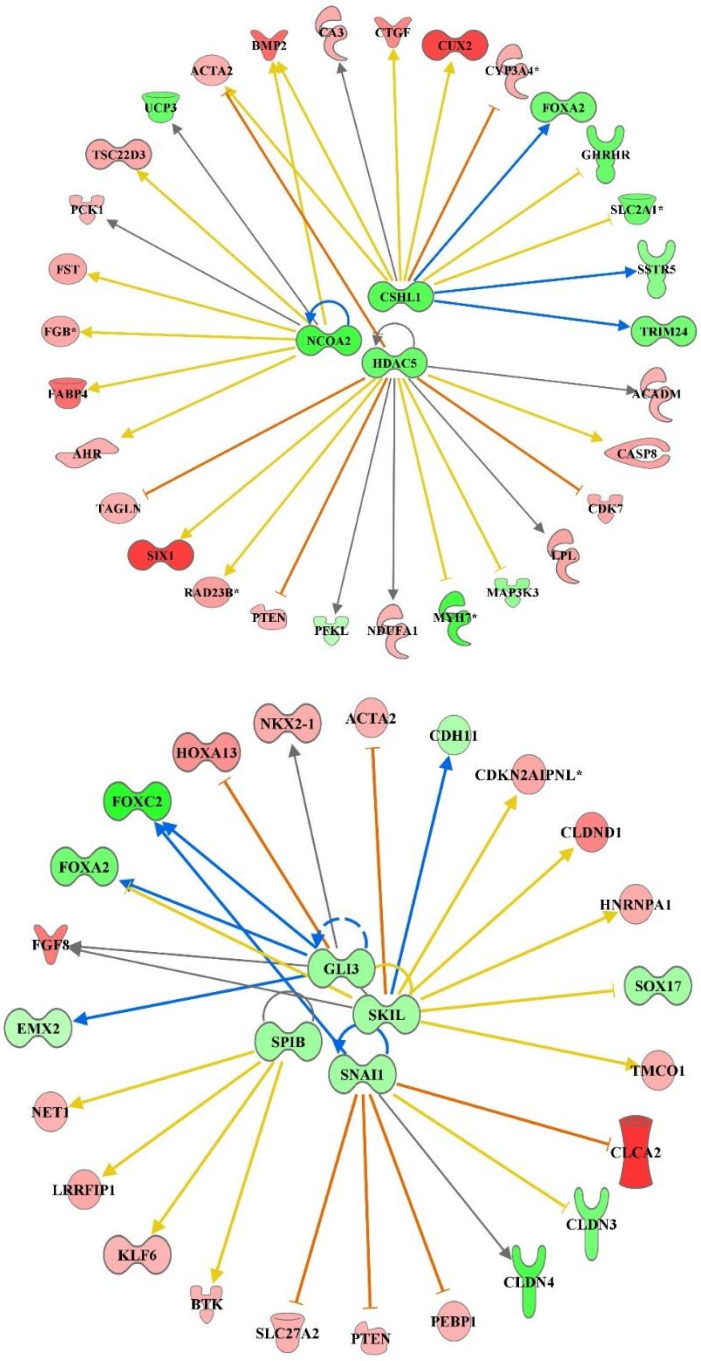
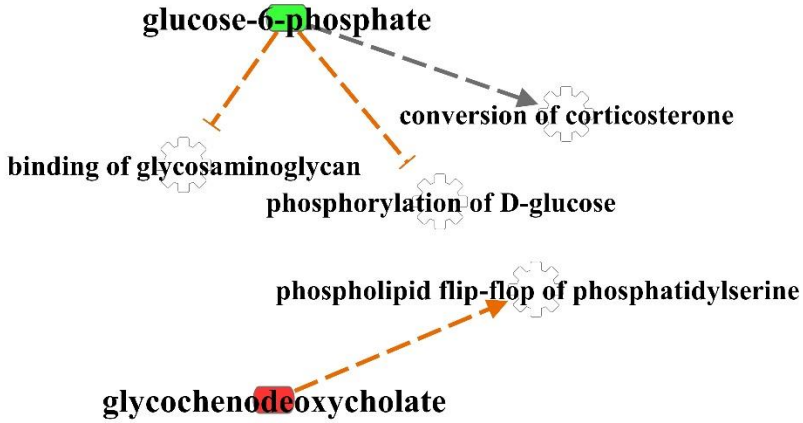
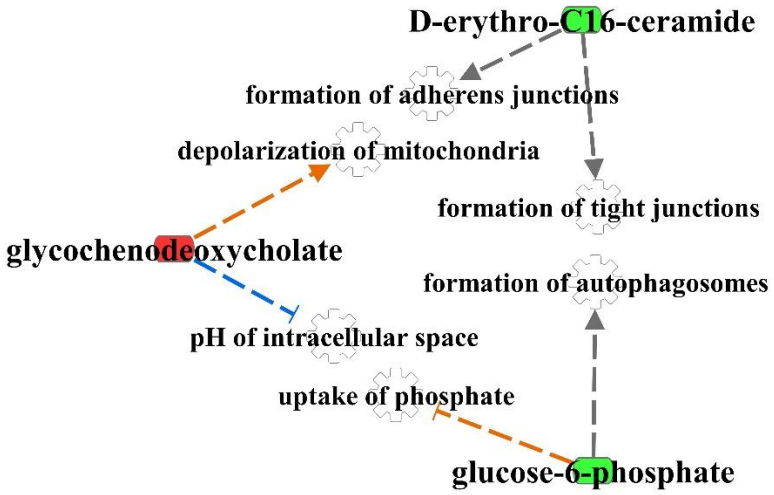


Figure 2.7: Representation of upstream analysis (A-B). The eight (7) molecules in the center are shown as down regulated transcription regulators (green), while the molecules in the periphery are the target genes from our differentially expressed gene (DEG) list.



A: Carbohydrate Metabolisms



B: Cellular Functions

Figure 2.8: Representation of Biochemical compounds in the form of metabolic (A: carbohydrate and lipid metabolism) and non-metabolic (B: cellular functions) networks in K vs. OVE.

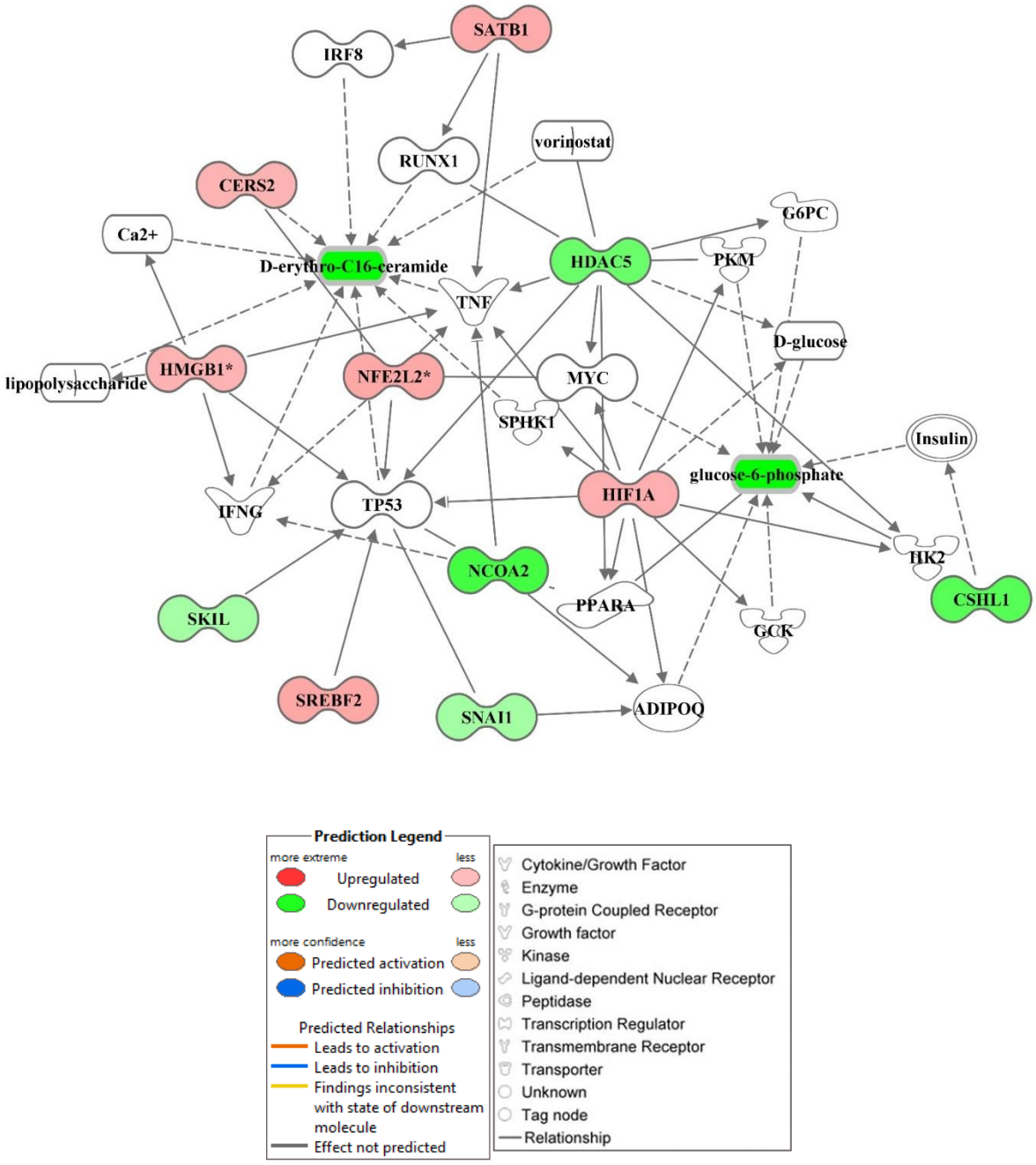


Figure 2.9: Integration of transcriptomic and metabolomics datasets. The network shows the interaction between transcription regulators and biochemical compounds in K vs. OVE group.

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CHAPTER # 3

Hepatic metabolomics and transcriptomics in prepartal dairy cows supplemented with Smartamine M and MetaSmart during the transition period.

Abstract

Supplementation with Smartamine M (SM) and MetaSmart (MS) during the transition period improves postpartal dry matter intake, milk production, and blood neutrophil immune function. In the current study we used metabolomics and transcriptomics to provide a more holistic view of the adaptations induced on the liver by dry period nutrition. Liver from cows fed a control high-energy diet without (OVE) or with SM or MS were used. Metabolomics was performed via LC-MS and GC-MS (Metabolon Inc.) and transcriptomics using a whole-transcriptome bovine microarray (Agilent). From a total of 313 biochemical compounds identified, metabolomics analysis ($P \leq 0.10$) revealed a total of 20, 21, and 48 compounds affected by SM vs. OVE, MS vs. OVE, and SM vs. MS, respectively. Comparing profiles in SM vs. OVE revealed that compounds up-regulated belong to the pentose, sterol, inositol, and purine metabolism pathways, while down-regulated compounds belong to secondary bile acid, arginine and proline, purine and pyrimidine, and eicosanoid metabolism pathways. In MS vs. OVE, the compounds up-regulated belong to primary bile acid, pyrimidine, and lysolipid metabolism, while compounds down-regulated were linked with glycolysis, gluconeogenesis, urea cycle, sphingolipid, and pyruvate metabolism. Liver of MS vs. OVE cows had lower hydroxybutyrate

and lactate concentration. The transcriptomic analysis of these groups resulted in 922 (SM vs. OVE), 1,573 (MS vs. OVE) and 1,033 (SM vs. MS) differentially expressed genes (DEG, P ≤0.05). Bioinformatics analysis using the Dynamic Impact Approach (DIA) that SM vs. OVE resulted in a marked impact and activation of 'fatty acid biosynthesis, cyanoamino acid metabolism', 'O-glycan biosynthesis, and 'glycosaminoglycan biosynthesis'. In MS vs. OVE, however, among the top-5 most-impacted pathway there was marked inhibition of 'phenylalanine, tyrosine, and tryptophan biosynthesis' and 'phenylalanine' metabolism. 'Cyanoamino acid metabolism' and 'taurine and hypotaurine' metabolism were highly-impacted and activated pathways in MS vs. OVE. Unique responses in SM vs. MS included a marked activation of 'fatty acid biosynthesis, 'glycosphingolipid metabolism, 'valine, leucine, and isoleucine biosynthesis', and 'sulfur metabolism'. Preliminary data interpretation suggests MS and SM induce distinct changes on the metabolome and transcriptome phenotype of the prepartal liver. The functional relevance of such changes remains to be determined.

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Introduction

In dairy cows, protein nutrition is utilized in two dependent ways. First, the protein contents of dairy ration (dietary proteins) become the main source of nitrogen (N) for microbial protein synthesis in the rumen. Second, this microbial protein becomes the source of amino acids for various biological functions such as maintenance, growth, reproduction and above all milk protein synthesis (Lee et al., 2015). Rumen also serve as a medium for microbial protein synthesis from protein free diet (Virtanen, 1966), and help to increase milk protein synthesis (Hao et al., 2017). However, inadequate supply of essential amino acids may be a limiting factor to maintain a high milk protein or overall milk production in modern dairy cows (NRC, 2001). Several studies have been conducted to identify the limiting amino acids in high producing dairy cows during transition period. It was found that methionine, lysine and histidine are limiting amino acids during early phase of lactation (Broderic.Ga et al., 1974, Vanhatalo et al., 1999, Phillips et al., 2003). Studies has shown that rumen-protected methionine and lysine supplementation plays an important role to increase milk protein and production in high producing dairy cows (Socha et al., 2005).

Methionine is the first limiting amino acid identified during early lactation in dairy cows. In this scenario, rumen-protected methionine in the form of Smartamine M (SM) and MetaSmart (MS) is being considered as one of the beneficial supplementation in modern dairy ration to increase the overall milk yield and animal performance in periparturient dairy cows (Rulquin et al., 2006, Ordway et al., 2009, Osorio et al., 2013). Methionine supplementation, in addition to balance the 3:1 ratio of lysine to methionine, also plays a vital role in maintaining the hepatic metabolic functions during the negative energy balance (NEB). It has been reported that

methionine in the liver of transition dairy cows acts as a lipotropic agent by stimulating very low-density lipoprotein (VLDL) synthesis and consequently increase the hepatic transport of triacylglycerides (TG) (Martinov et al., 2010). Methionine supplementation has also been shown to induce the synthesis of glutathione by providing the cysteine source (Martinov et al., 2010).

In our current study, the dairy cows were overfed with moderate energy diet without any supplementation (OVE) and with methionine supplementation in the form of SM and MS to different groups of cows (Adisseo Inc.). We hypothesize that supplementing the moderate energy diet with SM and MS would help to improve the dry matter intake (DMI), milk yield and milk protein concentration as shown in (Osorio et al., 2013). In addition to these expectations, the current study is aimed to provide a holistic view of hepatic metabolomics and transcriptomics data integration using recent bioinformatics and systems biology approaches. The objective of the study was to utilize the metabolomics and transcriptomics data along with Bioinformatics techniques to uncover response of long-term rumen-protected methionine supplementation in terms of preparing the liver for the onset of lactation.

Materials and Methods

Experimental design and dietary treatments

The procedure for this protocol (#09214) was approved by The Institutional Animal Care and Use Committee (IACUC) of the University of Illinois (Urbana). The experiment was conducted as a randomized complete block design as explained elsewhere (Osorio et al., 2013). For this study, we selected a subset of 18 cow that were divided into three main groups such as

cows that were overfed moderate energy diet (OVE, n=6), OVE plus supplemented with Smartamine M (SM, n=6), and OVE plus supplemented with MetaSmart (MS, n=6). All cows received the same far-off diet (1.24 Mcal/kg of DM; 14.3% CP) from −50 to −22 d before expected calving, a close-up diet (1.54 Mcal/kg of DM; 15.0% CP) from −21 d to calving, and fresh cow lactation diet from calving (1.75 Mcal/kg of DM; 17.5% CP) through 30 days in milk (DIM). Supplements of methionine were top-dressed from −21 to 30 DIM. The experimental design of the current study is shown in the Figure 3.1.

Liver biopsies and RNA extraction

Liver tissue samples were collected via puncture biopsies (Dann et al., 2006) from cows under local anesthesia at approximately 0730 hour once prepartum on d −10 (±3 d), and then postpartum on d 7 and 21. The tissue samples were stored in liquid nitrogen immediately and then at 80°C until further RNA extraction, microarrays and metabolomics analysis. We used liver samples from –10 d prepartum for our metabolome and transcriptome experiment. Total RNA was extracted from the liver samples using established protocol in our laboratory. Briefly, liver tissue sample was weighed (~55 milligrams on average) and straightway put inside a 2 ml centrifuge tube (Corning Inc. ®, Cat. No. 430052, Corning, NY, USA), with 1 ml of Qiazol reagent to proceed with RNA extraction. This extraction procedure also utilizes chloroform (Ambion® Cat. No. 9720, Austin, TX, USA), which removes residual DNA. Any residual genomic DNA was removed from RNA with DNase enzyme using miRNeasy Mini Kit columns (Qiagen, Hilden, Germany). RNA concentration was measured using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The purity of RNA (A260/A280) for all samples was above 2.0. The quality of RNA was evaluated using the

Agilent Bioanalyzer system (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). The average RNA integrity number (RIN) for all samples was around 6.9.

Metabolomics

Metabolomics analysis was performed by Metabolon Company (Metabolon Inc. NC). The liver tissue samples were prepared according to the sample preparation guidelines. Briefly, the 500 milligram (mg) of the liver tissue per sample was weighed, packed in dry ice and then shipped to the company. The metabolomics analysis was performed using mass spectrometry coupled with gas chromatography (GC-MS) and liquid chromatography (LC-MS). Three groups were used for the current study, which are OVE, SM and MS. Each group has 313 total identified biochemical compounds for the analysis purpose.

Transcriptomics

For transcriptome analysis, we used ~44 K bovine (v2) gene expression Agilent microarray platform. The microarrays experiment was performed according to our laboratory's established protocol and the instructions provided by Agilent technologies. The complete microarrays hybridization design is shown in the Figure 3.2. For the current study, we used OVE, SM and MS groups for the respective pairwise comparisons. The detailed description of the microarrays experiment is provided elsewhere (Shahzad et al., 2015). Briefly, the RNA with 200 nanograms (ng) per sample was used for cDNA synthesis. The cDNA was reverse transcribed to cRNA and then used for cy3 or cy5 fluorescent dye labeling according to the manufacturer's instructions. Purification of the labeled cRNA was performed with RNeasy mini

spin columns (Qiagen, cat# 74104), and it was subsequently eluted in 30 μL of DNase-RNase-free water. The eluted cRNA concentration was measured using NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA) to confirm the manufacturer's recommended criteria for yield and specific activity of at least 0.825 μg and ≥ 6 respectively. The labelled cRNA was fragmented using 10X blocking Agent and 25X fragmentation buffer and then the reaction was stopped using 2X GEx hybridization buffer. The samples were loaded onto the Agilent bovine microarray's slides and were hybridized in a rotating hybridization oven at 65°C for 17 hours. After that slides were washed and scanned using a GenePix 4000B scanner (Axon Instruments Inc., Sunnyvale, CA) and GenePix Pro v.6.1 software. Resulting spots with substandard features were flagged and excluded from the subsequent analysis.

Statistical Analysis

From metabolomics data, total 313 biochemical compounds were used for statistical analysis. The data was normalized in terms of raw area counts. Each biochemical compound from the raw values was rescaled to set the median value equal to 1. The missing values were imputed with the minimum value. Following the log transformation and imputation of missing values, with the minimum observed value for each compound, we used a mixed procedure of SAS (SAS Institute Inc., Cary, NC) to identify the biochemical compounds that affected significantly between the experimental groups. The biochemical compounds with $p \leq 0.10$ were short listed for subsequent analysis.

For microarray's statistical analysis, data from 12 arrays (24 samples) was used. The oligo IDs with bad flags (-100) were removed before normalization. The data was log

transformed and then corrected across dye and array effects using loess normalization and array centering method. After normalization, a mixed procedure of SAS was used. The statistical model included dietary treatments as a fixed effect. The raw *p* values were adjusted for the number of genes tested using Benjamini and Hochberg's false discovery rate (FDR; Benjamini and Hochberg, 1995) to account for multiple comparisons. However, there were not enough differentially expressed genes (DEG) using corrected *p* values, so we used raw $p \leq 0.05$ and fold change (FC) $\geq |1.5|$ for the evaluation purpose. For this study we selected three main comparisons which are SM vs. OVE, MS vs. OVE and SM vs. MS.

Pathways analysis

For metabolomics analysis, each biochemical compound was annotated with its corresponding sub-pathway. The results were further furnished with *p*- and fold change (FC) values resulting from SAS analysis. Post statistical analysis of the transcriptomics data was conducted using the Dynamic Impact Approach (DIA) (Bionaz et al., 2012) to identify the most impacted Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways within different groups. With a $p \leq 0.05$ and FC $\geq |1.5|$, we obtained 710, 786 and 601 DEG for SM vs. OVE, MS vs. OVE and SM vs. MS comparisons respectively. The DIA was run on the DEG to obtain the KEGG categories, sub-categories and their respective pathways. As an input, we provided a list of DEG consisting of Oligo IDs, Entrez gene IDs, *p* values and FC values for each comparison. For the analysis purpose, a minimum of 30% annotated genes on the microarray versus the whole genome were selected as described elsewhere (Bionaz et al., 2012). This gives us the results of each comparison in two distinct columns. The first column contains the impact values (overall perturbation) represented in blue colored horizontal bars, whereas the second column contains

the flux (direction of the impact) values represented in colors ranging from green (inhibited) to red (induced).

Network analysis and data integration

The network analysis for both metabolomics and transcriptomics data was conducted using Ingenuity Pathway Analysis (IPA) software. The metabolomics data consisting of biochemical compounds was annotated with PubChem identifiers (https://pubchem.ncbi.nlm.nih.gov/). For network constructions, a list of 13, 18 and 26 significantly affected ($p \leq 0.10$) biochemical compounds for SM vs. OVE, MS vs. OVE and SM vs. MS respectively was used along with their FC values. For transcriptomics network reconstruction, a list of DEG with a $p \leq 0.05$ and FC $\geq |1.5|$ was uploaded to run the core analysis. From the analysis results, we used upstream transcription regulators and their downstream target genes. The data integration was performed using transcription regulators and biochemical compounds with the help of 'Path Explorer' tool in IPA. The connections with both direct and indirect links were considered for the analysis.

Results and Discussion

Expression patterns of metabolome and transcriptome datasets

Both groups have different expression patterns based on the available biochemical compounds and the number of DEG. The expression patterns for both datasets are described in the following sections.

Metabolomics. The Figure 3.3 shows the overall expression pattern for metabolomics dataset. Here we have highlighted the number of significantly affected metabolites with $p \leq 0.05$, between 0.05 and 0.10, and $p \leq 0.10$ using different color scales. The figure shows that there were more number of biochemical compounds that appeared in SM vs. MS ($p \leq 0.10$) as compared to other comparisons. The results indicated 16, 26 and 36 compounds in SM vs. OVE, MS vs. OVE and SM vs. MS. However, these compounds were further annotated with PubChem identifiers for IPA analysis. The total numbers were reduced to 13, 18 and 26 respectively for each comparisons due to unavailability of complete annotation of these compounds in the PubChem identifier database.

Transcriptomics. For transcriptomics analysis, we selected $p \leq 0.05$ and FC $\geq |1.5|$ criteria. Using this criteria, we obtained 710, 786, and 601 number of DEG for SM vs. OVE, MS vs. OVE and SM vs. MS as shown in the Figure 3.4. The figure shows up- and down-regulation patterns of the DEG across all the comparisons. To provide an overview of the most affected DEG across the three comparisons, we have provided three tables (Tables 3.1-3.3) with $p \leq 0.01$ and FC $\geq |3.0|$. The tables enlist gene symbols, their descriptions and FC values.

Metabolomics pathways

For the discussion purpose, we selected biochemical compounds with their $p \leq 0.05$ as cut off. Using this cut off, we obtained 5, 16 and 17 compounds for SM vs. OVE, MS vs. OVE

and SM vs. MS respectively as shown in the Figures 3.5 and 3.6. In SM vs. OVE, it was observed that gamma-glutamylglycine and inosine biochemical compounds were up regulated, while the compounds related to dipeptide (leucylaspartate and glycylisoleucine) and secondary bile acid metabolism (glycodeoxycholate) were down regulated (SM vs. OVE, Figure 3.5). Inosine is involved in activation of liver enzymes glycogen phosphorylase (Camara-Artigas et al., 1997).

A more pronounced effect was observed in cows supplemented with MS as compared with OVE, where the compounds related to lysolipids, pentose metabolism, energy metabolism and secondary bile acid metabolism were up regulated and dipeptides, gluconeogenesis and pyruvate metabolism were down regulated (MS vs. OVE, Figure 3.5). These results indicate a shift of energy source from carbohydrate to lipid molecules.

On the other hand, while comparing SM group with MS (Figure 3.6), we found that compounds related to gluconeogenesis, amino sugar, and fatty acids were activated in SM group, whereas the compounds related to primary and secondary bile acids, tryptophan metabolism, urea cycle, purine metabolism, and lysolipids were activated in MS group.

Summary of KEGG pathways

The Dynamic Impact Approach (DIA) was used for functional enrichment analysis of the DEG. It provides results in the form of KEGG categories, sub-categories and pathways. We have included categories, and sub-categories under this section, as these results summarize the overall expression pattern of the DEG. The Figure 3.7 includes five main categories, which are ‘Metabolism’, ‘Genetic Information Processing’, ‘Environmental Information Processing’,

‘Cellular Processes’ and ‘Organismal Systems’. The summary results indicate the overall trend of the pathways under a particular comparison.

KEGG pathways analysis

For KEGG pathways analysis, we selected top 10 pathways (Figure 3.8) from each comparison for the discussion purpose.

SM vs. OVE. Under this comparison, we observed that ‘Energy metabolism’, ‘Lipid Metabolism’ and related pathways were induced as compared to ‘Metabolism of other Amino Acids’, ‘Carbohydrate’ and related pathways. These include ‘Cyanoamino acid metabolism’, ‘Taurine and hupotaurine metabolism’, ‘Glycoshphingolipid synthesis – ganglio series’, ‘Inositol phosphate metabolism’, and ‘Arachidonic acid metabolism’ that were deactivated in cows supplemented with SM group compared to OVE. Among these, taurine and hypotaurine are nonprotein sulfur-containing amino acids, and are considered as antioxidants (Aruoma et al., 1988). They become active under liver injury and act as hepatoprotective molecules (Acharya and Lau-Cam, 2010). Glycosphingolipids are types of glycolipids that are made up of ceramide backbone which is covalently attached to a glycan moiety (D'Angelo et al., 2013). The glycoshphingolipids (ganglio series) play an important role in membrane-protein modulation and cell–cell communication during cellular development processes. The inhibition of these pathways suggest normal functioning of the liver. In contrast, ‘Nitrogen metabolism’, and ‘Synthesis and degradation of ketone bodies’ were among the activated pathways. In cows fed with moderate energy diet with methionine supplementation, the nitrogen metabolism was induced overall for energy synthesis. It is used in two different ways. First, it is absorbed in the

form of ammonia, and then converted into urea by the liver. Second, it is utilized as glucose precursor for gluconeogenesis (Reynolds, 1992). The importance of this pathway is also shown during feed restriction in high yielding dairy cows. The restricted energy diet lead to poor nitrogen utilization and insufficient amino acid uptake (Eriksson, 2010). It has been reported that ketone bodies inhibit protein degradation and glucose synthesis and utilization in tissues (Holtenius and Holtenius, 1996). The activation of 'synthesis and degradation of ketone bodies' suggests a mechanism of energy shift from glucose and amino acids to fatty acids utilization for energy requirements. This mechanism is also supported by our metabolomics results, as we found several metabolites from carbohydrate and amino acid metabolisms that were down regulated.

MS vs. OVE. MetaSmart supplementation induced 'Digestive secretion', 'Cell Growth and Death' and related pathways. Whereas, 'Replication and Repair', 'Metabolism of Other Amino Acids', and related pathways were inhibited in MS vs. OVE. Among the activated pathways, 'Riboflavin metabolism', 'Bile Secretion', 'Salivary secretion', and 'Vitamin digestion and secretion' are included. The riboflavin (vitamin B2) and other vitamin digestion related pathways are essential for many metabolic functions such as fatty acid metabolism, amino acid metabolism, citrate cycle, and electron transport chain (Powers et al., 2012). The riboflavin deficiency may lead to metabolic disorders, immune dysfunction and abnormal development (Thakur et al., 2016). The bile secretion of both primary bile acids and secondary bile acids was stimulated due to MS supplementation as indicated by metabolomics results (Figure 3.5). Among the inhibited KEGG pathways, 'Base excision repair', 'Cyanoamino acid metabolism', and 'One carbon pool by folate' are included. The 'base excision repair' pathway was inhibited in cows supplemented with MS, in spite of the activation of cell growth related pathways. However, the

‘Nucleotide excision repair’ (results not shown) was activated in the same group. The one carbon pool constitutes folate and methionine metabolisms (Locasale, 2013). This pathway provides an important source of methyl donor for DNA methylation. The interruption in this pathway may lead to abnormal cell progression and growth (Xu and Chen, 2009). The inhibition of this pathway in our results may also suggest a source of methyl donor that might be through methionine metabolism due to the availability of sufficient methionine source MS supplementation.

SM vs. MS. When comparing the two sources of methionine supplementations, we found that pathways related to ‘Immune system’, ‘Development’, ‘Metabolism of Cofactor and vitamins’, ‘Signal Transduction’, ‘Signaling molecules and interaction’ were induced in SM group, whereas the pathways related to ‘Glycan biosynthesis and metabolism’, and ‘Digestive System’ (Bile secretion) were induced in MS group. Among the immune system, ‘Intestinal immune network for IgA production’ and ‘Antigen processing and presentation’ were activated. The first pathway is helpful in maintaining host immunity by producing IgA from B cells against foreign pathogens (Ko and Chang, 2015). The later pathway involves T-cell activation for antigen recognition, ubiquitylation and ultimately degradation of external pathogens (Vyas et al., 2008). Among the pathways induced in MS group, bile secretion is of prior importance in our study which has already been discussed earlier.

Networks of biochemical compounds and transcription regulators

The network analysis of biochemical compounds is shown in Figures 3.9-3.11 and of transcription regulators is shown in Figure 3.12. We found most of the biochemical compounds

associated with cellular functions in SM vs. OVE. These include uracil, inosine, and 5'-CMP (Cytidine 5'-monophosphate), which are involved in replication, proliferation and growth processes (Figure 3.9). The elevated level of uracil is associated with damaged cells as shown in human plasma cells, and serves as a milk biomarker in lactating dairy cows (Bi et al., 2000, Melzer et al., 2013). The activation of inosine is involved in nucleic acid synthesis, gene expression, signaling and ultimately lead to cell proliferation and differentiation (Dzidic et al., 2006). The 5'-CMP along with other nucleic acid metabolites has been identified as a by-product in milk samples of dairy cows (Tiemeyer et al., 1984). It has also been shown to be involved in DNA synthesis in the bovine mammary gland (Sheffield, 1987). The up regulation of 5'-CMP in the liver is involved in DNA synthesis and hence indicate cellular proliferation mechanism. The network analysis revealed 6 transcription regulators, of which 5 (BCOR, GMNN, USF1, ID3 and KLF5) were up regulated while one (PPRC1) was down regulated (Figure 3.12A). Among these, KLF5 (Kruppel-like factor 5) is involved in both promoting and suppressing cellular proliferation. In bovine adipose tissue, it has been shown as a potent regulator of lipogenic/adipogenic transcription activity (Schmitt et al., 2011).

In MS vs. OVE, we have found the involvement of the biochemical compounds in carbohydrates, lipids, and several cellular related functions. In carbohydrate and lipid metabolism, we discovered chenodeoxycholic acid and NADH as up regulated ones (Figure 3.10A). Chenodeoxycholic acid is synthesized from cholesterol and is a part of primary bile acid (Russell, 2003). It is usually conjugated with either glycine or taurine (Tsai et al., 2011). The bile acids usually facilitate lipid digestion by making micelles in the liver. The lactic acid and phosphoenolpyruvate were down regulated in our analysis. The Figure 3.10A shows that deactivation of these compounds is involved in activation of 'release of glycerol' molecule. This

mechanism suggests the synthesis of glucose from glycerol as a substrate instead of lactic acid or phosphoenolpyruvate. These compounds in addition to indican and nicotinamide-beta-riboside were also involved in cellular functions (Figure 3.10B). Their main functions include activation and suppression of proliferation and apoptosis. The network analysis of DEG, revealed three transcription regulators (Figure 3.12B). These include TBX5, EPAS1 and FOXC2, all of which were downregulated. The down regulation of these genes along with other transcriptomics and metabolomics results suggest an inhibition of replication, repair and growth related mechanisms.

The comparison of SM vs. MS shows upregulation of arachidonic acid, linoleic acid, docosahexaenoic acid and phosphoenolpyruvate and downregulation of NADH and arginine in the carbohydrate and lipid metabolism (Figure 3.11A). The biochemical compounds linoleic acid, arachidonic acid, docosahexaenoic acid and guanosine were up regulated, while arginine, chenodeoxycholic acid, glycolic acid, indican and taurochenodeoxycholate were down regulated within cellular function category (Figure 3.11B). The arachidonic acid, linoleic acid, and docosahexaenoic acid are considered as essential fatty acids, and are important in growth, development and several other cellular functions. In rodents, it has been shown that sufficient supply of arachidonic acid and docosahexaenoic acid prevents hepatic steatosis (Le et al., 2012). Guanosine is involved in activation of cellular functions such as proliferation and inhibition of necrosis, apoptosis and cell death as shown in the figure. We have found four transcription regulators (GLI2, FOXJ1, KDM5B and SMAD4) among the DEG in SM vs. MS (Figure 3.12C). All of these transcription regulators were up regulated. Among these, GLI2 (Glioblastoma 2) is involved in regulation of hedgehog signaling (Ochoa et al., 2010), whereas SMAD4 is involved in wnt signaling and posttranslational modifications (Wilkinson et al., 2008).

Integration of metabolome and transcriptome datasets

The data integration of transcription regulators and biochemical compounds indicates direct and indirect links between genes and metabolites. Figure 3.13 (A-C) shows the networks of biochemical compounds (filled in colors, red=up regulated and green=down regulated) and the DEG (encircled, red=up regulated and green=down regulated). The compounds and genes that are not colored are the predicted ones and serves as the main connecting links between them. The data integration summarizes the results from the networks of metabolome and transcriptome.

Conclusion

Supplementation of rumen-protected methionine elicits modest but distinct alterations in the liver at both transcriptome and metabolome levels. Some of these unique alterations might have averted the detrimental effects of energy overfeeding prepartum on susceptibility to ketosis. Compared with healthy OVE cows, SM supplementation has important effects on regulating liver regeneration and metabolism, environmental stimuli, synthesis and degradation of ketone bodies, and nitrogen metabolism. Supplementation with MS has important effects on gene expression, glucose and lipid synthesis, and primary and secondary bile acids metabolism. The results from DIA and IPA core analysis highlighted the role of several pathways and metabolites that are involved in carbohydrate metabolism, lipid metabolism, cell signaling, growth, and proliferation. The networks underscore the linkages among different metabolites and their downstream functions that are closely related with carbohydrate, lipid, and cellular functions. Integration analysis of transcription regulators and metabolites revealed alterations in protein synthesis, apoptosis, cell growth, cell proliferation, glucose metabolism and lipid metabolism.

Figures and Tables

Table 3.1: A list of differentially expressed genes with $p \leq 0.01$ and fold change (FC) $\geq |3|$ in SM vs. OVE group. The table is sorted descending order based on the FC values.

Symbol	Description	SM vs. OVE
GBP6	guanylate binding protein family, member 6	17.58
LOC100126815	MHC class I-like family A1	6.72
ULBP3	UL16 binding protein 3	6.06
ANO3	anoctamin 3	5.35
STK32C	Serine/threonine-protein kinase 32C	5.21
SKP2	S-phase kinase-associated protein 2 (p45)	5.06
LOC522938	Uncharacterized protein	4.72
CXCL9	chemokine (C-X-C motif) ligand 9	4.65
PNMA2	paraneoplastic antigen MA2	4.59
ADARB2	adenosine deaminase, RNA-specific, B2	4.53
CACNA1G	calcium channel, voltage-dependent, T type, alpha 1G subunit	4.45
ABL1	c-abl oncogene 1, non-receptor tyrosine kinase	4.33
NEB	Nebulin - Oryctolagus cuniculus	4.25
NTS	Neurotensin	4.21
GPX3	glutathione peroxidase 3 (plasma)	4.11
PARD3B	par-3 partitioning defective 3 homolog B	4.09
PABPC5	poly(A) binding protein, cytoplasmic 5	3.94
SGOL2	shugoshin-like 2 (S. pombe)	3.83
FBXW7	F-box and WD repeat domain containing 7	3.81
GMEB1	glucocorticoid modulatory element binding protein 1	3.74
LCORL	ligand dependent nuclear receptor corepressor-like	3.72

Table 3.1 (Cont.)

IRX4	iroquois homeobox 4	3.68
LOC507049	T-cell receptor beta chain V region	3.67
DDIT4L	DNA-damage-inducible transcript 4-like	3.61
CNTNAP2	contactin associated protein-like 2	3.54
ULBP27	UL16-binding protein 27	3.50
CATSPER4	cation channel, sperm associated 4	3.50
LOC509124	olfactory receptor 9G4	3.40
KIR3DL1	killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1	3.34
EDN2	endothelin 2	3.16
SLC22A2	solute carrier family 22 (organic cation transporter)	3.09
GPX3	glutathione peroxidase 3 (plasma)	3.07
IZUMO1	izumo sperm-egg fusion 1	3.01
DAGLA	diacylglycerol lipase, alpha	-3.01
NCAPD3	non-SMC condensin II complex, subunit D3	-3.01
LOC789869	Zinc finger and BTB domain-containing protein 8	-3.13
ADAR	adenosine deaminase, RNA-specific	-3.16
SBSN	suprabasin	-3.21
LOC783655	olfactory receptor 5AC1-like	-3.22
HYAL3	hyaluronidase 3 mRNA	-3.23
PDZRN3	PDZ domain containing ring finger 3	-3.27
DYRK3	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 3	-3.31
ENTPD6	ectonucleoside triphosphate diphosphohydrolase 6 (putative)	-3.36
KLHL12	kelch-like 12 (Drosophila)	-3.38
TGM3	transglutaminase 3 (E polypeptide, protein-glutamine-gamma-glutamyltransferase)	-3.43
DDX54	DEAD (Asp-Glu-Ala-Asp) box polypeptide 54	-3.49

Table 3.1 (Cont.)

GGT7	gamma-glutamyltransferase 7	-3.51
SEMA4A	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4A	-3.51
OR5AS1	olfactory receptor, family 5, subfamily AS, member 1	-3.68
SOX7	SRY (sex determining region Y)-box 7	-3.68
LOC505465	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein	-3.70
VSTM5	V-set and transmembrane domain containing 5	-3.77
TINAG	tubulointerstitial nephritis antigen	-3.79
SLC26A3	solute carrier family 26, member 3	-3.84
LOC526294	olfactory receptor-like protein DTMT	-3.89
TMPRSS13	transmembrane protease, serine 13	-3.89
RBM14	RNA binding motif protein 14	-4.14
HIST1H2AA	histone cluster 1, H2aa	-4.16
HOXC11	homeobox C11	-4.18
FAM20B	family with sequence similarity 20, member B	-4.22
MRPL42	mitochondrial ribosomal protein L42 (MRPL42), nuclear gene encoding mitochondrial protein	-4.40
C29H11orf84	chromosome 29 open reading frame, human C11orf84	-4.48
CCDC36	coiled-coil domain containing 36	-4.65
RGS3	regulator of G-protein signaling 3	-4.68
ALK	Tyrosine-protein kinase receptor	-4.72
SHCBP1L	SHC SH2-domain binding protein 1-like	-5.10

Table 3.2: A list of differentially expressed genes with $p \leq 0.01$ and fold change (FC) $\geq |3|$ in MS vs. OVE group. The table is sorted in descending order based on the FC values.

Symbol	Description	MS vs. OVE
CCNE2	cyclin E2	6.55
NCKAP5L	NCK-associated protein 5-like	5.58
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	4.91
PBK	PDZ binding kinase	4.67
PAG2	pregnancy-associated glycoprotein 2	4.64
OR1K1	olfactory receptor, family 1, subfamily K, member 1	4.28
ADCY2	adenylate cyclase 2	4.22
ADCY7	adenylate cyclase 7	4.16
ACE3	Uncharacterized protein	3.78
POLR3G	polymerase (RNA) III (DNA directed) polypeptide G (32kD)	3.76
MAGED4B	melanoma antigen family D, 4B	3.71
GPR133	G protein-coupled receptor 133	3.69
GPX8	glutathione peroxidase 8	3.65
HMMR	hyaluronan-mediated motility receptor (RHAMM)	3.42
LRAT	lecithin retinol acyltransferase (phosphatidylcholine--retinol O-acyltransferase)	3.39
GLRA3	glycine receptor, alpha 3	3.29
LOC541022	Uncharacterized protein	3.28
KRT25	keratin 25	3.27
STXBP5L	syntaxin binding protein 5-like	3.23
LOC617417	Uncharacterized protein	3.20
GLIPR2	GLI pathogenesis-related 2	3.17
BUB1B	budding uninhibited by benzimidazoles 1 homolog beta	3.09
LOC100848433	uncharacterized LOC100848433	3.03
IL23R	interleukin 23 receptor	3.02
MAML3	mastermind-like 3	-3.07
RAB20	RAB20, member RAS oncogene family	-3.11
RHBDD2	rhomboid domain containing 2	-3.19
NRXN1	neurexin 1	-3.21
CCDC116	coiled-coil domain containing 116	-3.26
ZBED2	zinc finger, BED-type containing 2	-3.42
LOC789869	Zinc finger and BTB domain-containing protein 8B-like	-3.46
GTPBP4	GTP binding protein 4	-3.56
GON4L	gon-4-like	-3.64
ZNF446	zinc finger protein 446	-3.67

Table 3.2 (Cont.)

LOC515619	olfactory receptor Olr149	-3.73
LHFPL5	lipoma HMGIC fusion partner-like 5	-3.73
MYOF	myoferlin	-3.77
TRIM67	tripartite motif containing 67	-3.79
EVX1	even-skipped homeobox 1	-3.97
ZNF35	zinc finger protein 35	-4.08
RGS17	regulator of G-protein signaling 17	-4.27
ZBTB32	zinc finger and BTB domain containing 32	-4.31
ALK	Tyrosine-protein kinase receptor	-4.38
CSRNP1	cysteine-serine-rich nuclear protein 1	-4.40
TMEM246	transmembrane protein 246	-4.49
SLC39A4	solute carrier family 39 (zinc transporter), member 4	-4.74
PARP4	poly (ADP-ribose) polymerase family, member 4	-4.77
FAM102B	family with sequence similarity 102, member B	-4.89
KLK6	kallikrein-related peptidase 6	-5.00
NCAM1	neural cell adhesion molecule 1	-5.06
ABL2	v-abl Abelson murine leukemia viral oncogene homolog 2	-5.07
MAU2	MAU2 chromatid cohesion factor homolog	-5.45
FAM116B	family with sequence similarity 116, member B	-5.48
LOC528412	multidrug resistance-associated protein	-5.54

Table 3.3: A list of differentially expressed genes with $p \leq 0.01$ and fold change (FC) $\geq |3|$ in SM vs. MS group. The table is sorted in descending order based on the FC values.

Symbol	Description	SM vs. MS
ACTL6A	actin-like 6A	4.56
CATSPER4	cation channel, sperm associated 4	4.56
EVX1	even-skipped homeobox 1	4.51
CACNA1G	calcium channel, voltage-dependent, T type, alpha 1G subunit	4.45
TFAP2C	transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)	4.36
HAVCR2	hepatitis A virus cellular receptor 2	4.32
SKP2	S-phase kinase-associated protein 2 (p45)	4.31
CSRNP1	cysteine-serine-rich nuclear protein 1	4.13
RNF112	ring finger protein 112	4.13
AADAC	arylacetamide deacetylase (esterase)	4.08
IRX4	iroquois homeobox 4	4.07
CYTIP	cytohesin 1 interacting protein	4.05
STK32C	Rep: Serine/threonine-protein kinase 32C	4.05
UVRAG	UV radiation resistance associated gene	3.90
RANBP17	RAN binding protein 17	3.87
NTS	neurotensin	3.86
ZNF446	zinc finger protein 446	3.79
SLC8A1	solute carrier family 8 (sodium/calcium exchanger), member 1	3.76
NOTCH2	notch 2	3.63
AARS	alanyl-tRNA synthetase	3.61
SATB2	SATB homeobox 2	3.54
IFITM5	interferon induced transmembrane protein 5	3.52
ZBTB32	zinc finger and BTB domain containing 32	3.51
TNNT3	Troponin T, fast skeletal muscle	3.50
MLKL	mixed lineage kinase domain-like	3.49
CORT	cortistatin	3.48
CLCN6	chloride channel 6	3.47
GPM6A	glycoprotein M6A	3.46
CD86	CD86 molecule	3.46
NLGN2	neuroligin 2	3.40
WDR52	WD repeat domain 52	3.39
AKAP10	A kinase (PRKA) anchor protein 10, nuclear gene encoding mitochondrial protein	3.33
GIT2	G protein-coupled receptor kinase interacting ArfGAP 2	3.31

Table 3.3 (Cont.)

KIR3DL1	killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1	3.31
ADM	adrenomedullin	3.26
LOC509124	olfactory receptor 9G4	3.26
NEB	Rep: Nebulin - Oryctolagus cuniculus	3.26
EIF5A2	eukaryotic translation initiation factor 5A2	3.18
KDM5B	lysine (K)-specific demethylase 5B	3.17
TNFAIP8L3	tumor necrosis factor, alpha-induced protein 8-like 3	-3.02
FTSJD1	FtsJ methyltransferase domain containing 1	-3.13
GLIPR1L1	GLI pathogenesis-related 1 like 1	-3.27
KIF18A	kinesin family member 18A	-3.28
DBX1	developing brain homeobox 1	-3.42
ITGB4	integrin, beta 4	-3.42
R3HDM2	R3H domain containing 2	-3.44
NCKAP5L	NCK-associated protein 5-like	-3.46
FAM83H	family with sequence similarity 83, member H	-3.46
ICA1	islet cell autoantigen 1, 69kDa	-3.48
RGS3	regulator of G-protein signaling 3	-3.51
VSTM5	V-set and transmembrane domain containing 5	-3.51
PPP1R42	protein phosphatase 1, regulatory subunit 42	-3.55
LAMB2	laminin, beta 2 (laminin S)	-3.55
INTU	inturned planar cell polarity effector homolog (Drosophila)	-3.63
CEP112	coiled-coil domain containing 46	-3.67
LOC751563	prolactin-related protein 12	-3.72
PLEKHA8	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 8	-3.76
ANKS3	ankyrin repeat and sterile alpha motif domain containing 3	-3.81
ECT2	epithelial cell transforming sequence 2 oncogene	-3.83
ZMAT1	zinc finger, matrin-type 1	-3.83
MRPL42	mitochondrial ribosomal protein L42, nuclear gene encoding mitochondrial protein	-3.89
HYAL3	hyaluronidase 3 mRNA, partial cds.	-4.03
WDR69	WD repeat domain 69	-4.04
PSPH	phosphoserine phosphatase	-4.10
ZSWIM4	zinc finger, SWIM-type containing 4	-4.12
GLIPR2	GLI pathogenesis-related 2	-4.16
ZNF619	zinc finger protein 619	-4.59
LSP1	lymphocyte-specific protein 1	-4.72
PBK	PDZ binding kinase	-5.39

Table 3.3 (Cont.)

DDX54	DEAD (Asp-Glu-Ala-Asp) box polypeptide 54	-5.62
CDKN3	cyclin-dependent kinase inhibitor 3	-5.65
H2B	Histone H2B type 1	-5.96

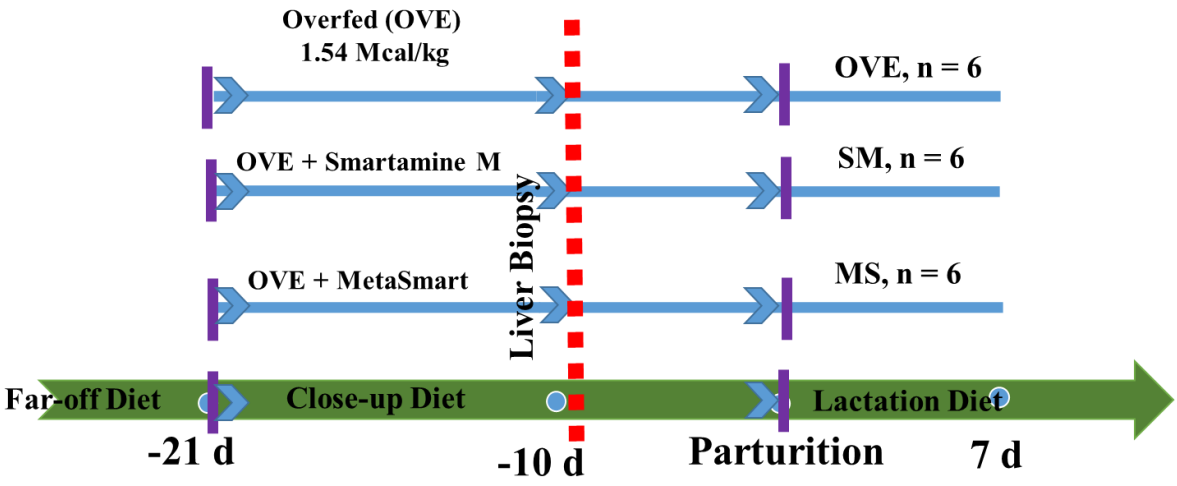


Figure 3.1: Experimental design. The tissue biopsies were taken at -10 d relative to parturition across the three groups: overfed with moderate energy diet (OVE), OVE plus Smartamine M (SM) and OVE plus MetaSmart (MS) supplements.

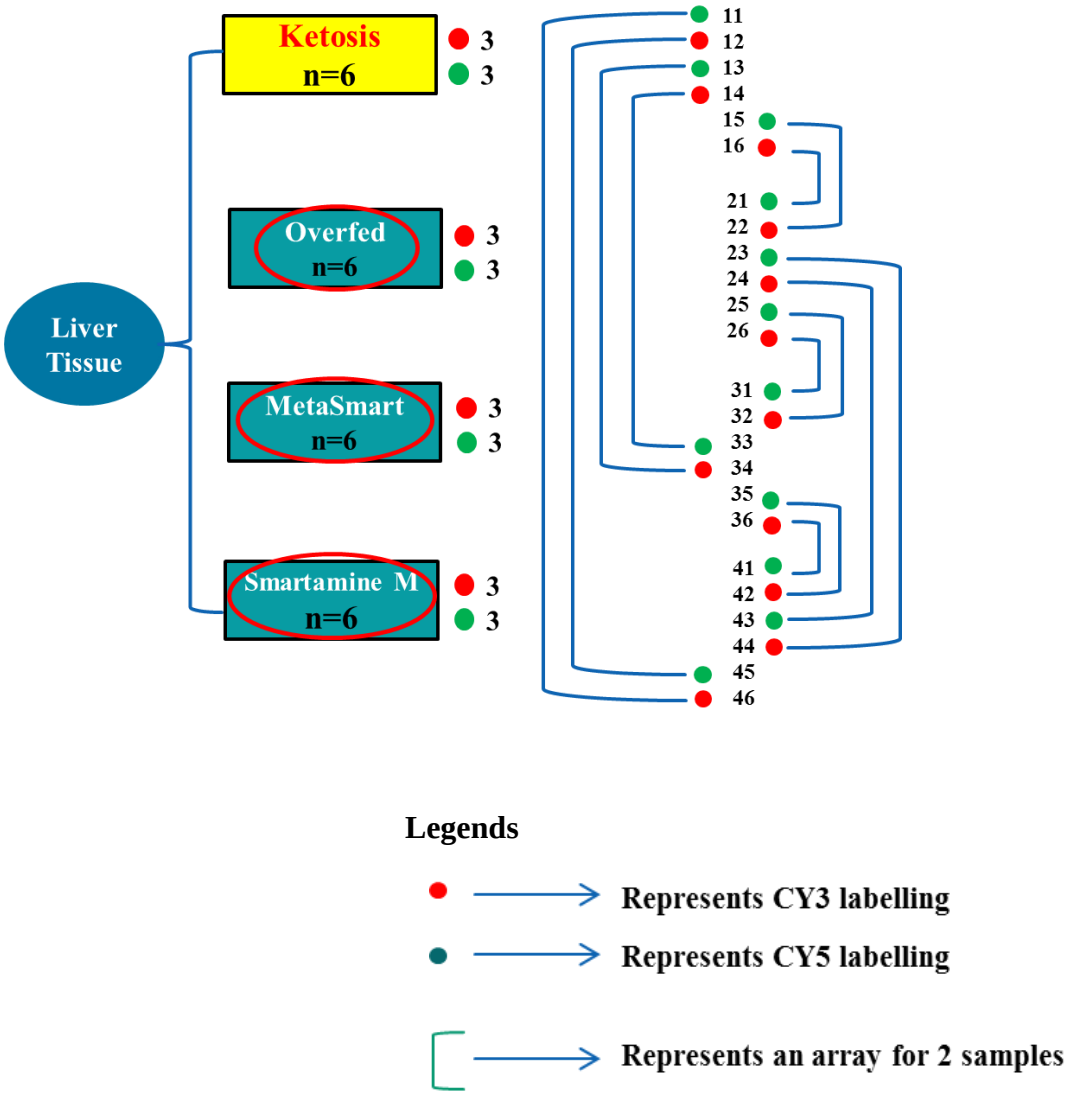


Figure 3.2: The complete microarray's hybridization design is shown in the figure. A two color (red and green) hybridization plan was used. The Overfed with moderate energy diet (OVE), Smartamine M (SM) and MetaSmart (MS) groups encircled with red are used in the current chapter.

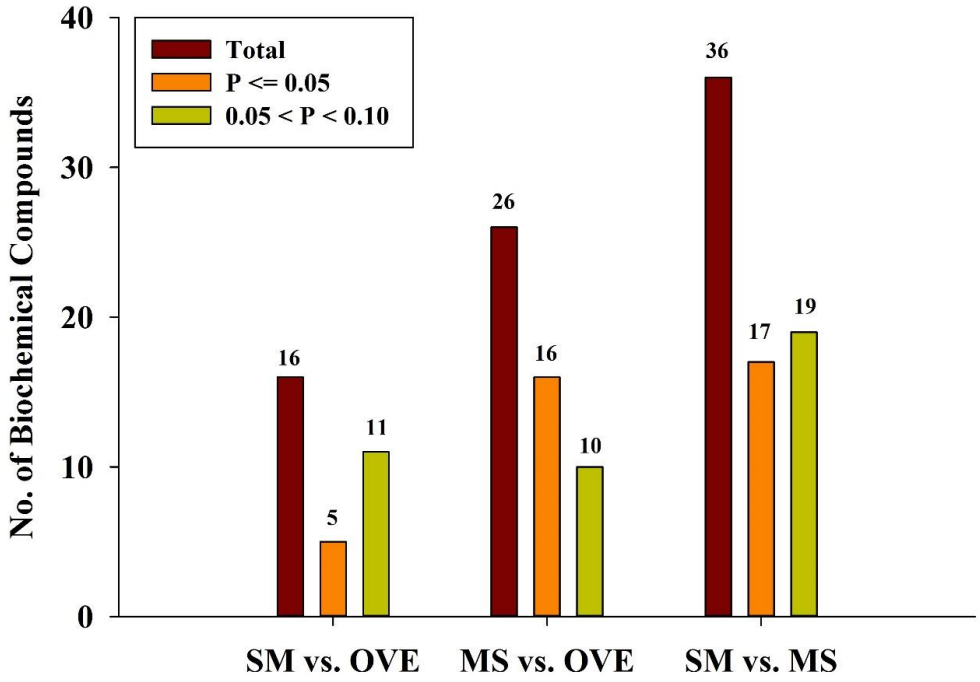


Figure 3.3: No. of significantly affected metabolites with $p \leq 0.05$, between 0.05 to 0.10 and total $p \leq 0.10$ for the respective comparisons.

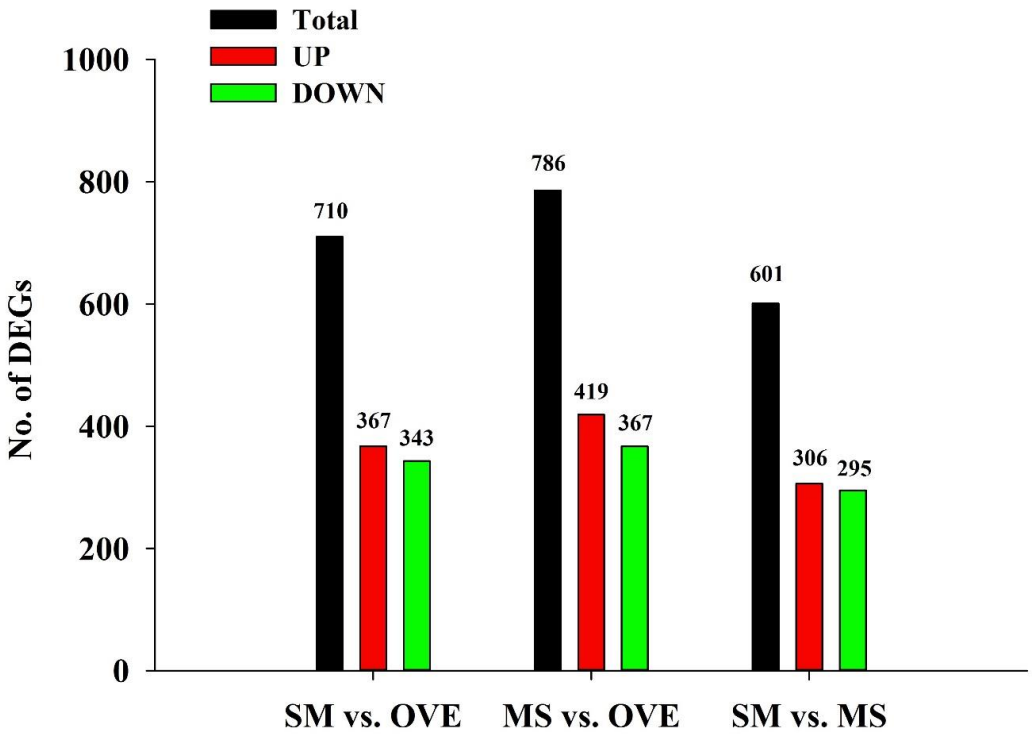


Figure 3.4: No. of differentially expressed genes (DEG) with $p \leq 0.05$ and FC $\geq |1.5|$ are shown by vertical bars across the three comparisons SM vs. OVE, MS vs. OVE and SM vs. MS. The y-axis represents the number of DEG, whereas x-axis represents the comparisons.

Biochemical Name	Sub Pathway	SM vs. OVE	
		p -value	FC
leucylaspartate	Dipeptide	0.01	0.67
glycodeoxycholate	Secondary Bile Acid Metabolism	0.01	0.60
gamma-glutamylglycine	Gamma-glutamyl Amino Acid	0.02	1.28
glycylisoleucine	Dipeptide	0.03	0.67
inosine	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.04	1.26
		MS vs. OVE	
glycylisoleucine	Dipeptide	0.00	0.54
glycerate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.01	0.76
leucylaspartate	Dipeptide	0.01	0.69
phosphoenolpyruvate (PEP)	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.01	0.68
2-linoleoylglycerophosphocholine*	Lysolipid	0.01	1.47
tyrosylglycine	Dipeptide	0.02	0.75
xylonate	Pentose Metabolism	0.02	1.65
chenodeoxycholate	Primary Bile Acid Metabolism	0.02	1.41
cyclic adenosine diphosphate-ribose	Purine Metabolism, Adenine containing	0.03	1.53
pro-hydroxy-pro	Urea cycle; Arginine and Proline Metabolism	0.03	0.80
N-palmitoyl-D-erythro-sphingosine	Sphingolipid Metabolism	0.03	0.77
3-phosphoglycerate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.03	0.77
glycylleucine	Dipeptide	0.04	0.78
nicotinamide riboside*	Nicotinate and Nicotinamide Metabolism	0.04	1.19
ribitol	Pentose Metabolism	0.04	1.41
taurolithocholate	Secondary Bile Acid Metabolism	0.04	1.36

Legends

***p* -value**	***p* < 0.05**	
FC	**FC<-1.0**	**FC ≥ 1.0**

Figure 3.5: Significantly affected biochemical compounds with $p < 0.05$ are shown for overfed cows with moderate energy diet (OVE), Smartamine M (SM) and MetaSmart (MS) comparisons. The column represented with FC (fold change) indicates up- and down-regulation, while the column represented with *p*-value indicates the respective significant values.

Biochemical Name	Sub Pathway	SM vs. MS	
		p -value	FC
taurolithocholate	Secondary Bile Acid Metabolism	0.00	0.62
xanthosine	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.01	0.81
3-indoxyl sulfate	Tryptophan Metabolism	0.01	0.77
chenodeoxycholate	Primary Bile Acid Metabolism	0.01	0.68
glycerate	Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	0.02	1.26
N-acetylglucosamine	Aminosugar Metabolism	0.02	1.61
gamma-glutamylglycine	Gamma-glutamyl Amino Acid	0.02	1.29
arginine	Urea cycle; Arginine and Proline Metabolism	0.02	0.74
stearidonate (18:4n3)	Polyunsaturated Fatty Acid (n3 and n6)	0.02	3.56
xylitol	Pentose Metabolism	0.03	0.74
13-HODE + 9-HODE	Fatty Acid, Monohydroxy	0.03	1.53
cyclic adenosine diphosphate-ribose	Purine Metabolism, Adenine containing	0.04	0.67
17,18-DiHETE	Eicosanoid	0.04	0.78
1-dihomo-linoleoylglycerophosphocholine (20:2n6)*	Lysolipid	0.04	0.47
10-heptadecenoate (17:1n7)	Long Chain Fatty Acid	0.05	1.77
docosahexaenoate (DHA; 22:6n3)	Polyunsaturated Fatty Acid (n3 and n6)	0.05	1.43
2-linoleoylglycerophosphoethanolamine*	Lysolipid	0.05	0.64

Legends

***p* -value**	***p* < 0.05**	
FC	**FC<-1.0**	**FC ≥ 1.0**

Figure 3.6: Significantly affected biochemical compounds with a $p < 0.05$ are shown for Smartamine M (SM) and MetaSmart (MS) comparison. The column represented with FC (fold change) indicates up and down regulation, while the column represented with *p*-value indicates the respective significant values.

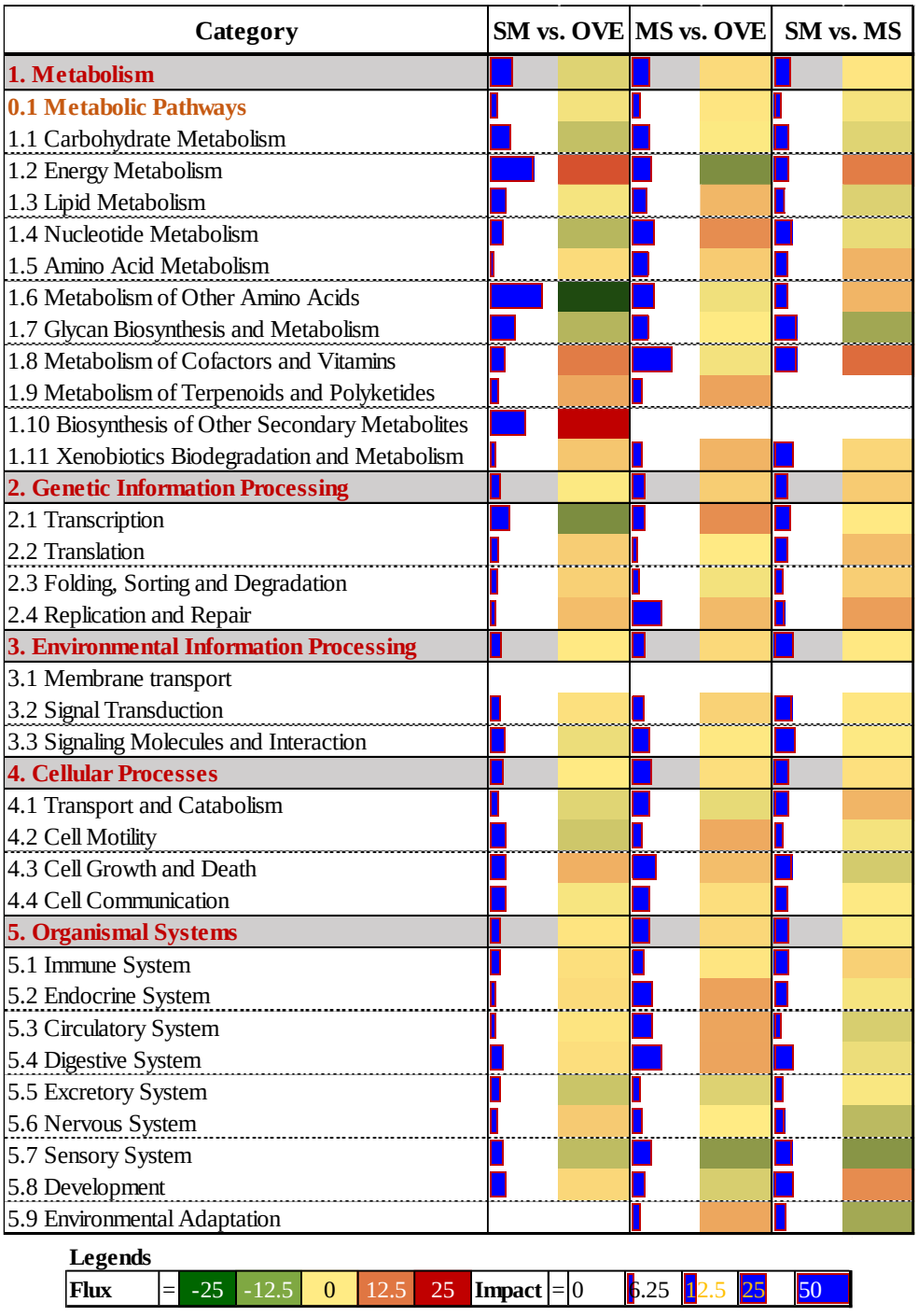


Figure 3.7: Summary of KEGG categories and subcategories for each comparison SM vs. OVE, MS vs OVE and SM vs. MS. The first column with blue colored horizontal bars under each comparison represents the impact values, whereas the second column represents the direction of the impact (flux) ranging from colors green (-25 to 0) to red (1 to +25).

Sub Category	Pathways	SM vs. OVE	
		Impact	Flux
1.6 Metabolism of Other Amino Acids	Cyanoamino acid metabolism		
1.6 Metabolism of Other Amino Acids	Taurine and hypotaurine metabolism		
1.2 Energy Metabolism	Nitrogen metabolism		
1.7 Glycan Biosynthesis and Metabolism	Glycosphingolipid biosynthesis - ganglio series		
1.7 Glycan Biosynthesis and Metabolism	Glycosaminoglycan biosynthesis - chondroitin sulfate		
1.1 Carbohydrate Metabolism	Inositol phosphate metabolism		
1.3 Lipid Metabolism	Synthesis and degradation of ketone bodies		
1.10 Biosynthesis of Other Secondary Metabolites	Caffeine metabolism		
1.3 Lipid Metabolism	Arachidonic acid metabolism		
1.6 Metabolism of Other Amino Acids	Selenoamino acid metabolism		
		MS vs. OVE	
		Impact	Flux
1.8 Metabolism of Cofactors and Vitamins	Riboflavin metabolism		
2.4 Replication and Repair	Base excision repair		
5.4 Digestive System	Bile secretion		
5.4 Digestive System	Salivary secretion		
1.6 Metabolism of Other Amino Acids	Cyanoamino acid metabolism		
5.4 Digestive System	Vitamin digestion and absorption		
1.8 Metabolism of Cofactors and Vitamins	One carbon pool by folate		
4.1 Transport and Catabolism	Regulation of autophagy		
4.3 Cell Growth and Death	Oocyte meiosis		
5.7 Sensory System	Taste transduction		
		SM vs. MS	
		Impact	Flux
1.7 Glycan Biosynthesis and Metabolism	Glycosaminoglycan degradation		
5.1 Immune System	Intestinal immune network for IgA production		
5.8 Development	Dorso-ventral axis formation		
5.1 Immune System	Antigen processing and presentation		
1.8 Metabolism of Cofactors and Vitamins	Riboflavin metabolism		
3.2 Signal Transduction	Notch signaling pathway		
3.3 Signaling Molecules and Interaction	Cell adhesion molecules (CAMs)		
3.2 Signal Transduction	Calcium signaling pathway		
5.4 Digestive System	Bile secretion		
1.7 Glycan Biosynthesis and Metabolism	Other glycan degradation		

Legends

Flux	=	-25	-12.5	0	12.5	25	Impact	=	0	6.25	12.5	25	50

Figure 3.8: The top 10 most impacted KEGG pathways along with their respective sub-categories are shown for the three comparisons SM vs. OVE, MS vs. OVE and SM vs. MS. The first column for each comparison represents the impact values, whereas the second column represents the direction of the impact (flux).

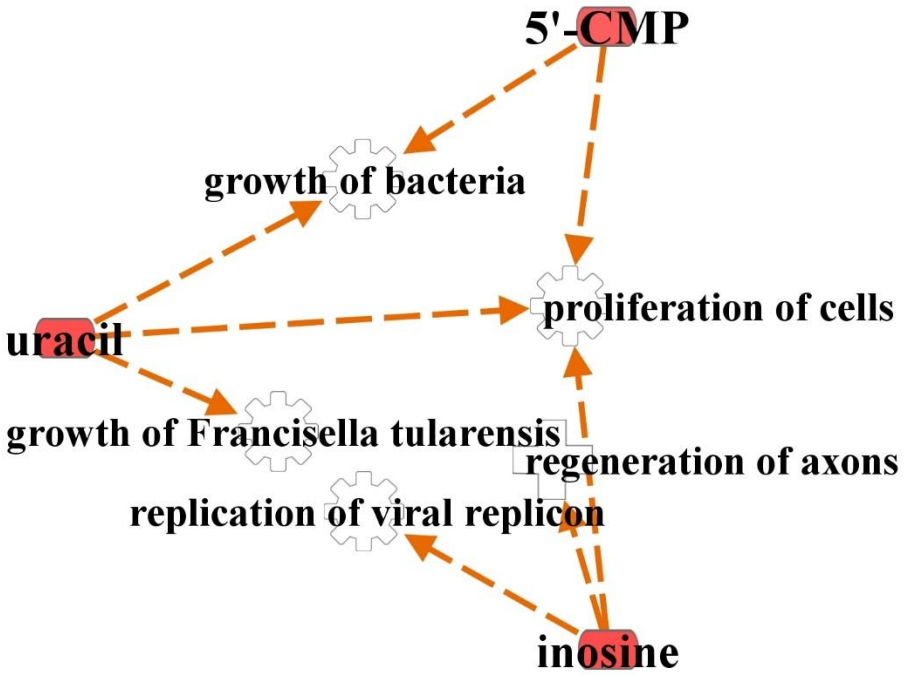
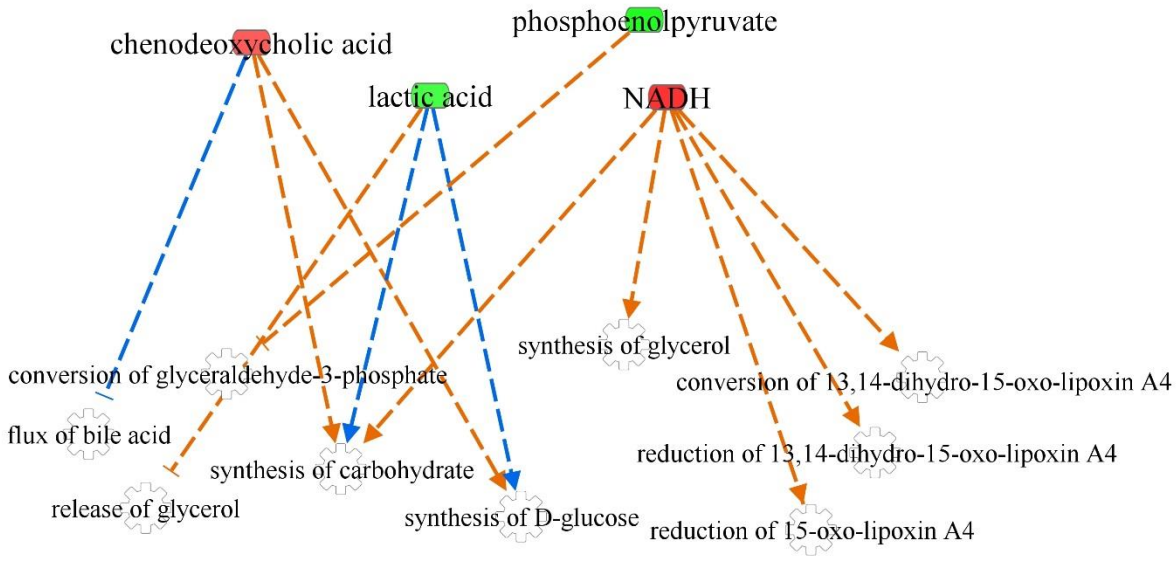
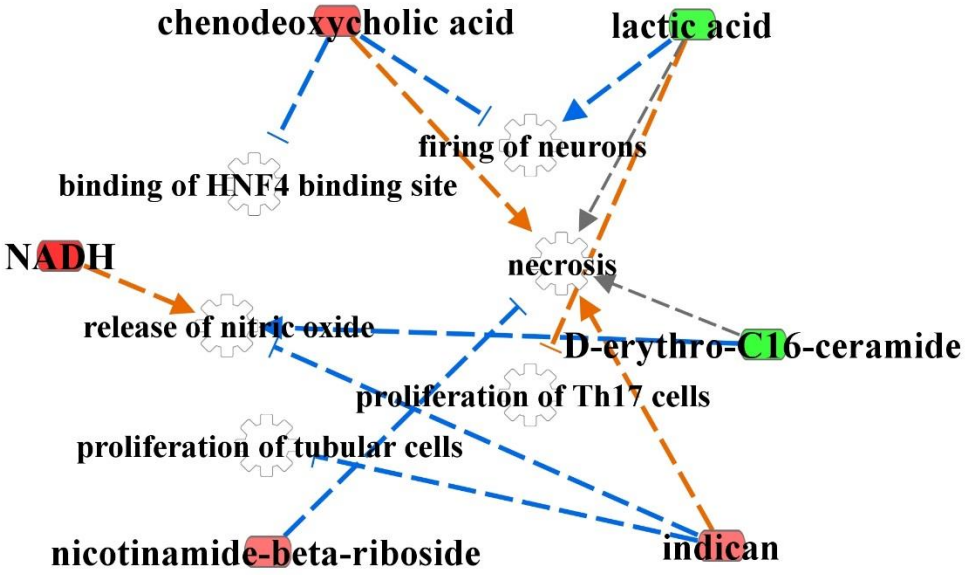


Figure 3.9: SM vs. OVE: The network of biochemical compounds and their cellular functions. The red color indicates the up regulation of the biochemical compounds.

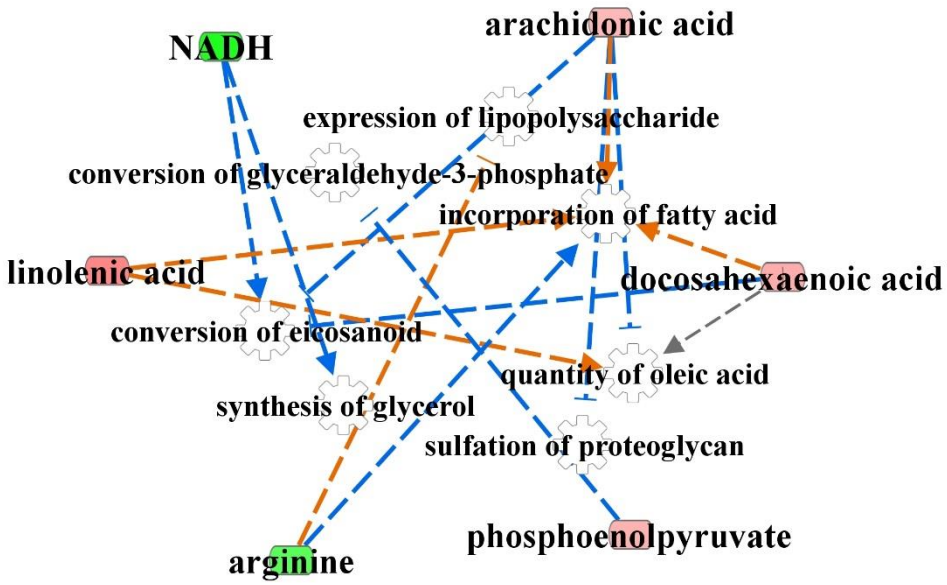


A). Carbohydrate and Lipid Metabolism

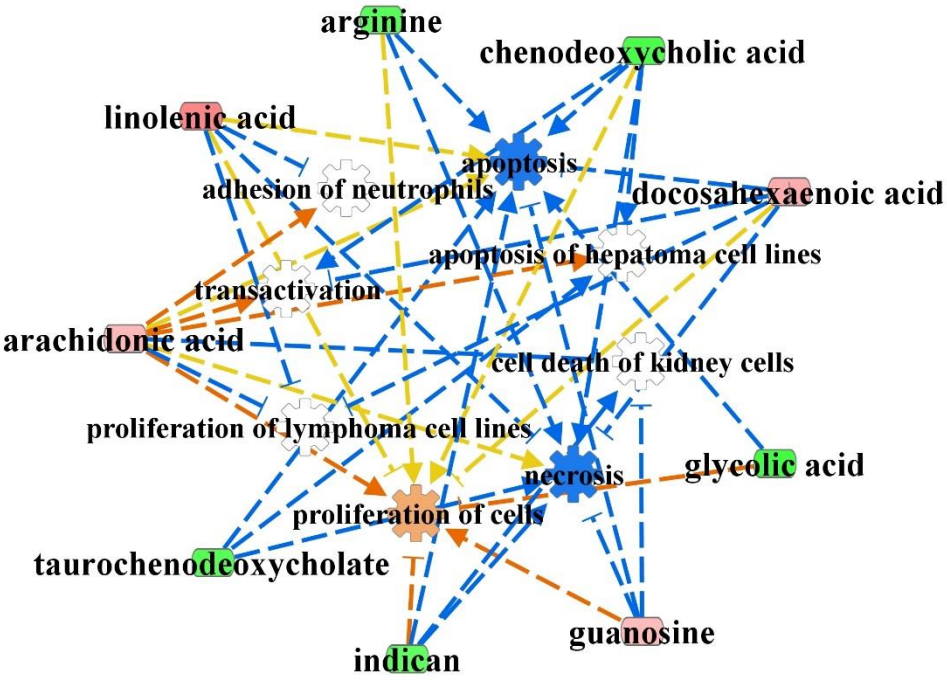


B). Cellular Functions

Figure 3.10: MS vs. OVE: (A) Carbohydrate and Lipid Metabolism, (B) Cellular Functions.



A). Carbohydrate and Lipid Metabolism



B). Cellular Functions

Figure 3.11: SM vs. MS: (A) Carbohydrate and Lipid Metabolism (B) Cellular Functions.

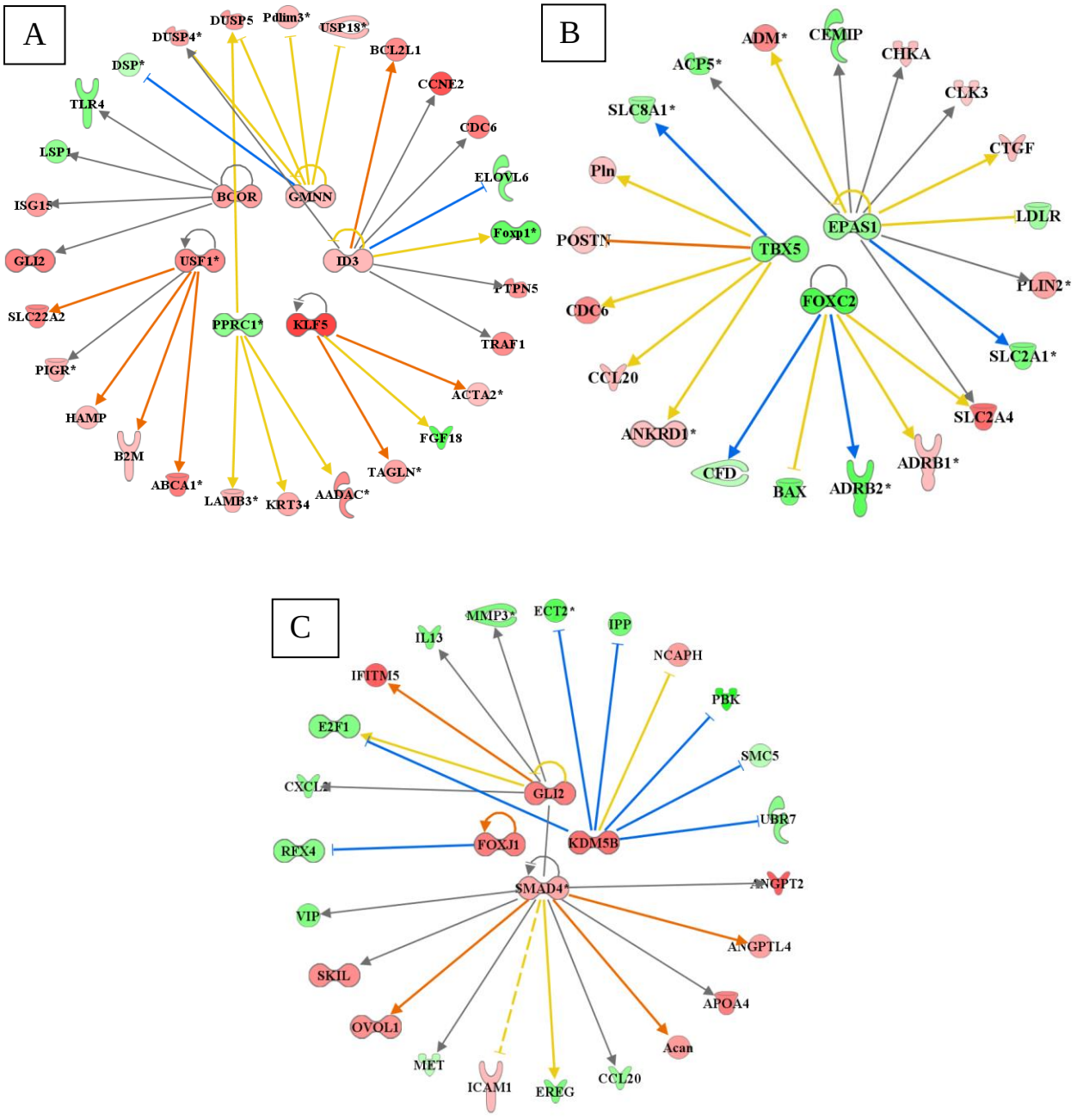


Figure 3.12: Transcription regulator networks: (A). SM vs. OVE, (B). MS vs. OVE, (C). SM vs. MS.

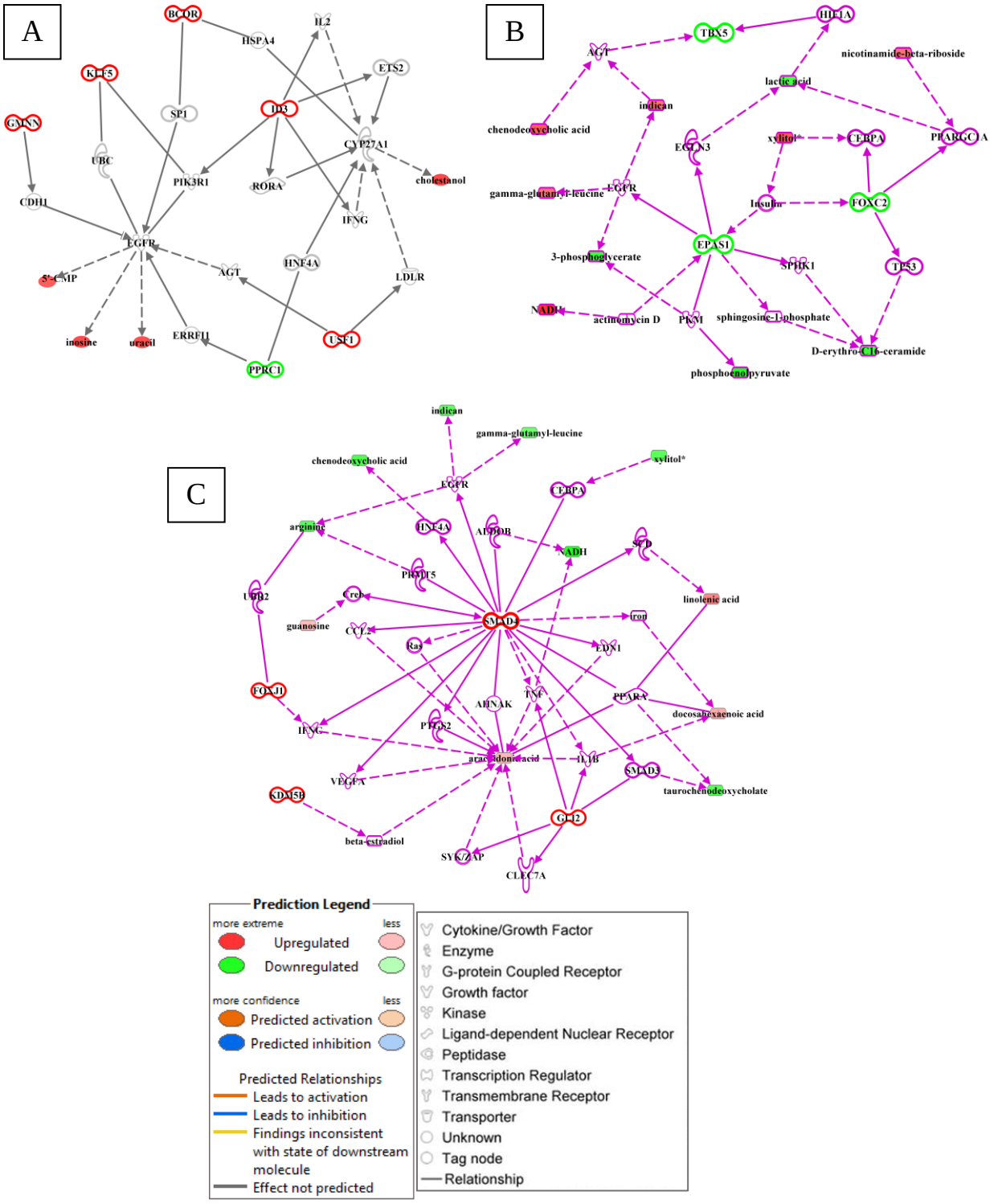


Figure 3.13: Data integration of transcription regulators and biochemical compounds: (A). SM vs. OVE, (B). MS vs. OVE, (C). SM vs. MS.

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CHAPTER # 4

A comparative analysis of metabolomics and transcriptomics from prepartal liver of cows developing ketosis postpartum and healthy cows supplemented with Smartamine M and MetaSmart during the transition period

Abstract

Cows overfed energy during the dry period are most-susceptible to developing ketosis postpartum. Supplementation with Smartamine M (SM) and MetaSmart (MS) during the transition period improves postpartal dry matter intake and resulted in fewer cases of clinical ketosis postpartum. Metabolomics (GC-MS, LC-MS; Metabolon Inc.) and transcriptomics (44K-whole-transcriptome microarray; Agilent) analyses were performed in liver tissue harvested at -10 d relative to parturition from cows that were healthy on 7 d postpartum or were diagnosed with clinical ketosis (K, n = 8). From -21 d to calving all cows consumed a higher-energy diet without (developed K) or with SM (n = 8) and MS (n = 8) (clinically healthy). From 313 identified biochemical compounds, metabolomics analysis ($P \leq 0.10$) revealed 34 or 33 affected in the comparison of K vs. SM or K vs. MS. Comparing profiles in K vs. SM revealed 13 compounds up-regulated and 21 down-regulated. Among the up-regulated compounds most belong to bile acid, fatty acid, branched-chain amino acid, and arginine and proline metabolism. Among the down-regulated compounds, there were several lysolipids and di-carboxylic acids along with components of pentose, purine, and sphingolipid metabolism. Citrate was markedly

lower in liver of K vs. SM. In the comparison of K vs. MS, 7 compounds were up-regulated and 26 were down-regulated. The up-regulated compounds are intermediates of glycolysis/gluconeogenesis/pyruvate, histidine, glycine/serine/threonine, and fatty acid metabolism. Among down-regulated compounds 7 were lysolipids but also citrate, squalene, several pentoses, and purines were affected. Analysis of transcriptomics data resulted in 834 or 1,261 differentially expressed genes (DEG, $P \leq 0.05$) in K vs. SM or K vs. MS. Bioinformatics analysis using the Dynamic Impact Approach (DIA) revealed a strong activation in K vs. MS of Notch, Hedgehog, and TGF-beta signaling pathways along with 'steroid biogenesis'. In contrast, 'synthesis and degradation of ketone bodies' was markedly inhibited. The pathway response in K vs. SM was less pronounced in part due to the fewer number of DEG. For example, the Hedgehog signaling pathway was highly-impacted but moderately activated; whereas, the 'renin-angiotensin system' was the most-impacted and markedly inhibited. Preliminary data analysis suggests that supplemental MS and SM elicit distinct metabolomics and transcriptomics responses in liver before calving. Cows developing K post-partum also had a distinct molecular phenotype compared with those supplemented with methionine. The functional relevance of these differences remains to be determined.

JAM Reference:

K. Shahzad, J. S. Osorio, D. N. Luchini and J. J. Loor. 2014 Journal of Dairy Science, 97(E-Suppl. 1): 713.

Introduction

Ketosis, a metabolic disorder during early lactation, if not taken into consideration before calving, may lead to metabolism related complicacies and huge financial loss in dairy industry. This metabolic disorder may lead to liver dysfunction, reduced immunity, and decreased reproductive performance (Li et al., 2012, Shin et al., 2015). Proper nutritional managements during the dry period may help cows to prevent the spread of metabolic disorders (Gerloff, 2000). A substantial research has been conducted in this regard to avoid ketosis development postpartum in periparturient dairy cows (Grummer, 1995, Drackley, 1999, Loor et al., 2007a). Supplementing diet with limiting amino acids such as lysine (Lys) or methionine (Met) to fine-tune the Lys : Met ratio or rumen-protected choline have been proved as a powerful tool to avoid ketosis development in high producing dairy cows (Lima et al., 2012, Osorio et al., 2013, Jacometo et al., 2017).

A common objective of the previous studies dealing with methionine supplementation was to obtain the optimal level of methionine in the metabolizable protein (MP) (Armentano et al., 1997, Rulquin and Delaby, 1997) and to alleviate the burden of non-esterified fatty acids (NEFA) in the liver (Martinov et al., 2010), and consequently to increase the milk yield and dry matter intake (DMI). In this perspective, Addisseo Company (Adisseo Inc. Antony, France) has developed commercial forms of methionine supplementations, such as Smartamine M (SM) and MetaSmart (MS). These commercial products have been employed by several studies e.g., (Ordway et al., 2009, Chen et al., 2011) and have an overall positive impact on dairy cow's health and performance. The metabolic profiling in cows fed with moderate energy diet without any supplementation and supplemented with SM and MS diets have been reported by (Osorio et

al., 2013) which indicates that methionine supplementation plays an important role in increasing the DMI and increased milk protein synthesis. In addition to this study, a detailed analysis of blood and tissue biomarkers revealed glutathione synthesis, reduction in proinflammatory signaling, increased very low-density lipoprotein (VLDL) secretion, and improved immune response (Osorio et al., 2014b). Gene expression profiling of targeted genes using quantitative PCR highlights the improvement in glutathione metabolism, inflammation, oxidative stress and epigenetics (Osorio et al., 2014a). In the study, it was also found that cows fed with high-energy diet during the dry period become more susceptible to ketosis. However, supplementation with SM and MS during the dry period improves postpartal health, increased DMI and fewer cases of clinical ketosis in dairy cows.

There is a still limited information available at omics level, linking the biological phenomena to the etiology of ketosis development. In the current study, we selected dairy different groups of cows fed with moderate energy diet and supplemented with SM and MS to provide sufficient amount of methionine in the MP. We took liver tissue biopsies at -10 d before calving and performed the comparative analysis of transcriptome and metabolome using microarray and mass spectrometry coupled with gas chromatography (GC-MS) or liquid chromatography (LC-MS) to unravel the underlying mechanism involved in the ketogenic process.

The objective of the study was to evaluate hepatic metabolome and transcriptome profiles in healthy cows supplemented with SM or MS versus cows fed with unsupplemented moderate-energy diet that developed ketosis (K) postpartum. We have used the microarray and metabolome profiling techniques and bioinformatics techniques to unravel the complex mechanism involved in ketosis development using retrospective approach.

Materials and Methods

Experimental design and dietary treatments

The Institutional Animal Care and Use Committee (IACUC) of the University of Illinois (Urbana) approved the procedure for this protocol (#09214). The experiment was conducted as a randomized complete block design as explained elsewhere (Osorio et al., 2013). All cows received the same far-off diet (1.24 Mcal/kg of DM; 14.3% CP) from −50 to −21 d before expected calving, a close-up diet (1.54 Mcal/kg of DM; 15.0% CP) from −21 d to calving, and fresh cow lactation diet from calving (1.75 Mcal/kg of DM; 17.5% CP) through 30 days in milk (DIM). Supplements of methionine were top-dressed from −21 to 30 DIM. For this study, we selected a subset of 18 cows, which are cows fed with moderate energy diet and developed ketosis (K, n=6), the cows that were fed moderate energy diet and supplemented with Smartamine M (SM, n=6), or MetaSmart (MS, n=6). The experimental design is shown in the Figure 4.1.

Liver biopsies and RNA extraction

Liver tissue samples were collected via puncture biopsy (Dann et al., 2006) from cows under local anesthesia at approximately 0730 hour once prepartum on d −10 (±3 d), and then postpartum on d 7 and 21. Tissue samples were stored in liquid nitrogen immediately and then at -80 C until RNA extraction. We used liver samples from –10 d prepartum for the current metabolome and transcriptome analysis. Total RNA was extracted from the liver samples using established protocol in our laboratory. Briefly, liver tissue sample was weighed (~55 milligram

on average) and straightway put inside a 2 ml centrifuge tube (Corning Inc. ®, Cat. No. 430052, Corning, NY, USA), with 1 ml of Qiazol reagent to proceed with RNA extraction. This extraction procedure also utilizes chloroform (Ambion® Cat. No. 9720, Austin, TX, USA), which removes residual DNA. Any residual genomic DNA was removed from RNA with DNase using miRNeasy Mini Kit columns (Qiagen, Hilden, Germany). RNA concentration was measured using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The purity of RNA (A260/A280) for all samples was above 2.0. The quality of RNA was evaluated using the Agilent Bioanalyzer system (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). The average RNA integrity number (RIN) value for all samples was around 6.9.

Metabolomics

Metabolon Company (Metabolon Inc. NC) performed Metabolomics analysis. The liver tissue samples were prepared according to the sample preparation guidelines. Briefly, the 500 milligram (mg) of the liver tissue per sample was weighed, packed in dry ice and then shipped to the company. The metabolomics analysis was performed using GC-MS and LC-MS. A total of 313 biochemical compounds (metabolites) were identified in this analysis. For the current study, we used SM, MS and K groups for the respective comparisons.

Transcriptomics

For transcriptomics analysis, we used ~44 K bovine (v2) gene expression Agilent microarray platform. The microarrays experiment was performed according to our laboratory's

established protocol and the instructions provided by Agilent technologies. The experiment was performed using four groups, which are OVE, K, SM and MS. The complete microarrays hybridization design is given in the Figure 4.2, but for the current study, we used SM, MS and K groups to evaluate supplementation and postpartal dairy health. The detailed description of the microarray experiment is provided in the above chapters 2-3 and elsewhere (Shahzad et al., 2015).

Statistical analysis

For metabolomics analysis, a total of 313 biochemical compounds were used for statistical analysis. The data was normalized in terms of raw area counts. Each biochemical compound from the raw values was rescaled to set the median value equal to 1.0. The missing values were imputed with the minimum value. Following the log transformation and imputation of missing values with the minimum observed value for each compound, we used a mixed procedure of SAS (SAS Institute Inc., Cary, NC) to identify the biochemical compounds that differed significantly between the experimental groups. A list of significantly affected biochemical compounds with $p \leq 0.10$ was selected for evaluation purpose.

For microarray's statistical analysis, a data from 12 arrays (24 samples) was used. The oligo IDs with bad flags (-100) were removed before normalization. The data was log transformed and then corrected across dye and array effects using loess normalization and array centering method. After normalization, a mixed procedure of SAS was used. The statistical model included dietary treatments as a fixed effect. The raw *p*-values were adjusted for the number of genes tested using Benjamini and Hochberg's false discovery rate (FDR; Benjamini

and Hochberg, 1995) to account for multiple comparisons. However, there were not enough differentially expressed genes (DEG) under this criteria, so we used a $p \leq 0.05$ and fold change (FC) $\geq |1.5|$ cut offs for the evaluation purpose.

Pathways analysis

For metabolomics analysis, each biochemical compound was annotated with its corresponding sub pathway. The results are further furnished with *p*- and fold change (FC) values resulting from SAS analysis. We obtained a total of 1,021 (K vs. SM) and 771 (K vs. MS) DEG with a $p \leq 0.05$ and FC $\geq |1.5|$. For transcriptome analysis, a dynamic impact approach (DIA) tool was used to unravel the Kyoto Encyclopedia of Genes and Genome (KEGG) pathways. A list of DEG along with their Oligo IDs, Entrez gene IDs, *p* values and FC values was used as an input. For the analysis, a minimum of 30% annotated genes in the microarray versus the whole genome were selected as described elsewhere (Bionaz et al., 2012). The DIA was run on the selected DEG to obtain the impact and flux values for each KEGG categories, sub-categories and their respective pathways. The impact values reflect the overall perturbation, while the flux values reflect the overall direction of a pathway, thus allowing us to evaluate transcriptome profiles in a more holistic fashion.

Network analysis and data integration

The network analyses of both metabolome and transcriptome datasets were conducted using IPA software. The data from the Metabolon's biochemical compounds was annotated with PubChem identifiers provided online (https://pubchem.ncbi.nlm.nih.gov/). For metabolomics

network reconstructions, a list of 13 (K vs. SM) and 43 (K vs. MS) significantly affected biochemical compounds ($p \leq 0.10$) was used along with their FC values. For transcriptomic network reconstructions, a list of DEG with a $p \leq 0.05$ and FC $\geq |1.5|$ was used to upload into the IPA to run the core analysis. We used upstream transcription regulators and their downstream target genes from the IPA core analysis results. The data integration was performed using transcription regulators and biochemical compounds using both direct and indirect pathways using 'Path Explorer' tool in IPA. The results were exported in the form of publishable qualities.

Results and Discussion

There are relatively fewer studies conducted dealing with the role of ketosis development in the liver of transition dairy cows, suggesting the mechanism of nutritional management using amino acid supplementations (McCarthy et al., 1968, Waterman and Schultz, 1972, Osorio et al., 2013). These studies were aimed in treating ketosis by means of dietary treatments in the form of rumen protected methionine or choline supplementation. Supplemental rumen-protected methionine elicits modest but distinct response in the liver at both transcriptome and metabolome levels (Vailati-Riboni et al., 2017). Some of these unique alterations might help avert the detrimental effects of energy overfeeding prepartum, such as to avoid ketosis susceptibility postpartum.

Expression patterns of metabolomics and transcriptomics datasets

From metabolomics analysis of K vs. SM, we obtained a total of 13 out of 313 biochemical compounds with $p \leq 0.10$. The Figure 4.3 shows the overall patterns of the metabolites with respect to different *p*-values. Among these, 7 were up regulated while the 6 were down regulated. In K vs. MS, we found 43 biochemical compounds with $p \leq 0.10$ (full results are not shown). Out of these, 5 were up regulated, while the 38 compounds were down regulated. In Figure 4.4, we have provided a list of the biochemical compounds and their sub-pathways along with their respective $p \leq 0.05$ and fold change values.

Within transcriptome analysis (Figure 4.5), we obtained more of DEG (1,021) in K vs. SM comparison as compared with K vs. MS (771). However, in both cases, the number of up regulated DEG were lower than the down regulated DEG as shown in the figure. For both DIA and IPA analysis, we used $p \leq 0.05$ and FC $\geq |1.5|$. A lists of DEG with $p \leq 0.01$ and FC $\geq |3.0|$ is provided in the Tables 4.1 and 4.2. These tables provide the overall expression pattern in terms of gene symbols, their descriptions and the respective fold change values.

Summary of KEGG pathways

The Figure 4.6 shows the summary of KEGG pathways in terms of categories and subcategories. The results illustrate an overall inhibition pattern of pathways in ketotic group compared with both SM and MS supplemental groups. The carbohydrate metabolism was shown as the most affected sub-category in both comparisons. However, the energy metabolism was induced moderately in K vs. MS group. It can also be seen that the pathways under the genetic information processing were slightly induced as compared to the other categories. These results display an overall trend of cellular growth, proliferation and apoptosis mechanisms.

Pathways and networks analyses

The metabolomics and transcriptomics analysis results as pathways and networks levels are discussed in the following sections.

K vs. SM. Among the metabolomics results (Figure 4.4), fatty acid metabolism (butyrylglycine), primary bile acid (glycochenodeoxycholate) and urea cycle (N-delta-acetylornithine) were activated in the ketotic group. Whereas, the lysolipids, fatty acid-dicarboxylate, TCA cycle and purine metabolism were overall inhibited. From the DIA analysis of KEGG pathways, we found 9 out 10 pathways that were deactivated (Figure 4.7). These pathways belong to subcategories such as carbohydrate metabolism, metabolism of other amino acids, excretory system, glycan biosynthesis and metabolism, development, and signal transduction. The activated pathway includes 'glycosphingolipid biosynthesis- ganglio series'.

The network analysis of biochemical compounds revealed the functional enrichment of adenosine and citric acid in carbohydrate and lipid metabolism (Figure 4.8A). On the other hand, along with these compounds, guanosine, hypoxanthine and glycochenodeoxycholate showed their involvement in cellular functions as shown in the Figure 4.8B. The network analysis revealed five transcription regulators of which two (PAX7 and NFYB) were up regulated and three (ARNT, FOSL2 and HDAC5) were down regulated (Figure 4.10A). These transcription regulators were involved in cell proliferation, differentiation and developmental processes (Dey et al., 2011, Fan et al., 2014).

The previous work from our group has shown that transcription of genes encoding enzymes of fatty acid synthesis, including acetyl-CoA carboxylase, fatty acid desaturase, and stearoyl-CoA desaturase, is suppressed during ketosis (Loor et al., 2007b). In the current study,

the metabolic results indicate an increased demand for short-chain acyl-CoA dehydrogenase butyrylglycine in ketotic group compared with SM group. Together, these results suggest that fatty acid metabolism in the K group was shifted to mitochondrial fatty acid oxidation which feeds acetyl-CoA into ketogenesis. The other carbohydrate related metabolites and pathways were also down regulated in the ketotic group. These include citrate, 'Fructose and mannose metabolism', and 'Pentose phosphate pathway' (Zhang et al., 2013, White, 2015). The primary bile acids, such as glycochenodeoxycholate was increased in K vs. SM suggesting NADPH oxidase-dependent hepatocyte shrinkage through ceramide (Becker et al., 2007). This process further impairs the bile formation in the liver (Keitel et al., 2008).

The metabolism of other amino acids such as cyanoamino acid, taurine and hypotaurine was inhibited in the ketotic group. In contrast, it is activation in SM group plays an important role in dairy health. The taurine is synthesized from the methionine or cysteine or hypotaurine in the liver and is involved in several processes such as osmoregulation, calcium utilization, and most importantly bile acid conjugation (Brand et al., 1998).

K vs. MS. In this group, all of the biochemical compounds were down regulated except the two metabolites (1-methylimidazoleacetate and 4-imidazoleacetate) that belong to histidine metabolism. The compounds that were down regulated belong to pentose metabolism, purine metabolism, tryptophan metabolism, lysolipids, fatty acids, nicotinate and nicotinamide metabolism, and sphingolipid metabolism as shown in the Figure 4.4. The DIA analysis of KEGG pathways revealed inhibition of all of the top ten pathways except the 'Nitrogen metabolism'. The inhibited pathways belong to the sub-categories such as carbohydrate metabolism, metabolism of cofactors and vitamins, glycan biosynthesis and metabolism, and development.

The network analysis of the biochemical compounds revealed NAD^+, NADH and phosphoenolpyruvate association with carbohydrate metabolism (Figure 4.9A). On the other hand, NAD^+, NADH, along with nicotinamide-beta-riboside, indicant, and alpha-hydroxyglytarate were involved in cellular functions (Figure 4.9B). The networks analysis revealed three transcription regulators from which one was up regulated (EPAS1), and the other two were down regulated (EHF and LEF1) in the ketotic group of cows (Figure 4.10B).

Pentose metabolism, which draws carbons from the pentose phosphate pathway, showed a difference between the ketosis and supplemental groups. For instance, the pentose sugars ribose and ribulose were significantly elevated in the MS group compared to animals that experienced ketosis. Similarly, pentose alcohol xylitol and ribitol were decreased in K vs. MS comparison. The DIA results indicate the inhibition of 'Riboflavin metabolism' (Figure 4.7). The decreased ribitol level may be associated with riboflavin deficiency (Lankinen et al., 2011). Additionally, it has been indicated that ribitol via pentose phosphate pathway is used for energy synthesis by entering into glycolysis (Zhang et al., 2013). Interestingly, xylitol can reduce ketone production in dairy cattle potentially through its ability to stimulate energy production via glucose-dependent pathway and its stimulation of insulin release, both act to suppress ketogenesis in the liver (Sakai et al., 1996, Mizutani et al., 2003, Toyoda et al., 2008). A hypothesis stemming from the prior literature and the distinct increase of xylitol following the SM and MS treatments is that internal xylitol produced under methionine supplementation limits liver ketone production.

The nitrogen metabolism was the only pathway among the top ten pathways in the ketotic group that was activated. It is characterized into main categories based on the nitrogen source: nitrogen from microbial protein, and nitrogenous compounds and has an important role in blood

and milk in the form of blood urea nitrogen (BUN) and milk urea nitrogen (MUN) (Bahrami-Yekdangi et al., 2014). In a study of human cancer patients, it was reported that the ketogenic diet has no significant impact on nitrogen metabolism (Fearon et al., 1988). However, in our results, the activation of this pathway may suggest a greater nitrogen utilization in the ketotic group of cows as compared with MS supplemented group.

Integration of metabolome and transcriptome datasets

The data integration using biochemical compounds and transcription regulators unraveled the connecting links between these molecules. Figure 4.11 shows the integration between the biochemical compounds and transcription regulators. In K vs. SM (Figure 4.11A), the data integration shows the immune related molecules such as MHC class I and NFkB complexes that were linked with FOSLA2 and PAX7 transcription regulators. In several studies, it has been reported that immune system is usually compromised during the ketosis leading to several other health related disorders, and production losses (Osorio et al., 2013, Sordillo, 2016). In K vs. MS (Figure 4.11B), all of the shown biochemical compounds were down regulated except phosphoenolpyruvate. Among the transcription regulators, EPAS1 (Endothelial PAS domain-containing protein 1) was up regulated. This gene is also known as hypoxia-inducible factor and is activated under low oxygen conditions (Schonenberger and Kovacs, 2015). The overall study shows that supplementing SM or MS when lysine is adequate, helps to improve the health of dairy cows postpartum and ultimately increase the voluntary DMI and milk production. This also helps to maintain the efficiency of metabolic and non-metabolic pathways in the liver.

Conclusion

The genome-wide metabolomics and transcriptomics profiling study was conducted to determine the metabolic and genetic level changes occurring in the liver of transition cows and to examine the role of SM or MS in treating the metabolic disorders. The comparison of liver profiles from cows representing various rations, supplements, and ketosis groups revealed several key metabolic and genomic level differences. The increase of the pentose alcohol xylitol in the MS and SM groups relative to the ketotic group was interesting because xylitol is a useful as ketotic therapeutic agent due to its ability to stimulate insulin secretion and to reduce circulating fatty acids. However, it is not clear that endogenously produced xylitol has similar effects or it achieves levels high enough to mimic the effects of exogenous xylitol. The ketotic group showed more pronounced inhibition of KEGG related pathways and metabolites as compared with SM and MS supplemental groups. The network analysis using both metabolomics and transcriptomics datasets revealed distinct features of ketogenesis in the ketotic group. Altogether, these results showed that both SM and MS affected the metabolic functions of the liver. Both SM and MS appeared to have a definitive impact on liver functions that were distinct from the liver phenotypes displayed during ketosis development. This retrospective study may help to diagnose and prevent the ketosis development at pre-calving stages.

Figures and Tables

Table 4.1: A list of differentially expressed genes with $p \leq 0.01$ and fold change (FC) $\geq |3|$ in K vs. SM group. The table is ranked based on the FC values.

Symbol	Description	K vs. SM
CHRDL1	chordin-like 1	8.82
PI3	peptidase inhibitor 3, skin-derived (SKALP)	7.63
CCDC77	coiled-coil domain containing 77	6.30
CCDC42B	coiled-coil domain containing 42B	6.20
OR10K1	olfactory receptor, family 10, subfamily K, member 1	5.20
LOC526294	olfactory receptor-like protein DTMT	4.99
LAMA1	laminin, alpha 1	4.92
C25H7orf43	chromosome 7 open reading frame 43	4.88
PBK	PDZ binding kinase	4.80
PLCG2	phospholipase C, gamma 2	4.63
EPHB1	EPH receptor B1	4.56
MGC138914	uncharacterized LOC512219	4.26
PSPH	phosphoserine phosphatase	4.24
PYROXD1	pyridine nucleotide-disulphide oxidoreductase domain 1	4.19
VSTM5	V-set and transmembrane domain containing 5	4.17
LPL	lipoprotein lipase	4.04
ATXN3	ataxin 3	4.04
KIF18A	kinesin family member 18A	3.82
DPY19L3	dpy-19-like 3	3.78
KRT9	keratin 9	3.72
SLC22A16	solute carrier family 22 (organic cation/carnitine transporter), member 16	3.71
ACACB	acetyl-CoA carboxylase beta	3.65
GRM7	glutamate receptor, metabotropic 7	3.39
WDR69	WD repeat domain 69	3.38
PRL	prolactin	3.34
RGS3	regulator of G-protein signaling 3	3.29
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	3.24
CACNA1D	calcium channel, voltage-dependent, L type, alpha 1D subunit	3.23
CCDC70	coiled-coil domain containing 70	3.22
KRT5	keratin 5	3.18
LOC100848685	caspase-10	3.11
CNR1	cannabinoid receptor 1 (brain)	3.03
LOC615101	melanoma-associated antigen B17-like	-3.01

Table 4.1 (Cont.)

GYG1	glycogenin 1	-3.02
ADH1C	alcohol dehydrogenase 1C (class I), gamma polypeptide	-3.04
RAE1	ribonucleic acid export 1	-3.12
ZNF527	zinc finger protein 527	-3.14
VWC2L	von Willebrand factor C domain containing protein 2-like	-3.14
CD99L2	CD99 molecule-like 2	-3.16
ZNF384	zinc finger protein 384	-3.23
STK11IP	serine/threonine kinase 11 interacting protein	-3.27
CREBL2	cAMP responsive element binding protein-like 2	-3.30
RALGDS	ral guanine nucleotide dissociation stimulator	-3.30
GPX3	glutathione peroxidase 3 (plasma)	-3.31
LOC524074	regulatory factor X, 6	-3.31
DCST2	DC-STAMP domain containing 2	-3.34
HCN1	hyperpolarization activated cyclic nucleotide-gated potassium channel 1	-3.34
HPCAL1	hippocalcin-like 1	-3.35
LOC100850459	phosphodiesterase 7A	-3.35
SATB2	SATB homeobox 2	-3.37
CORT	cortistatin	-3.37
LOC522938	Uncharacterized protein	-3.39
HSH2D	hematopoietic SH2 domain containing	-3.42
MED12	mediator complex subunit 12	-3.47
SLC48A1	solute carrier family 48 (heme transporter), member 1	-3.48
CENPE	centromere protein E	-3.48
ABHD16A	abhydrolase domain containing 16A	-3.48
HDAC5	histone deacetylase 5	-3.48
GALC	galactosylceramidase	-3.50
BTNL9	butyrophilin-like 9	-3.51
FLOT1	flotillin 1	-3.51
PLLP	plasmolipin	-3.53
DUSP15	dual specificity phosphatase 15	-3.55
PYGO2	pygopus family PHD finger 2	-3.62
CDCA8	cell division cycle associated 8	-3.66
RIPK3	receptor-interacting serine-threonine kinase 3	-3.67
LOC509124	olfactory receptor 9G4	-3.67
STK32C	Serine/threonine-protein kinase 32C	-3.69
DDX3Y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	-3.78
BAD	BCL2-associated agonist of cell death	-3.81
PLOD3	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	-3.83

Table 4.1 (Cont.)

STON1	stonin 1	-3.84
KIAA0922	KIAA0922 ortholog	-3.84
CIB2	calcium and integrin binding family member 2	-3.86
IRAK3	interleukin-1 receptor-associated kinase 3	-3.89
PRELP	prolargin precursor	-3.89
C1QTNF3	C1q and tumor necrosis factor related protein 3	-3.94
IRX4	iroquois homeobox 4	-3.94
DLGAP5	discs, large (Drosophila) homolog-associated protein 5	-3.97
MAPK3	mitogen-activated protein kinase 3	-4.01
TUBD1	tubulin, delta 1	-4.02
HOXA5	homeobox A5	-4.05
LOC100335205	T-cell receptor gamma chain C region C10.5	-4.09
PROK2	prokineticin 2	-4.09
DST	dystonin, transcript variant 1	-4.25
RAPGEF4	Rap guanine nucleotide exchange factor (GEF) 4	-4.38
ADORA2B	adenosine A2b receptor	-4.42
WDR6	WD repeat domain 6	-4.52
PMM1	phosphomannomutase 1	-4.53
NCOA3	nuclear receptor coactivator 3	-4.53
SLC25A39	solute carrier family 25, member 39	-4.76
RALGAPA2	akt substrate AS250	-4.77
MYOZ2	myozenin 2	-4.89
NSG1	neuron specific gene family member 1	-4.91
LOC517722	olfactory receptor 2T27	-4.97
EML3	echinoderm microtubule associated protein like 3	-5.06
FAM214B	KIAA1539 ortholog	-5.10
ABL1	c-abl oncogene 1, non-receptor tyrosine kinase	-5.16
GPX3	glutathione peroxidase 3 (plasma)	-5.20
ABHD1	abhydrolase domain containing 1	-5.29
KIF27	kinesin family member 27	-5.63
NCOA2	nuclear receptor coactivator 2	-5.77
COBRA1	cofactor of BRCA1	-5.93
FBP2	fructose-1,6-bisphosphatase 2	-6.28
CPXM2	carboxypeptidase X (M14 family), member 2	-7.75
GPX3	glutathione peroxidase 3 (plasma)	-8.63

Table 4.2: A list of differentially expressed genes with $p \leq 0.01$ and fold change (FC) $\geq |3|$ in K vs. MS group. The table is ranked based on the FC values.

Symbol	Description	K vs. MS
LOC100847724	WAP four-disulfide core domain protein 18-like	11.32
ZNF35	zinc finger protein 35	6.35
CCDC77	coiled-coil domain containing 77	6.07
FAM102B	family with sequence similarity 102, member B	5.48
PI3	peptidase inhibitor 3, skin-derived (SKALP)	5.43
CSRNP1	cysteine-serine-rich nuclear protein 1	5.05
BOLA-N	MHC class I antigen	5.04
NLGN2	neuroligin 2	4.67
MSL1	male-specific lethal 1 homolog	4.22
CCDC42B	coiled-coil domain containing 42B	4.11
CLCA2	chloride channel accessory 2	4.08
KRT5	keratin 5	3.98
BOLA	MHC class I heavy chain	3.81
CACNA1D	calcium channel, voltage-dependent, L type, alpha 1D subunit	3.58
JSP.1	MHC Class I JSP.1	3.57
PCBP3	poly(rC) binding protein 3	3.56
MAML3	mastermind-like 3 (Drosophila)	3.55
EIF2C1	eukaryotic translation initiation factor 2C, 1	3.45
PARP4	poly (ADP-ribose) polymerase family, member 4	3.39
EPHB1	EPH receptor B1	3.38
GIT2	G protein-coupled receptor kinase interacting ArfGAP 2	3.34
LOC526294	olfactory receptor-like protein DTMT	3.30
KRT9	keratin 9	3.18
SPATA17	spermatogenesis associated 17	3.09
LOC100848685	caspase-10	3.08
XPNPEP2	X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound	-3.01
ADH1C	alcohol dehydrogenase 1C (class I), gamma polypeptide	-3.02
CD99L2	CD99 molecule-like 2	-3.03
RIPK3	receptor-interacting serine-threonine kinase 3	-3.04
ABL1	c-abl oncogene 1, non-receptor tyrosine kinase	-3.05
TMTC2	transmembrane and tetratricopeptide repeat containing 2	-3.11
MTMR14	myotubularin related protein 14	-3.14
TMEM206	transmembrane protein 206	-3.20
MBTPS1	membrane-bound transcription factor peptidase, site 1	-3.20
ITGB4	integrin, beta 4	-3.23

Table 4.2 (Cont.)

PDE4DIP	phosphodiesterase 4D interacting protein	-3.26
ZNF565	zinc finger protein 565	-3.34
RAE1	ribonucleic acid export 1	-3.34
STK11IP	serine/threonine kinase 11 interacting protein	-3.35
ADCY7	adenylate cyclase 7	-3.43
ACVR1C	activin A receptor, type IC	-3.49
LOC541022	Uncharacterized protein	-3.52
SOGA1	suppressor of glucose, autophagy associated 1	-3.55
CHAF1B	chromatin assembly factor 1, subunit B (p60)	-3.57
ANKS3	ankyrin repeat and sterile alpha motif domain containing 3	-3.58
IL23R	interleukin 23 receptor	-3.73
C8H9orf43	chromosome 8 open reading frame, human C9orf43	-3.75
LOC512548	antileukoproteinase	-3.76
HDAC5	histone deacetylase 5	-3.81
NABP2	oligonucleotide/oligosaccharide-binding fold containing 2B	-3.84
MAP3K4	mitogen-activated protein kinase kinase kinase 4	-3.87
BAD	BCL2-associated agonist of cell death	-3.91
DST	dystonin, transcript variant 1	-3.92
RALGDS	ral guanine nucleotide dissociation stimulator	-3.97
BIN1	bridging integrator 1	-4.00
MTMR9	myotubularin related protein 9	-4.02
KRT80	keratin 80	-4.05
FAM214B	KIAA1539 ortholog	-4.05
ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif, 9	-4.06
XIAP	X-linked inhibitor of apoptosis	-4.11
ACTL6A	actin-like 6A	-4.31
KIAA1009	KIAA1009 ortholog	-4.33
SLC25A39	solute carrier family 25, member 39	-4.35
DUSP15	dual specificity phosphatase 15	-4.37
NCKAP5L	NCK-associated protein 5-like	-4.38
IRAK3	interleukin-1 receptor-associated kinase 3	-4.38
NCAN	neurocan	-4.39
CEP112	coiled-coil domain containing 46	-4.41
PYGO2	pygopus family PHD finger 2	-4.42
DDX3Y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	-4.42
MAPK3	mitogen-activated protein kinase 3	-4.45
TPGS1	tubulin polyglutamylase complex subunit 1	-4.56
MEIS2	Meis homeobox 2	-4.67
ABHD16A	abhydrolase domain containing 16A	-4.68
CIB2	calcium and integrin binding family member 2	-4.69

Table 4.2 (Cont.)

EML3	echinoderm microtubule associated protein like 3	-4.79
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog	-4.94
ANXA11	annexin A11	-5.32
COBRA1	cofactor of BRCA1	-5.39
FBP2	fructose-1,6-bisphosphatase 2	-5.56
PIGT	phosphatidylinositol glycan anchor biosynthesis, class T	-7.42
FBP2	fructose-1,6-bisphosphatase 2	-9.11

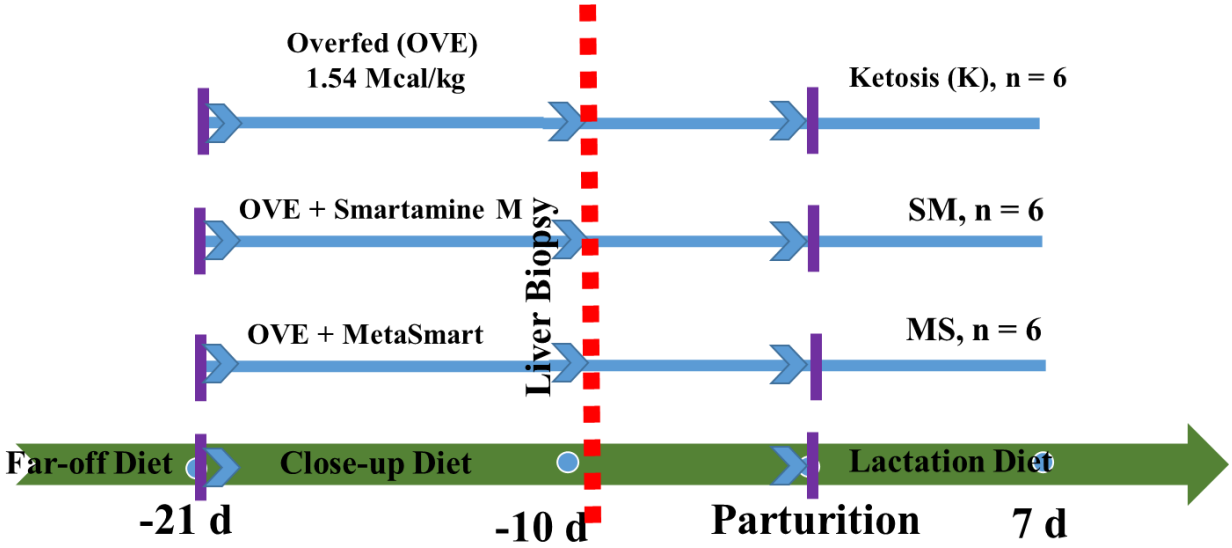


Figure 4.1: Experimental Design. The tissue biopsies were taken at -10 d relative to parturition. The cows were fed with moderate energy diet and developed Ketosis (K), supplemented with Smartamine M (SM) and MetaSmart (MS), and remain healthy postpartum.

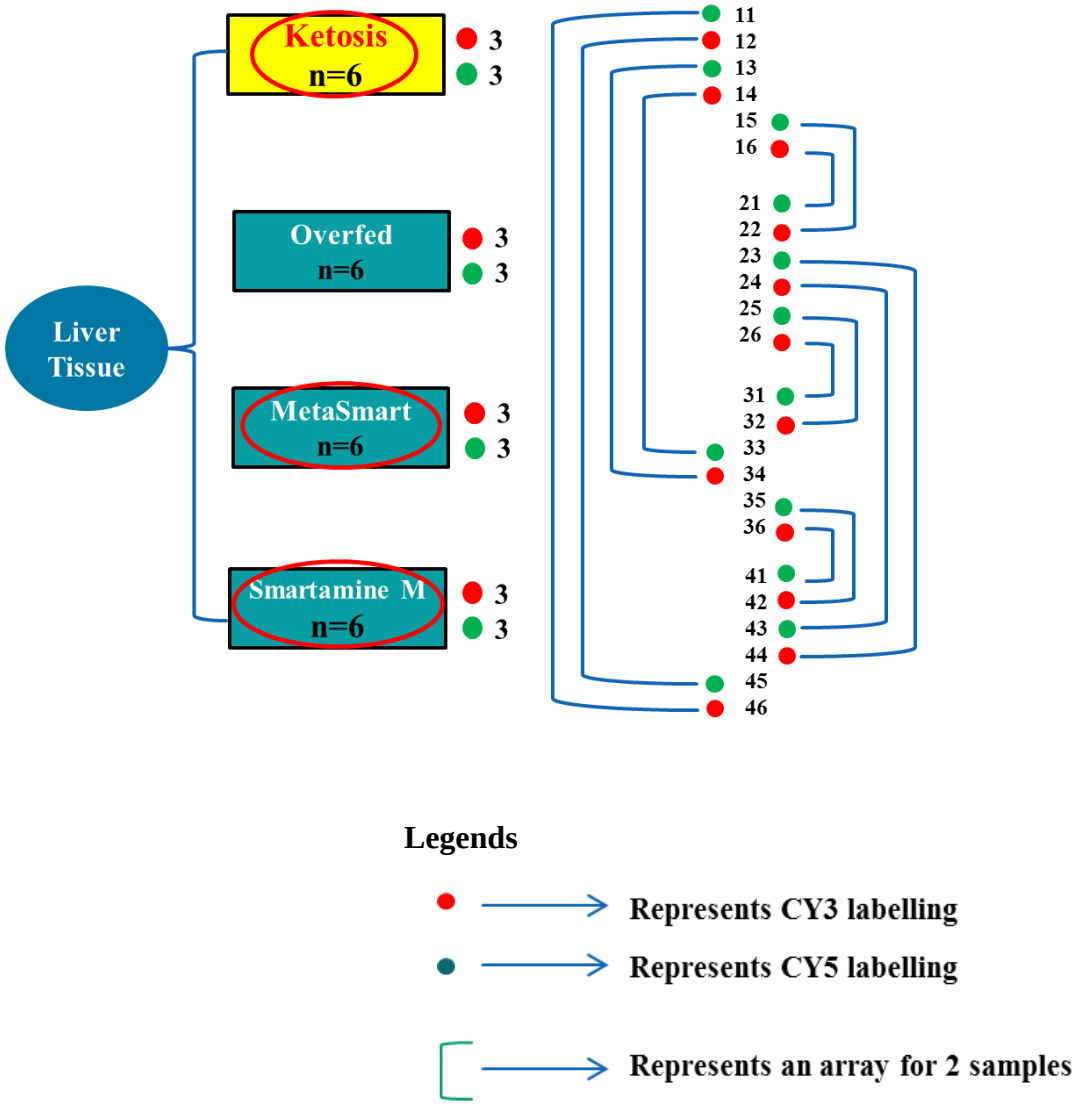


Figure 4.2: The complete microarray's hybridization design is shown in the figure. A two color (red and green) hybridization plan was used. Ketotic (K), Smartamine M (SM) and MetaSmart (MS) groups encircled with red are used in the current study.

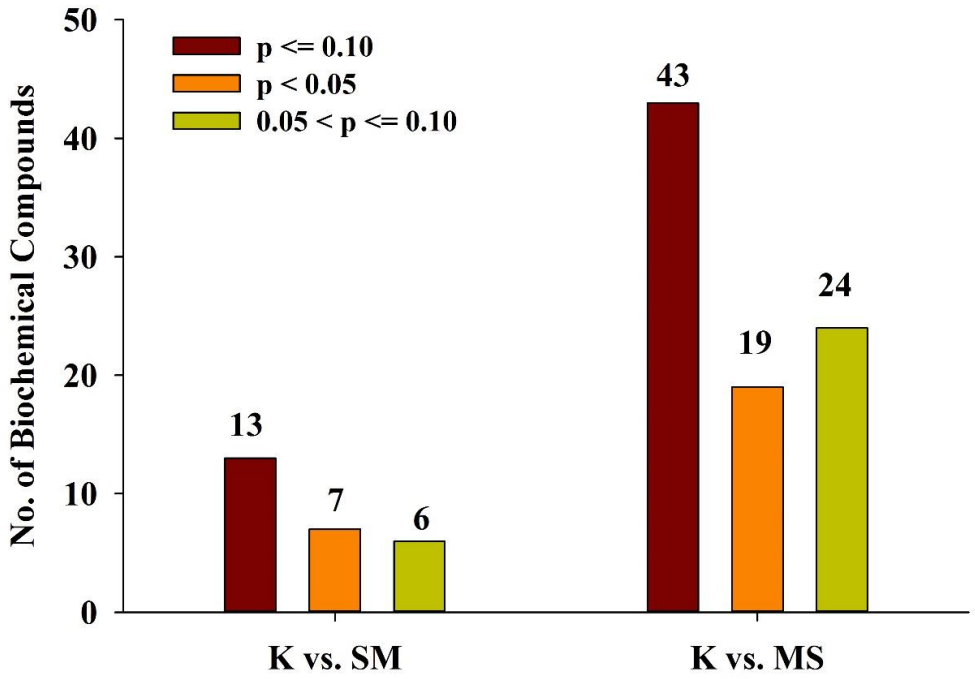


Figure 4.3: No. of significantly affected biochemical compounds with $p \leq 0.05$, between 0.05 to 0.10, and $p \leq 0.10$ for the two comparisons K vs. SM and K vs. MS.

Biochemical Name	Sub Pathway	K vs. SM	
		p -value	FC
butyrylglycine	Fatty Acid Metabolism (also BCAA Metabolism)	0.002	1.77
glycochenodeoxycholate	Primary Bile Acid Metabolism	0.015	3.09
1-palmitoylglycerophosphoglycerol*	Lysolipid	0.019	-1.48
N-delta-acetylornithine*	Urea cycle; Arginine and Proline Metabolism	0.032	1.34
2-hydroxyglutarate	Fatty Acid, Dicarboxylate	0.037	-1.49
citrate	TCA Cycle	0.039	-1.68
guanosine	Purine Metabolism, Guanine containing	0.045	-1.20
		K vs. MS	
xylitol	Pentose Metabolism	0.001	-1.70
xanthosine	Purine Metabolism, (Hypo)Xanthine/Inosine containing	0.003	-1.27
ribitol	Pentose Metabolism	0.010	-1.55
1-methylimidazoleacetate	Histidine Metabolism	0.011	2.02
ribulose	Pentose Metabolism	0.012	-1.47
3-indoxyl sulfate	Tryptophan Metabolism	0.013	-1.28
ribose	Pentose Metabolism	0.014	-1.40
2-linoleoylglycerophosphoethanolamine*	Lysolipid	0.015	-1.78
2-arachidonoylglycerophosphoinositol*	Lysolipid	0.017	-1.54
2-hydroxyglutarate	Fatty Acid, Dicarboxylate	0.020	-1.57
1-oleoylglycerophosphoethanolamine	Lysolipid	0.025	-1.83
nicotinamide riboside*	Nicotinate and Nicotinamide Metabolism	0.026	-1.20
1-margaroylglycerophosphoethanolamine*	Lysolipid	0.031	-2.35
1-linoleoylglycerophosphoethanolamine*	Lysolipid	0.032	-1.63
2-oleoylglycerophosphoethanolamine*	Lysolipid	0.037	-1.74
xylonate	Pentose Metabolism	0.041	-1.55
1-palmitoylplasmenylethanolamine*	Lysolipid	0.045	-2.25
4-imidazoleacetate	Histidine Metabolism	0.045	1.86
sphinganine	Sphingolipid Metabolism	0.046	-1.37

Legends

***p* -value**	***p* < 0.05**	
FC	**FC<-1.0**	**FC ≥ 1.0**

Figure 4.4: Significantly affected metabolites with $p \leq 0.05$ are shown for each comparison K vs. SM and K vs. MS. The first column for each comparison represents the *p*-values, while the second column represents the fold change (FC) values.

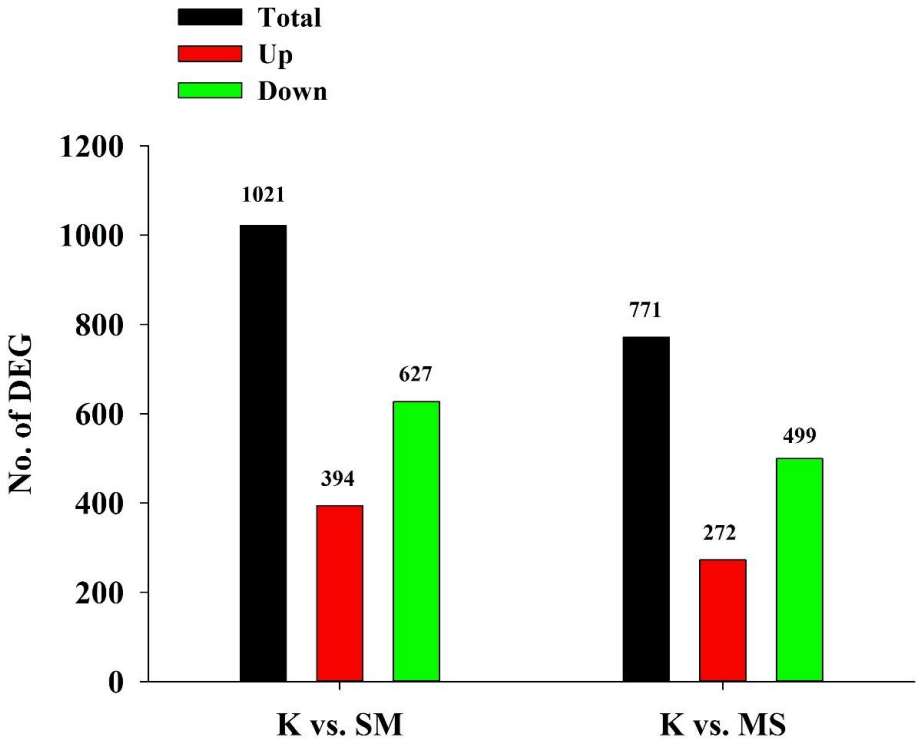


Figure 4.5: No. of differentially expressed genes (DEG) with $p \leq 0.05$ and fold change (FC) $\geq$ |1.5| are shown by vertical bars. The y-axis represents the number of DEG, whereas x-axis represents the comparisons K vs. SM and K vs. MS.

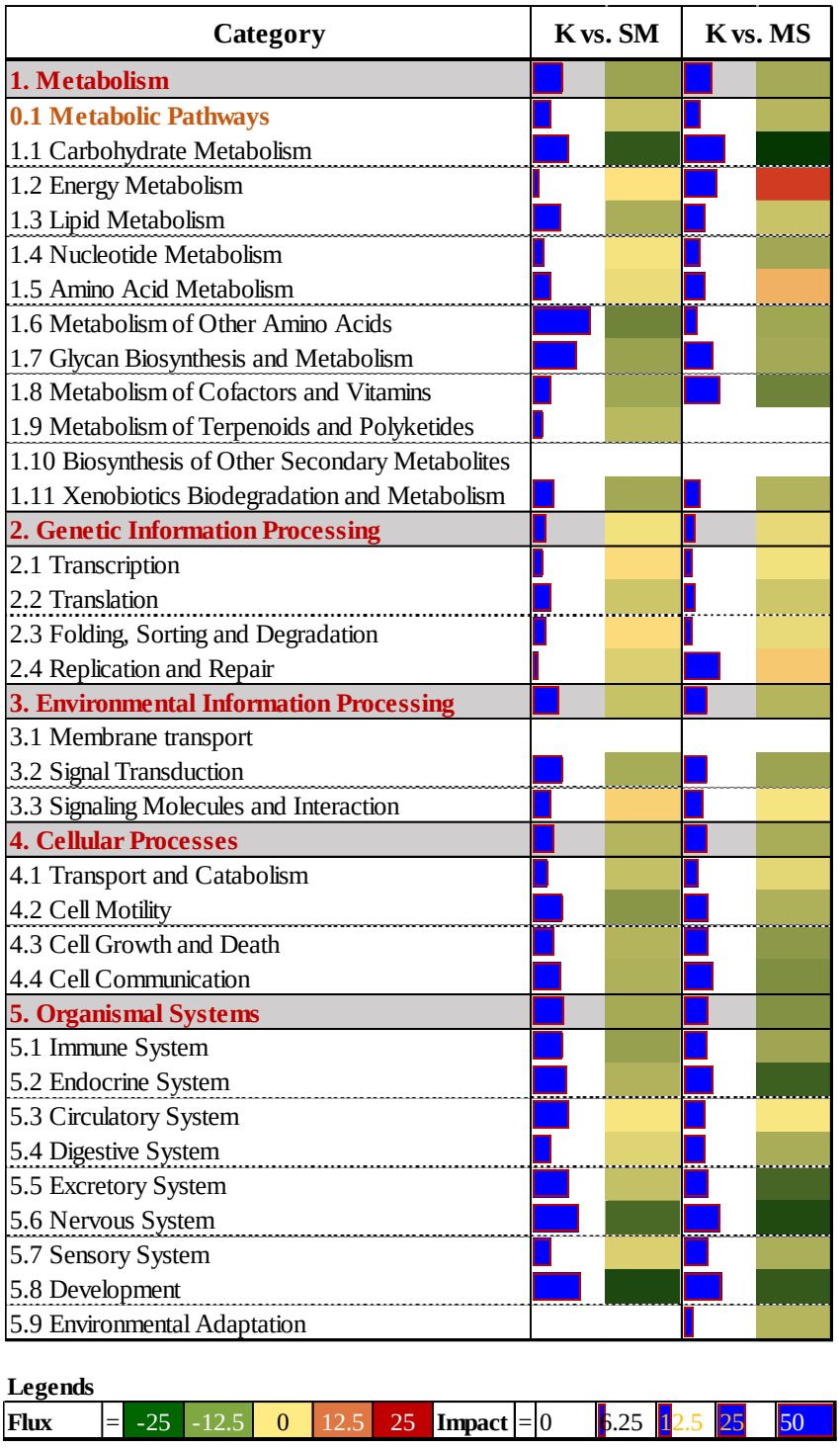
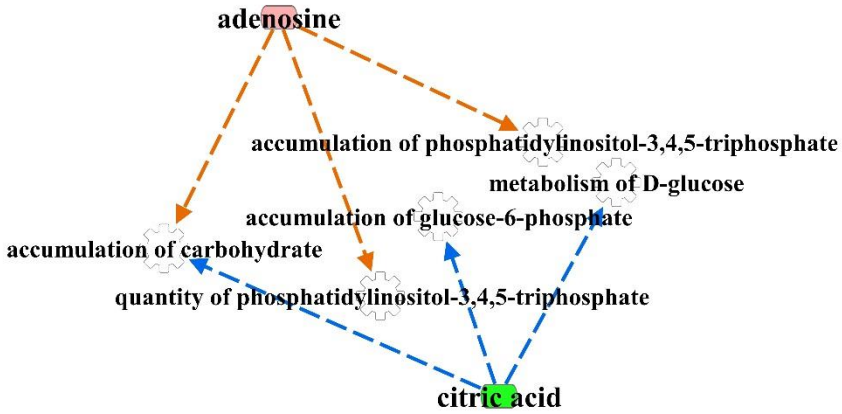


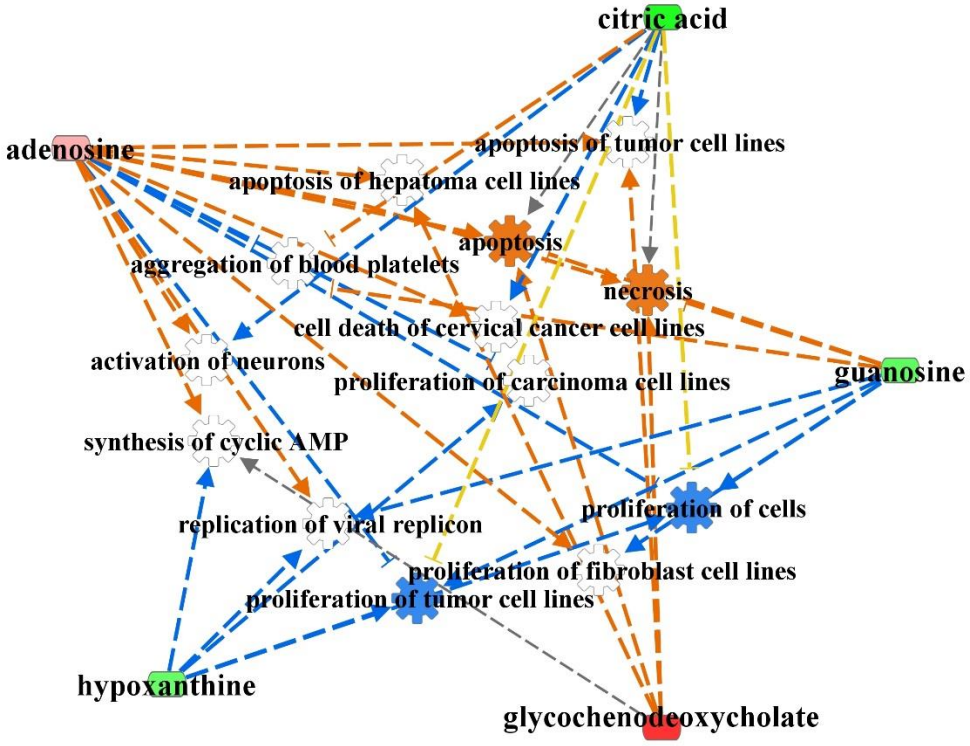
Figure 4.6: DIA KEGG summary encompassing the five main categories for each comparison K vs. SM and K vs. MS. The first column under each comparison represents the impact values, whereas the second column represents the direction of the impact (flux).

Sub Category	Pathways	K vs. SM	
		Impact	Flux
1.6 Metabolism of Other Amino Acids	Cyanoamino acid metabolism		
1.1 Carbohydrate Metabolism	Fructose and mannose metabolism		
5.5 Excretory System	Aldosterone-regulated sodium reabsorption		
1.7 Glycan Biosynthesis and Metabolism	Other glycan degradation		
1.7 Glycan Biosynthesis and Metabolism	Glycosphingolipid biosynthesis - globo series		
1.6 Metabolism of Other Amino Acids	Taurine and hypotaurine metabolism		
5.8 Development	Dorso-ventral axis formation		
3.2 Signal Transduction	ErbB signaling pathway		
1.1 Carbohydrate Metabolism	Pentose phosphate pathway		
1.7 Glycan Biosynthesis and Metabolism	Glycosphingolipid biosynthesis - ganglio series		
		K vs. MS	
		Impact	Flux
1.1 Carbohydrate Metabolism	Fructose and mannose metabolism		
1.8 Metabolism of Cofactors and Vitamins	Riboflavin metabolism		
1.1 Carbohydrate Metabolism	Pentose phosphate pathway		
1.7 Glycan Biosynthesis and Metabolism	Glycosylphosphatidylinositol(GPI)-anchor biosynthesis		
1.1 Carbohydrate Metabolism	Galactose metabolism		
5.8 Development	Dorso-ventral axis formation		
1.2 Energy Metabolism	Nitrogen metabolism		
1.7 Glycan Biosynthesis and Metabolism	Glycosphingolipid biosynthesis - ganglio series		
1.1 Carbohydrate Metabolism	Glycolysis / Gluconeogenesis		
1.7 Glycan Biosynthesis and Metabolism	Other glycan degradation		

Figure 4.7: The KEGG pathways along with their respective sub-categories are shown for K vs. SM and K vs. MS. The first column of each comparison represents the impact values (blue bars), whereas the second column (ranging from green to red) represents the direction of the impact (flux).

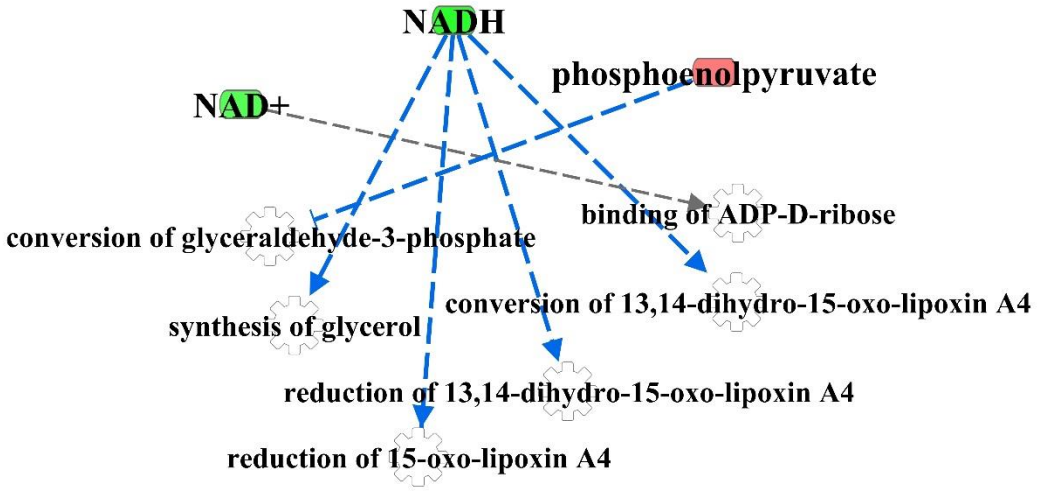


A). Carbohydrate and Lipid Metabolism

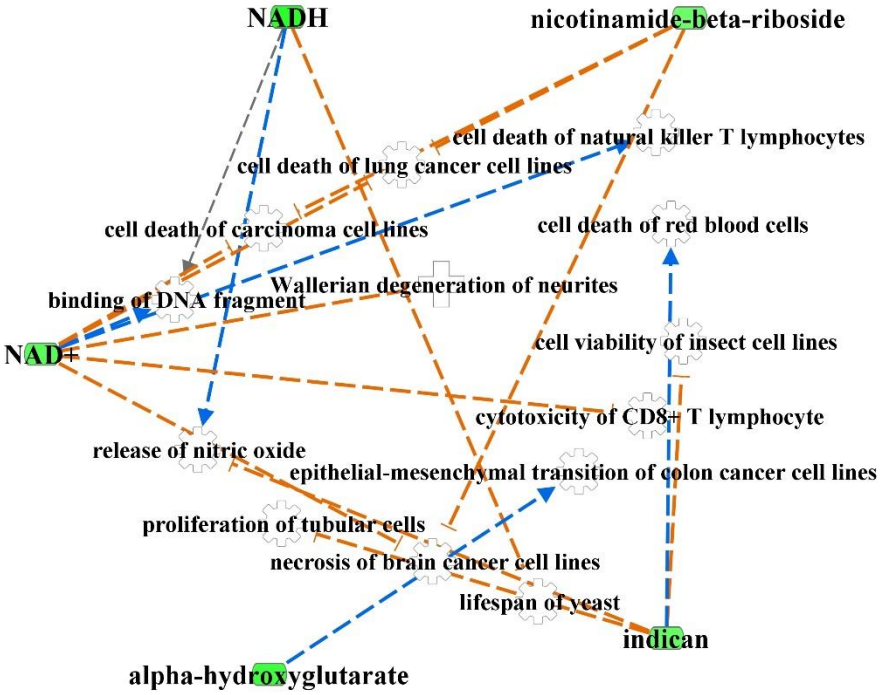


B). Cellular Functions

Figure 4.8: K vs. SM: (A) Carbohydrate and Lipid Metabolism (B) Cellular Functions.



A). Carbohydrate and Lipid Metabolism



B). Cellular Functions

Figure 4.9: K vs. MS: (A) Carbohydrate and Lipid Metabolism (B) Cellular Functions.

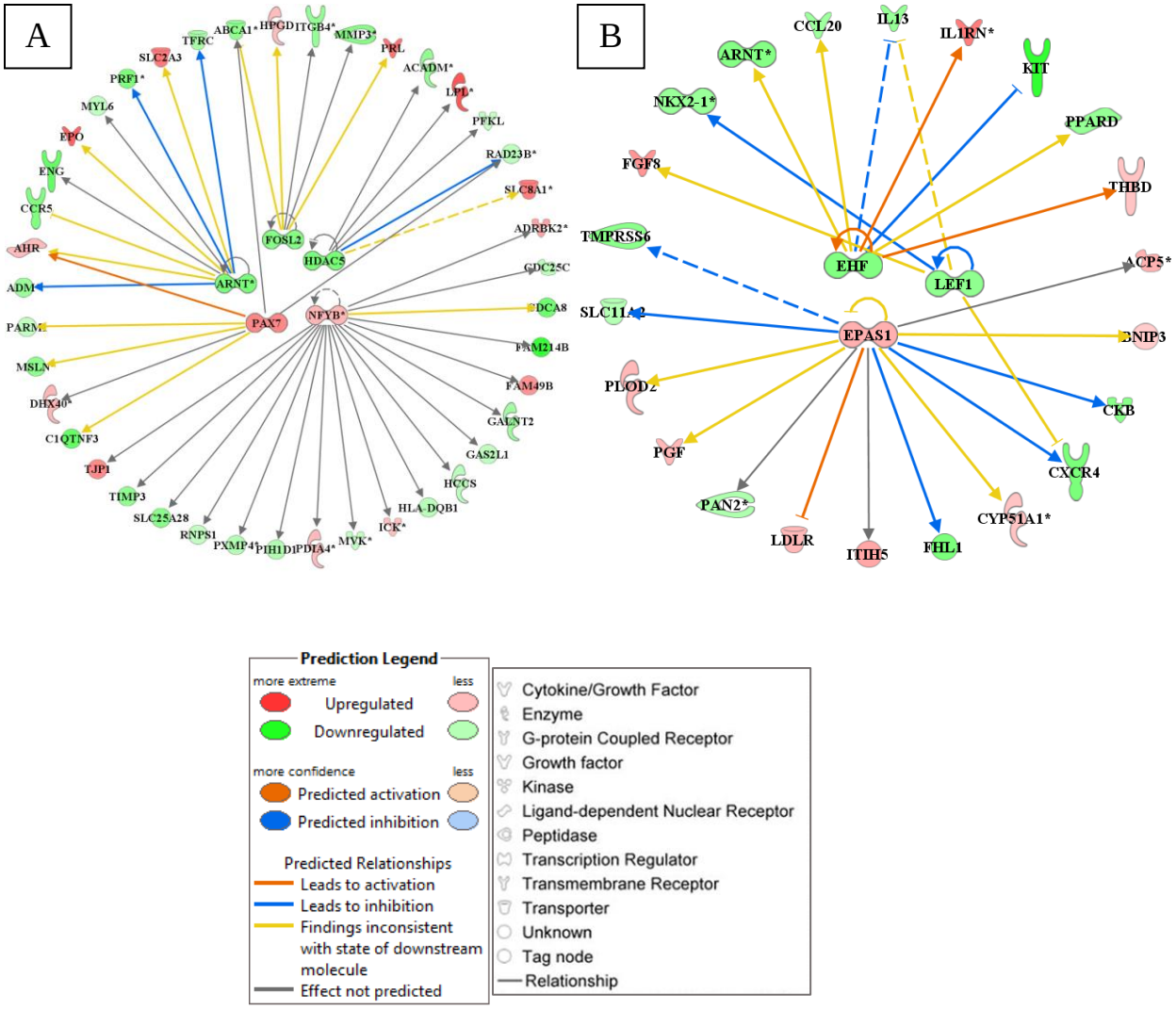


Figure 4.10: Transcription Regulators: (A) K vs. SM, (B) K vs. MS.

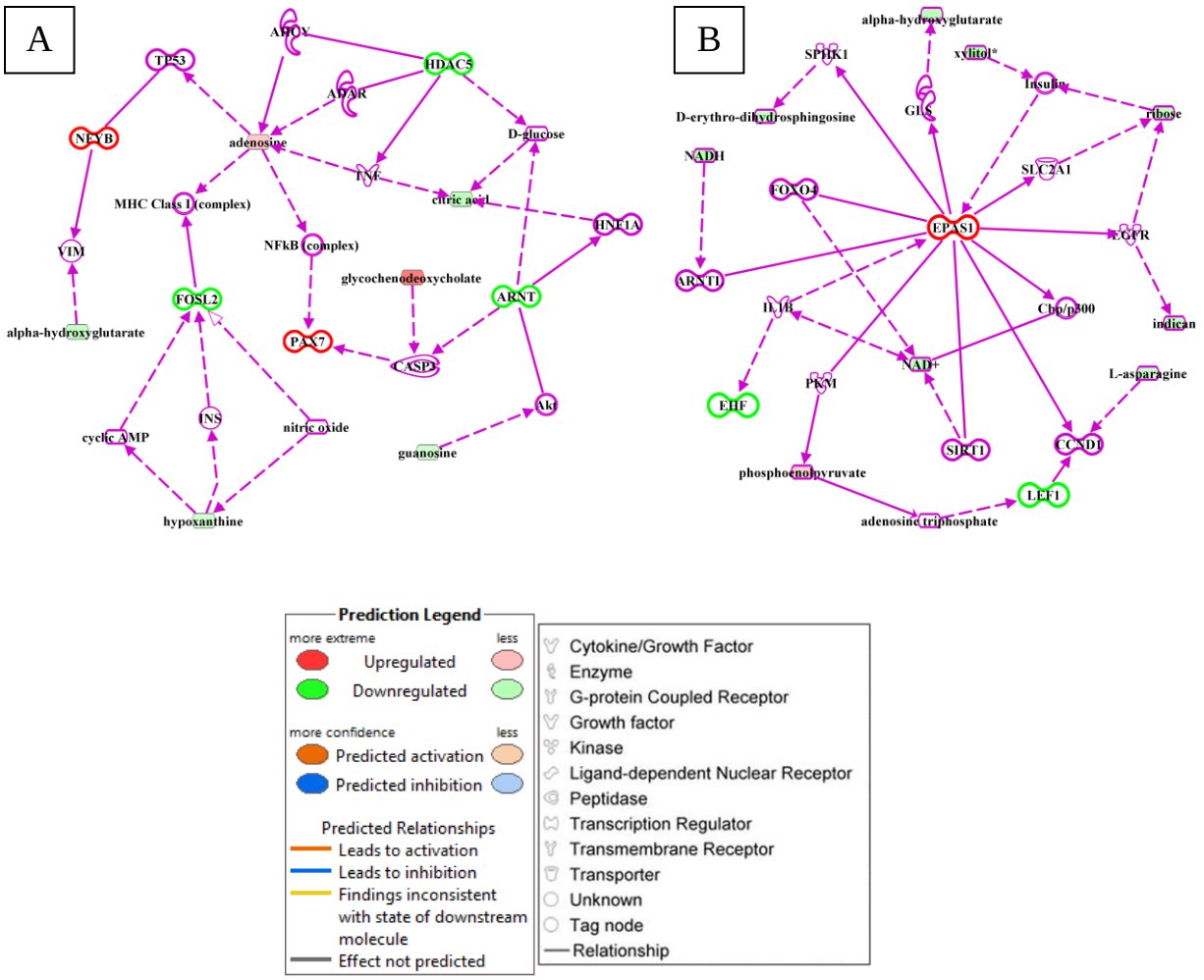


Figure 4.11: Data integration of transcription regulators and biochemical compounds of (A) K vs. SM, (B) K vs. MS.

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CHAPTER # 5

Analysis of transcription regulator gene networks in peripartal bovine liver during summer and spring seasons

Abstract

Thermal stress (TS) affects the health and productivity of dairy cows. We used gene network analysis on transcriptome data to uncover transcription regulators (TR) and their target genes during TS. Twelve multiparous Holstein dairy cows were used to harvest liver tissues at -30, 3, and 35 d relative to parturition during the spring (SP: March 28-April 30, n = 6) and summer (SU: June 15-July 02, n = 6). Mean temperature-humidity indexes for SP (day/night: below 72) and SU (day: 79.5±2.9, night: 70.1±4.7) were recorded. Transcriptomics was conducted using the 44-Agilent bovine microarrays. Statistical analysis with FDR $\leq$ 0.10 resulted in 618, 1,030 and 894 differentially expressed genes during SU vs. SP at -30, 3 and 35 d, respectively. Ingenuity pathway analysis (IPA) was used for gene network reconstructions. Among molecular and cellular functions, the IPA analysis identified cell death, survival, cellular growth and development as the most enriched functions. Carbohydrate metabolism was the most enriched at -30 and 3 d, while nucleic acid metabolism and cellular development were the most enriched at 3 and 35 d. A total of 6, 7 and 7 TR were identified at -30, 3 and 35 d. The IPA analysis uncovered HNF4A, MYC, and NCOA1 (-30, 3 and 35 d), STAT3, and RELA (-30 and 35 d), BCL6 (3 and 35 d), KAT2B (-30 d), and GATA2 (3 d) as key TR. Comparing SU vs. SP at -30d uncovered HNF4A and MYC (both triggered by RELA) as key TR. Both are linked with several downstream up-regulated target genes involved in oxidation of xenobiotic compounds

(CYP3A4), tryptophan catabolism (ACMSD1), arginine catabolism (ARG1), apoptosis regulation, and ER Calcium homeostasis (CFLAR, TMBIM6). In contrast, the down-regulated target genes were involved in cellular proliferation, anti-apoptotic activities, immune related disorders (CDKN1, LGALS1, TSPO), and liver disease (SERPINA1, FTH1). At 3 d, both HNF4A and MYC were down-regulated. Up-regulation of BCL6 was directly linked with the IL-6 dependent immune-response and cell growth. In contrast, BCL6 was associated with down-regulation of IL7R, IL13R1 and CXCL10-dependent immune responses. During lactation at 35 d, the up-regulation of RELA was associated with target genes involved in the activation of anti-inflammatory responses (CCL3, B2M), extracellular matrix breakdown (MMP1), regulation of cell cycle (MYC, PTEN, CASP8) and gluconeogenesis (PCK1). Preliminary evaluation of these results suggests that calving during the summer vs. spring is associated with the molecular phenotype of the liver.

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Introduction

Heat stress during the hot season adversely affects dairy health and productivity through length of photoperiod, month of parturition and by means of various physiological, behavioral, and metabolic level alterations (do Amaral et al., 2009, Bernabucci et al., 2010). The environmental factors influence the overall expression of hepatic genes that lead to metabolic and physiological adaptations in the body especially during the transition period. During this period, liver plays an essential role in regulating the homeostasis and preventing animals to suffer from adverse consequences (Febbraio, 2001).

It has been reported that the intensity of heat stress negatively influence the milk production during both dry period and early lactation (do Amaral et al., 2009, do Amaral et al., 2011). The overall feed intake is reduced leading to weight loss, dysfunction of immune system and decreased milk production (Rhoads et al., 2010). Despite the reduced feed intake in the heat stressed cows, it has been shown that fats are not mobilized from the adipose tissue due to the inactive lipolytic stimuli and altered homeorhesis (Baumgard and Rhoads, 2013). As a response, the level of non-esterified fatty acids (NEFA) is not increased during the heat stress (Shwartz et al., 2009), rather the pattern of endocrines is changed (Collier et al., 1982). In heat stressed cows, it has been revealed that metabolic pathways such as gluconeogenesis and cholesterol synthesis are inhibited, whereas the level of NEFA, and beta hydroxybutyrate (BHBA) is increased leading to lipid infiltration in the hepatic tissue (Basiricò et al., 2010). At this level, the immune system is also compromised due to abnormal inflammatory responses as indicated by do Amaral et al. (2011). The heat shock proteins have been shown to play a major role in eliciting the immune response under the increased heat stress conditions (Campisi et al., 2003). In this regard, the

neutrophils are the first line of defense that fight against external microbes (Kampen et al., 2004). The proper dairy management strategies could help to prevent the heat stress response in dairy cows. In the past, several studies have been focused on highlighting the role of heat stress and the causative physiological factors (do Amaral et al., 2009, Bernabucci et al., 2010, Rhoads et al., 2010), however, none of these have addressed the role of hepatic metabolic pathways in terms of network visualizations. In our first study using the same dataset (Shahzad et al., 2015), we have focused on analyzing the metabolic and non-metabolic pathways using dynamic impact approach (DIA) (Bionaz et al., 2012).

The current study is focused on analyzing the role of differentially expressed genes (DEG) by means of network constructions. Furthermore, we focused on the transcription regulators to draw the biological networks. The objective of the study was to conduct a network analysis of transcription regulators of DEG at different time points (-30, 3 and 35 d) in transition cows as a mean to uncover additional mechanisms involved in regulating the hepatic response during calving seasons.

Materials and Methods

Experimental design and liver biopsies

Twelve (12) Holstein multiparous dairy cows were enrolled in the study. The cows were assigned into two groups based on calving season: Spring (SP, March-April, n = 6) and summer (SU, June-July, n = 6). All of these cows were housed in a free stall cubicle barn and were provided with cooling-ventilation system and concrete roofing. Mean temperature-humidity

indices for SP (day/night, below 72) and SU (day, 79.5 ± 2.9; night, 70.1 ± 4.7) were recorded. There were no clinical health problems observed in cows at the time of calving, and none of the cows received any treatment for metabolic disorders. The biopsies were performed under local anesthesia. Liver tissue was harvested at -30 (±2), 3, and 35 d relative to parturition and immediately saved in liquid nitrogen for short term. The tissue samples were stored at -80^{o}C until RNA extraction and microarrays analysis.

RNA extraction and microarrays

The total RNA was extracted from the liver tissue using QIAzol Lysis reagent (Qiagen, Chatsworth, CA, USA) by following our standard laboratory protocols. During the procedure, the homogenate was separated into aqueous and organic phases by centrifugation. The RNA was precipitated from the aqueous phase by adding isopropanol. The isolated RNA was aliquoted in DNase-free water, quantified using NanoDrop ND-1000 (Thermo Fisher Scientific Inc., Waltham, MA) and then stored at −80°C until further experiment. The extracted RNA was processed for quality control using the Agilent Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). Based on RIN > 6.5, the total RNA was processed for microarray analysis. The whole procedure of microarray is already published by our group and available at (Shahzad et al., 2015).

Briefly, the microarrays experiment was conducted using the 44K-Agilent bovine (V2) gene expression microarray chips (Agilent Technologies Inc.; cat# G2519F-023647). A total of 200 ng of RNA per sample was used to generate first-strand cDNA, which was subsequently reverse transcribed to cRNA using a low-input quick amp labeling kit (Agilent Technologies

Inc.; cat# 5190–2306). The resulting cRNA was labeled with either Cy3 or Cy5 fluorescent dye. Purification of the labeled cRNA product was performed with RNeasy mini spin columns (Qiagen, cat# 74104), and it was subsequently eluted in 30 μL of DNase-RNase-free water. The eluted labeled cRNA was quantified in a NanoDrop to confirm the manufacturer's recommended criteria for yield and specific activity of at least 0.825 μg and ≥6. The labeled cRNA was fragmented and then hybridized to the microarray slide. During this step, 825 ng of Cy3 and Cy5 labeled cRNA samples were combined; mixed with 11 μL of 10X Blocking Agent (Agilent Technologies Inc.; cat# 5188–5281), 2.2 μL of 25× Fragmentation Buffer (Agilent Technologies Inc.; cat# 5185–5974), and nuclease-free water (to a final volume of 55 μL); and then fragmented at 60°C for 30 seconds. The reaction was then stopped by adding 55 μL of 2×GEx Hybridization Buffer (Agilent Technologies Inc.; cat# 5190–0403), and then samples were loaded onto the slides. The samples were hybridized in a rotating hybridization oven at 65°C for 17 hours. The slides were washed according to the given instructions and scanned using a GenePix 4000B scanner (Axon Instruments Inc., Sunnyvale, CA) and GenePix Pro v.6.1 software. Resulting spots with substandard features were flagged and then excluded from the subsequent analysis.

Statistical analysis

Statistical analysis of microarray data was performed using the SAS (SAS Institute Inc., Cary, NC). Data from a total of 18 microarrays were adjusted for dye and array effects using lowess normalization and array centering method. A MIXED procedure of SAS with repeated measures was used to the normalized $\log_2$-transformed adjusted ratios. The model included the fixed effects of time (−30, 3, and 35 d), season (SP and SU), and interaction of time × season. Cows were considered as uncorrelated random effect. The raw *p*-values against multiple

comparisons were adjusted using Benjamini and Hochberg's false discovery rate (FDR) method (Benjamini and Hochberg, 1995). Differences in transcript profiles were considered significant at an FDR-adjusted *p*-value ≤ 0.10 for the gene network analysis.

Gene network analysis

Ingenuity Pathways Analysis (IPA) software was used for gene network analysis. A list of DEG along with corrected *p* values and fold change values was uploaded into the software. A core analysis tool was run under the IPA utility. From the core analysis, we used upstream analysis results for transcription regulators and their network reconstructions. The results were retrieved and are discussed below.

Results and Discussion

Gene expression pattern

For the current study, we used three time points (-30, 3, and 35 d) from SU vs. SP comparison. An FDR ≤ 0.10 and $p \leq 0.10$ were used as cut offs for the analysis purpose. We obtained 618 (384 up and 234 down), 1,030 (483 up and 547 down) and 894 (364 up and 530 down) DEG at -30, 3, and 35 d of SU vs. SP, respectively as shown by vertical bars in the Figure 5.1. The large number of expression pattern underscores the marked effects of seasonal calving at different time points. Tables 5.1-5.3 show the overall expression patterns of DEG with $p \leq 0.01$ and FC ≥ 4.0 to provide an overall picture of the summer calving effects compared with spring calving. In our previous work, we have shown that the DEG expressed in SU vs. SP are

the most importantly related with carbohydrates, lipids and amino acids metabolism under the metabolic category, and with stress response and immune system under the non-metabolic category (Shahzad et al., 2015). In another review, it was highlighted that heat stress adversely impacts the reproductive performance by affecting oocyte maturation and embryonic developmental processes in cattle and buffalo (Dash et al., 2016). It has also been associated with reduced dry matter intake (DMI) and decreased milk production (Brown et al., 2016). The research indicates a greater impact of temperature variations above the animal's thermo-neutral zone over the hepatic gene expression.

Transcription regulators

The upstream analysis of transcription regulators identified 6 (5 up and 1 down), 7 (2 up and 5 down) and 7 (2 up and 5 down) DEGs at d -30, 3 and 35 during SU vs. SP as shown in the Table 5.4. We observed that most of the transcription regulators were down regulated postpartum as compared to prepartum in SU vs. SP.

Day -30.

During SU vs. SP, we found five transcription regulators (STAT3, MYC, HNF4A, KAT2B, and RELA) that were up regulated, and one (NCOA1) that was down regulated (Table 5.4). Among the up regulated transcription regulators, HNF4A and MYC (both triggered by RELA) appeared to be key transcription regulators. These two transcription regulators were linked with several downstream target genes that were involved in oxidation of xenobiotic compounds (CYP3A4) (Tirona et al., 2003), tryptophan catabolism (ACMSD1) (Huo et al.,

2015), arginine catabolism (ARG1) (Wang et al., 2011), apoptosis regulation, and ER Calcium homeostasis (CFLAR, TMBIM6) (Xu et al., 2008). The results indicate that amino acids metabolism was more induced in the cows calved during the summer season.

The networks of RELA and MYC are shown in Figure 5.2. Their down regulated target genes were involved in cellular proliferation, anti-apoptotic activities, immune related disorders (CDKN1, LGALS1, TSPO) (Holtan et al., 2009, Liu et al., 2009, Green et al., 2014), and liver disease (SERPINA1, FTH1) (Rebl et al., 2012). The NCOA1 (Nuclear receptor coactivator 1) was down regulated in our results. The main role of this gene is to facilitate the assembly of basal transcription factors and steroid hormone regulation (Chen et al., 2010, Walsh et al., 2012). The dysregulation of this gene indicates abnormal hormonal control in the hepatic tissue.

Day +3.

After calving, we found two transcription regulators (BCL6, and PLAGL1) that were up regulated, and five transcription regulators (MYC, NCOA1, STAT1, HNF4A, and ZEB1) that were down regulated during summer calving as compared with spring calving. The upregulation of BCL6 was directly linked with the IL-6-dependent immune-response and cell growth (Choi et al., 2013). In contrast, BCL6 was associated with down regulation of IL7R, IL13R1 and CXCL10-dependent immune responses (Figure 5.3). This process indicates an activation of IL-6 dependent immunity and antigen processing and presentation response, while deactivation of other immune related reactions (e.g., IL2, TLL1, and IL7R) as shown in Table 5.2 (Karnowski et al., 2012). On the other hand, HNF4A and MYC were down regulated. The down-regulation of these genes indicate lower rate of cell proliferation and differentiation after calving.

Day +35.

After 35 days of calving, we found a different response in gene expression as shown in the Tables 5.3 and 5.4. The upstream analysis uncovered seven transcription regulators, of which two were up regulated (MYC and RELA), and five were down regulated (TFAM, FUBP1, KAT2B, HNF4A and ZNF274). The networks of RELA and MYC are shown in the Figure 5.4. The upregulation of RELA is associated with target genes involved in activation of anti-inflammatory response and antigen processing and presentation (CCL3, B2M) (Lim et al., 2007, Boulaire et al., 2009), extracellular matrix breakdown (MMP1) (Mishra et al., 2010), regulation of cell cycle (MYC, PTEN, CASP8) (Haupt et al., 2003, Hoffman and Liebermann, 2008) and reduced gluconeogenesis (PCK1, and KAT2B) (Ravnskjaer et al., 2013, Zhang et al., 2015). The data also indicate a more pronounced effect of immune response as specified by CCL3, IL27, and IFI30 genes shown in Table 5.3. The RELA gene dependent CCL3 activation is also indicated in the Figure 5.4, which in turn was induced by MYC gene. It has been shown that IL27 is involved in an innate immune response and is a strong inducer of cytokines and chemokines (Guzzo et al., 2010).

These findings indicate that carbohydrate metabolism was enriched at -30 and 3 d, while nucleic acid metabolism, immune response and cellular development were enriched at 3 and 35 d in SU vs. SP calving groups.

Conclusion

Gene network analysis highlighted several upstream transcription regulators and their target genes that were affected during summer (SU) and spring (SP) calving seasons. Among

these, cellular replenishment, carbohydrate metabolism, and immune system were the most enriched and activated pathways in SU vs. SP calving groups at different time points during the transition into lactation. The upstream analysis identified 6, 7, and 7 transcription regulators at -30, +3, and +35 d relative to parturition. The analysis uncovered HNF4A, and MYC (-30, 3, and 35 d), KAT2B and RELA (-30 and 35 d), and BCL6 (3 d) as key transcription regulators. HNF4A was appeared as the highly interconnected transcription regulator at all the three time points among the other transcription regulators. The network analysis identified cell death, survival, cellular growth and developmental processes as the most-enriched cellular and molecular functions. Carbohydrate metabolism was enriched at -30 and 3 d, while nucleic acid metabolism, immune response and cellular development process were enriched at 3 and 35 d. The evaluation of the results suggest that the molecular phenotype of the liver differs between the two calving groups. The functional relevance of the gene networks and their changes merit more detailed mechanistic studies.

Tables and Figures

Table 5.1: A list of differentially expressed genes with $p \leq 0.01$ and fold change $\geq |4.0|$ at -30 d during SU vs. SP.

Symbol	Description	SU vs. SP(-30 d)
PRC1	protein regulator of cytokinesis 1	-13.28
NEU4	sialidase 4	-11.37
CD34	CD34 molecule	-9.93
GANC	glucosidase	-8.09
WDR66	WD repeat domain 66	-7.98
DEFB119	defensin, beta 119	-6.20
PPP1R42	protein phosphatase 1	-5.89
ZIC2	Zic family member 2	-5.59
HSDL1	hydroxysteroid dehydrogenase like 1	-5.51
ZNF839	zinc finger protein 839	-4.80
LOC781910	olfactory receptor Olr1353	-4.72
PTGIS	prostaglandin I2 (prostacyclin) synthase	-4.53
MAGEB10	melanoma antigen family B	4.11
IMP5	signal peptide peptidase like 2C	4.22
RIMS2	regulating synaptic membrane exocytosis 2	4.28
MAGEE2	melanoma antigen family E	4.56
CCDC170	coiled-coil domain containing 170	4.62
GNG13	guanine nucleotide binding protein (G protein)	4.64
IL22	interleukin 22	4.72
BARHL1	BarH-like homeobox 1	4.77
PAG2	pregnancy-associated glycoprotein 2	4.79
HSD3B1	hydroxy-delta-5-steroid dehydrogenase	4.83
LGI1	leucine-rich	4.89
GLP1R	Uncharacterized protein	5.63
CCDC60	coiled-coil domain containing 60	5.73
OR51Q1	olfactory receptor	6.18
PPP1R27	protein phosphatase 1	6.91
LPAR3	lysophosphatidic acid receptor 3	7.02
EPHX4	epoxide hydrolase 4	7.54
ZNF280B	zinc finger protein 280B	10.15

Table 5.2: A list of differentially expressed genes with $p \leq 0.01$ and fold change $\geq |4.0|$ at +3 d during SU vs. SP.

Symbol	Description	SU vs. SP (+3 d)
PVRL1	poliovirus receptor-related 1	-19.16
LRRC49	leucine rich repeat containing 49	-14.43
OR2D3	olfactory receptor	-14.25
GAB3	GRB2-associated binding protein 3	-13.04
CHAT	choline O-acetyltransferase	-12.79
DYDC2	DPY30 domain containing 2	-9.70
CHAMP1	chromosome alignment maintaining phosphoprotein 1	-8.95
RAB3C	RAB3C	-8.78
ZNF280B	zinc finger protein 280B	-8.10
ADCYAP1R1	adenylate cyclase activating polypeptide 1 (pituitary) receptor type I	-7.40
SHANK3	SH3 and multiple ankyrin repeat domains 3	-7.19
TARSL2	threonyl-tRNA synthetase-like 2	-6.97
NKX2-1	NK2 homeobox 1	-6.95
B3GALNT1	beta-1,3-N-acetylgalactosaminyltransferase 1	-6.84
LOC781146	lysozyme	-6.61
NCCRP1	non-specific cytotoxic cell receptor protein 1 homolog	-6.59
MEX3D	mex-3 RNA binding family member D	-6.49
AP2A1	adaptor-related protein complex 2	-6.49
LOC100850628	olfactory receptor 51L1	-6.34
RIMS2	regulating synaptic membrane exocytosis 2	-6.24
SPIC	Spi-C transcription factor (Spi-1/PU.1 related)	-6.22
MTMR12	myotubularin related protein 12	-6.11
IL2	interleukin 2	-6.09
HMMR	hyaluronan-mediated motility receptor (RHAMM)	-6.06
PCSK2	proprotein convertase subtilisin/kexin type 2	-6.05
SCN3A	sodium channel	-5.98
LPCAT1	lysophosphatidylcholine acyltransferase 1	-5.92
INPP5K	inositol polyphosphate-5-phosphatase K	-5.81
RBL2	retinoblastoma-like 2 (p130)	-5.80
FARS2	phenylalanyl-tRNA synthetase 2	-5.72
CDH23	cadherin-related 23	-5.71
OVCH2	ovochymase 2	-5.68
HSD3B1	hydroxy-delta-5-steroid dehydrogenase	-5.60
LHCGR	luteinizing hormone receptor isoform 2 mRNA	-5.60

Table 5.2 (Cont.)

COG2	component of oligomeric golgi complex 2	-5.58
TLL1	tolloid-like 1	-5.57
IL7R	interleukin 7 receptor	-5.52
ZPBP2	zona pellucida binding protein 2	-5.48
ADAMTS4	ADAM metallopeptidase with thrombospondin type 1 motif	-5.39
NUMA1	nuclear mitotic apparatus protein 1	-5.37
XPR1	xenotropic and polytropic retrovirus receptor 1	-5.33
GPC5	glypican 5	-5.19
LOC789367	olfactory receptor 10C1	-5.14
TOP3B	topoisomerase (DNA) III beta	-5.02
LOC782792	putative olfactory receptor 5AK3	-4.95
POU1F1	Bovine growth-hormone factor 1 (bGHF-1) mRNA	-4.93
SIGLEC14	sialic acid-binding Ig-like lectin 1	-4.70
GCNT2	glucosaminyl (N-acetyl) transferase 2	-4.64
CRISP1	cysteine-rich secretory protein 1	-4.59
SLC44A4	solute carrier family 44	-4.55
LOC618816	olfactory receptor 6C2	-4.46
WIPF2	WAS/WASL interacting protein family	-4.45
DCLK1	doublecortin-like kinase 1	-4.43
CEP152	centrosomal protein 152kDa	-4.41
TMPRSS5	transmembrane protease	-4.40
LOC751811	AV618187 ovary fetus cDNA clone E1OV015H10 5'	-4.38
BAI3	brain-specific angiogenesis inhibitor 3	-4.32
ATP13A5	ATPase type 13A5	-4.28
UGT2B15	Uncharacterized protein	-4.27
HTR1D	5-hydroxytryptamine (serotonin) receptor 1D	-4.26
NUDT18	nudix (nucleoside diphosphate linked moiety X)-type motif 18	-4.20
LEPR	leptin receptor	-4.16
GATA2	GATA binding protein 2	-4.14
ACP2	acid phosphatase 2	-4.10
CXCL10	chemokine (C-X-C motif) ligand 10	-4.08
NTS	neurotensin	-4.06
GNAO1	guanine nucleotide binding protein (G protein)	-4.05
ABI3BP	target of Nesh-SH3	-4.04
MORN3	MORN repeat containing 3	-4.03
LOC100336669	guanylate-binding protein 4	-4.03
ACADM	acyl-CoA dehydrogenase	4.06
NFIL3	nuclear factor, interleukin 3 regulated	4.10
SNW1	SNW domain containing 1	4.15
PAG7	pregnancy-associated glycoprotein 7	4.25

Table 5.2 (Cont.)

DGUOK	deoxyguanosine kinase	4.28
LOC617119	olfactory receptor 8B3	4.44
PPIF	peptidylprolyl isomerase F	4.49
MIS18A	MIS18 kinetochore protein homolog A	4.89
ZNF236	zinc finger protein 236	5.34
PLAGL1	pleiomorphic adenoma gene-like 1	5.72
PCDHB11	protocadherin beta 11	6.10
ADAM1B	a disintegrin and metallopeptidase domain 1b	7.84
SNCA	synuclein alpha	10.63

Table 5.3: A list of differentially expressed genes with $p \leq 0.01$ and fold change $\geq |4.0|$ at +35 d during SU vs. SP.

Symbol	Description	SU vs. SP (+35 d)
CHAMP1	chromosome alignment maintaining phosphoprotein 1	-20.00
DBR1	debranching RNA lariats 1	-10.86
PNMAL1	PNMA-like 1	-10.13
H4	histone H4	-9.78
LOC617119	olfactory receptor 8B3	-8.05
PAG7	pregnancy-associated glycoprotein 7	-7.52
LOC789612	uncharacterized LOC789612	-7.51
PCSK2	proprotein convertase subtilisin/kexin type 2	-6.86
DSC3	desmocollin 3	-6.77
LAMC2	laminin subunit gamma 2	-6.59
SUMF1	sulfatase modifying factor 1	-6.50
CACNA1E	calcium channel, voltage-dependent, R type, alpha 1E subunit	-6.41
ACTG1	actin gamma 1	-6.15
PGPEP1L	pyroglutamyl-peptidase I-like	-5.84
B3GALNT1	beta-1,3-N-acetylgalactosaminyltransferase 1	-5.78
TLX1	T-cell leukemia homeobox 1	-5.69
ABCA2	ATP binding cassette subfamily A member 2	-5.59
LOC100140839	vesicle transport protein SFT2A	-5.55
C13H20orf26	chromosome 13 open reading frame	-5.47
LRRTM4	leucine rich repeat transmembrane neuronal 4	-5.09
A2ML1	alpha-2-macroglobulin-like 1	-4.79
LOC617079	ATP-binding cassette transporter C4-like	-4.70
CXCL2	chemokine (C-X-C motif) ligand 2	-4.64
JPH1	junctophilin 1	-4.09
DEFB123	defensin beta 123	-4.03
MAB21L3	mab-21-like 3 (C. elegans)	4.00
ZIC2	Zic family member 2	4.05
IFI30	interferon, gamma-inducible protein 30	4.13
IL27	interleukin 27	4.13
CNKSR2	connector enhancer of kinase suppressor of Ras 2	4.25
TOP2A	topoisomerase (DNA) II alpha	4.38
CA12	carbonic anhydrase XII	4.47
LOC505451	olfactory receptor, family 1, subfamily J, member 2-like	4.47
NTS	neurotensin	4.48
NXPH3	neurexophilin 3	4.73

Table 5.3 (Cont.)

COLEC12	collectin sub-family member 12	4.95
UNCX	UNC homeobox	5.04
PAG1	phosphoprotein membrane anchor with glycosphingolipid microdomains 1	5.16
MYH8	myosin, heavy chain 8, skeletal muscle, perinatal	5.18
ASPG	asparaginase homolog	5.42
NOD1	nucleotide-binding oligomerization domain containing 1	5.42
LOC614175	uncharacterized LOC614175	5.65
CA7	carbonic anhydrase VII	5.71
TMEM247	transmembrane protein 247	5.71
CHST4	carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 4	5.92
MEX3D	mex-3 RNA binding family member D	6.11
WHSC1	Wolf-Hirschhorn syndrome candidate 1	6.28
LOC787249	olfactory receptor 1J4	6.48
LAMC2	laminin subunit gamma 2	6.48
LOC516467	olfactory receptor 4F3/4F16/4F29	6.50
LPAR2	lysophosphatidic acid receptor 2	6.55
LOC525599	butyrophilin family member	7.38
LOC788592	serine/threonine-protein phosphatase 4 regulatory subunit 3B-like	8.18
KCTD13	potassium channel tetramerisation domain containing 13	8.75
EGFLAM	EGF-like	8.84
VIL1	villin 1	9.19
LOC519492	olfactory receptor 10T2	9.23
CD34	CD34 molecule	9.97
ST14	suppression of tumorigenicity 14 (colon carcinoma)	11.16
CCL3	chemokine (C-C motif) ligand 3	11.77
HTR4	5-hydroxytryptamine (serotonin) receptor 4	12.02
TMEM54	transmembrane protein 54	12.03
NTN4	netrin 4	13.38
DES	desmin	22.24
LOC784254	carbonic anhydrase 1	175.06

Table 5.4: Upstream transcription regulators, their fold change values and target genes predicted by IPA software.

Upstream Regulator	Fold Change	Total No.	Target molecules in dataset
SU vs. SP Day -30			
NCOA1	-1.187	8	C3,CDKN1A,FGF2,HSD3B2,KDR,MYC,NCOA2,PRLR
RELA	1.148	13	B2M,BIRC3,CCL3,CDKN1A,CYP3A4,DGCR6,FGF2,HNF4A,IL2,MMP1,MYC,RELA,SMAD4
KAT2B	1.377	4	B2M,CDKN1A,KAT2B,PRLR
HNF4A	1.514	62	ACP2,ACSS3,ACVR1,ADSS,ALDH1A1,AMBP,ANAPC15,BAD,BAZ1B,BCS1L,BNIP1,C11orf58,C1orf109,C3,CD46,CDC23,CDKN1A,CLTA,CROT,CWC15,CYP3A4,DNPH1,ECD,FTSJ1,FURIN,GIN1,GPX1,GSS,GYS2,HMGB1,HMGB2,HNF4A,HNRNPA0,KIF22,MBL2,MINA,MRPL2,MYC,NDUFV1,NME1,NONO,PEF1,PHPT1,PKM,POLR1B,PRDX5,PRLR,PUS3,RAB3C,RBM39,RBM42,RUVBL2,SELRC1,SERPINA1,SLC38A4,SMAD4,TMBIM6,TMEM101,TUBB4A,UGT2A3,VTN,YIF1A
MYC	2.201	33	ACTA1,ARG1,ATP13A2,CDKN1A,CFLAR,Clu,CLUH,COX5B,CSDE1,DCTPP1,DNPH1,FTH1,GDI2,HLA-A,HSPD1,KDR,LGALS1,MINA,MYC,NCL,NME1,ODC1,PKM,POLR1B,PPP1R15A,RUVBL2,SERPINA1,SLC3A2,TIMP1,TSPO,USP54,VIM,ZIC2
STAT3	2.34	20	ARG1,C5,CD46,CDKN1A,CFLAR,CTLA4,CYP26A1,FGF2,HNF4A,IL22,IL6R,KAT2B,KDR,MYC,NKX2-1,RALGDS,STAT3,TIMP1,TNFSF10,VIM
SU vs. SP Day 3			
MYC	-2.999	49	ACTA1,ACTN1,ARG1,ASNS,ATP13A2,BCL6,C1QBP,CASP8,CDK4,CDKN1A,CFLAR,CLUH,COX5B,COX6B1,CPT1A,CSDE1,CXCL10,DBI,DCTPP1,DNPH1,FTH1,GDI2,GOT1,HAMP,HSPD1,KDR,MGST3,MIF,MINA,MYC,NBN,NME1,ODC1,PAICS,PCK1,PDK1,POLR1B,PPP1R15A,PRDX4,PTEN,RPL27,RPS12,RUVBL2,SERPINA1,SLC3A2,TIMP1,TSPO,UGT1A6,USP54
NCOA1	-2.111	9	C3,CDKN1A,FGF2,HSD3B2,KDR,MYC,NCOA2,PCK1,PRLR
STAT1	-1.953	16	ARG1,BAD,C3,CASP8,CCL3,CDKN1A,CFB,CXCL10,FGF2,GBP1,IL2,LY6E,MYC,Rnf213,STAT1,TNFSF10
HNF4A	-1.713	112	ACLY,ACOX2,ACP2,ACSL1,ACSS3,ACTN1,ACVR1,ADH6,ADSS,ALDH1A1,ALDH8A1,AMBP,ANAPC15,ARHGEF19,BAD,BAZ1B,BCL6,BCS1L,BNIP1,C11orf52,C11orf58,C11orf71,C1orf109,C3,CCDC47,CD46,CDC23,CDK12,CDKN1A,COX7A2,COX7C,CPT1A,CRYZ,CYP3A4,DBT,DDX10,DNPH1,ECD,F11,FTSJ1,GIN1,GOT1,GPX1,GSS,GYS2,HMGB2,HNF4A,HNRNPA0,IFI30,IMMT,KIF22,LAPTM4A,LMAN2L,LYPLA2,MBL2,MGST3,MINA,MRPL2,MRPL24,MRPS21,MUT,MYC,NARS2,NME1,NONO,NUDT2,NUDT5,PCK1,PDZK1,PEF1,PGM1,PLAA,POLR1B,PRCC,PRDX5,PRLR,PRPS1,PSMB7,PSME3,PUS3,RAB3C,RABAC1,RBM39,RBM42,REPIN1,RIOK3,RNF113A,RNF5,RSL24D1,RTFDC1,RUVBL2,SELRC1,SERPINA1,SLC17A2,SLC25A1,SLC38A4,SMAD4,SNW1,SSSCA1,STK19,STYXL1,SUCLG1,TFPT,THRAP3,TMEM101,TMEM115,TMEM208,TP53RK,UBL7,UGT1A6,UGT2A3,YIF1A
ZEB1	-1.571	5	ESRP1,ESRP2,IL2,LAMC2,RBL2
BCL6	2.213	12	BCL6,CCL3,CDKN1A,CXCL10,FGF2,FTH1,IL13RA1,IL6R,IL7R,MCM3AP,MYC,PTEN
PLAGL1	5.717	3	ADCYAP1R1, CDKN1A,PTEN
SU vs. SP Day 35			
TFAM	-1.856	3	ACADM, ACADS, ACOX1
FUBP1	-1.738	2	CCNH, MYC
KAT2B	-1.565	4	B2M, HLA-B,KATB, PRLR
HNF4A	-1.465	97	ACLY,ACOX2,ACP2,ACSL1,ACTN1,ACVR1,AHSG,ALDH1A1,ALDH8A1,AMBP,ARHGEF19,BAD,BAZ1B,BCL6,BCS1L,BNIP1,C11orf52,C11orf58,C1orf109,C3,CCDC47,CD46,CDK12,CLTA,COQ10B,COX7C,CPT1A,CROT,CRYZ,CWC15,CYP3A4,DBT,ECD,F11,FURIN,GOT1,HMGB1,HMGB2,HNF4A,IFI30,IMMT,LAMTOR2,LARP4,LMAN2L,MBL2,MGST3,MINA,MRPL2,MRPL24,MRPS21,MUT,MYC,NARS2,NDUFV1,NME1,NONO,NUDT5,PALMD,PCK1,PDZK1,PEF1,PHPT1,PKM,PLAA,POLR1B,PRCC,PRDX5,PRLR,PRMT7,PSMB7,PUS3,RBM42,RIOK3,RNF113A,RSL24D1,RTFDC1,RUVBL2,SEC11A,SERPINA1,SLC25A1,SLC38A4,SMAD4,SNW1,STK19,STYXL1,TFPT,THRAP3,TMBIM6,TMEM101,TMEM208,TP53RK,UBL7,UGT1A6,UGT2A3,UQCC1,VTN,YIF1A
ZNF274	-1.377	1	HAMP
RELA	1.503	13	B2M,CASP8,CCL3,CFB,CYP3A4,DGCR6,HNF4A,MMP1,MYC,PCK1,PTEN,RELA,SMAD4
MYC	2.681	49	ACTA1,ACTB,ACTN1,ADK,ARG1,ASNS,ASS1,BCL6,CASP8,CD48,CDK4,Clu,COX5B,CPT1A,CSDE1,FTH1,GDI2,GOT1,HAMP,HLA-A,HNRNPA1L2,HSPD1,KDR,LGALS1,MGST3,MIF,MINA,MYC,NBN,NME1,ODC1,PAICS,PCK1,PDK1,PKM,POLR1B,PPP1R15A,PRDX4,PTEN,ROCK2,RPL27,RUVBL2,SERPINA1,TIMP1,TSPO,UGT1A6,USP54,VIM,ZIC2

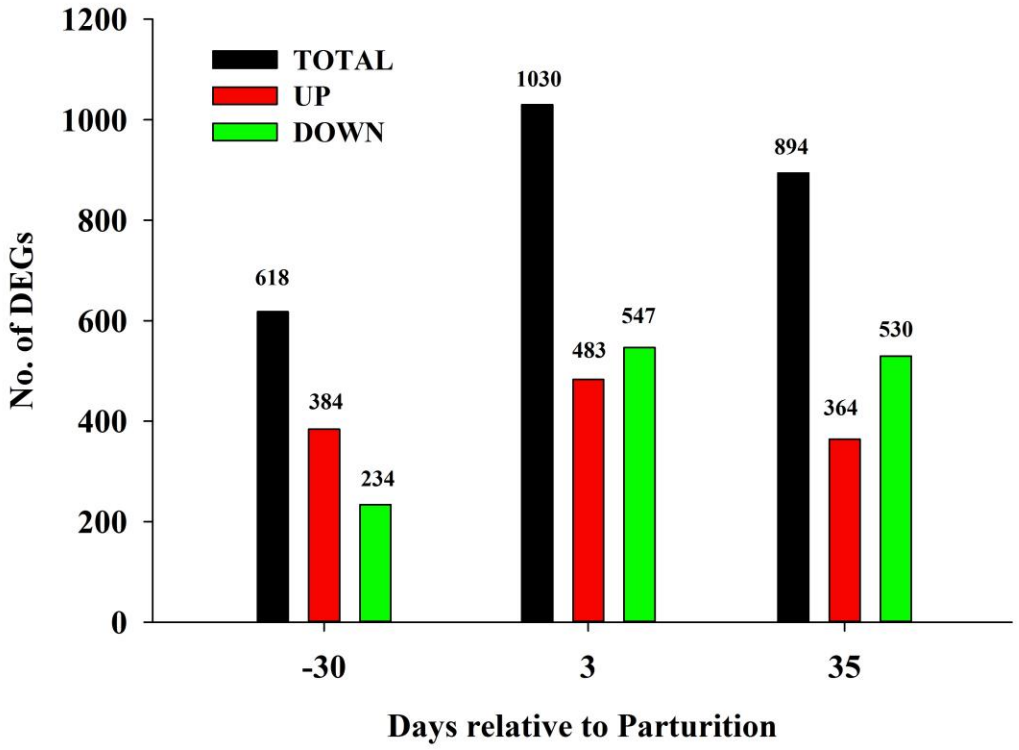

Figure 5.1: Differentially expressed genes (DEG) in SU vs. SP comparison are shown for each time point (-35, 3 and 30 d) relative to parturition. The X-axis represents the days relative to parturition, while the Y-axis represents the DEG that were up or down regulated at each time point.

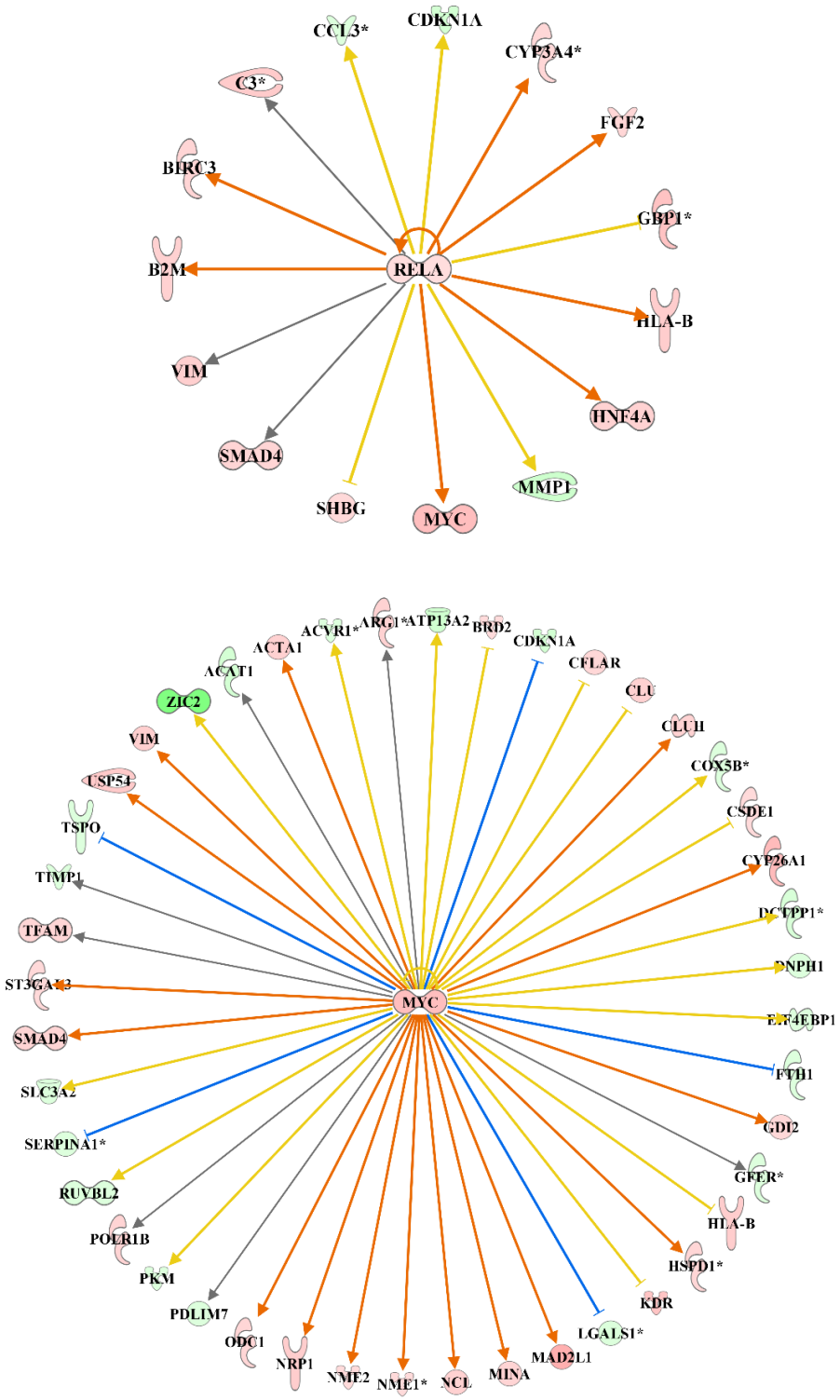

Figure 5.2: Transcription regulator network of **RELA** and **MYC** genes and their targets at -30 d during the SU vs. SP.

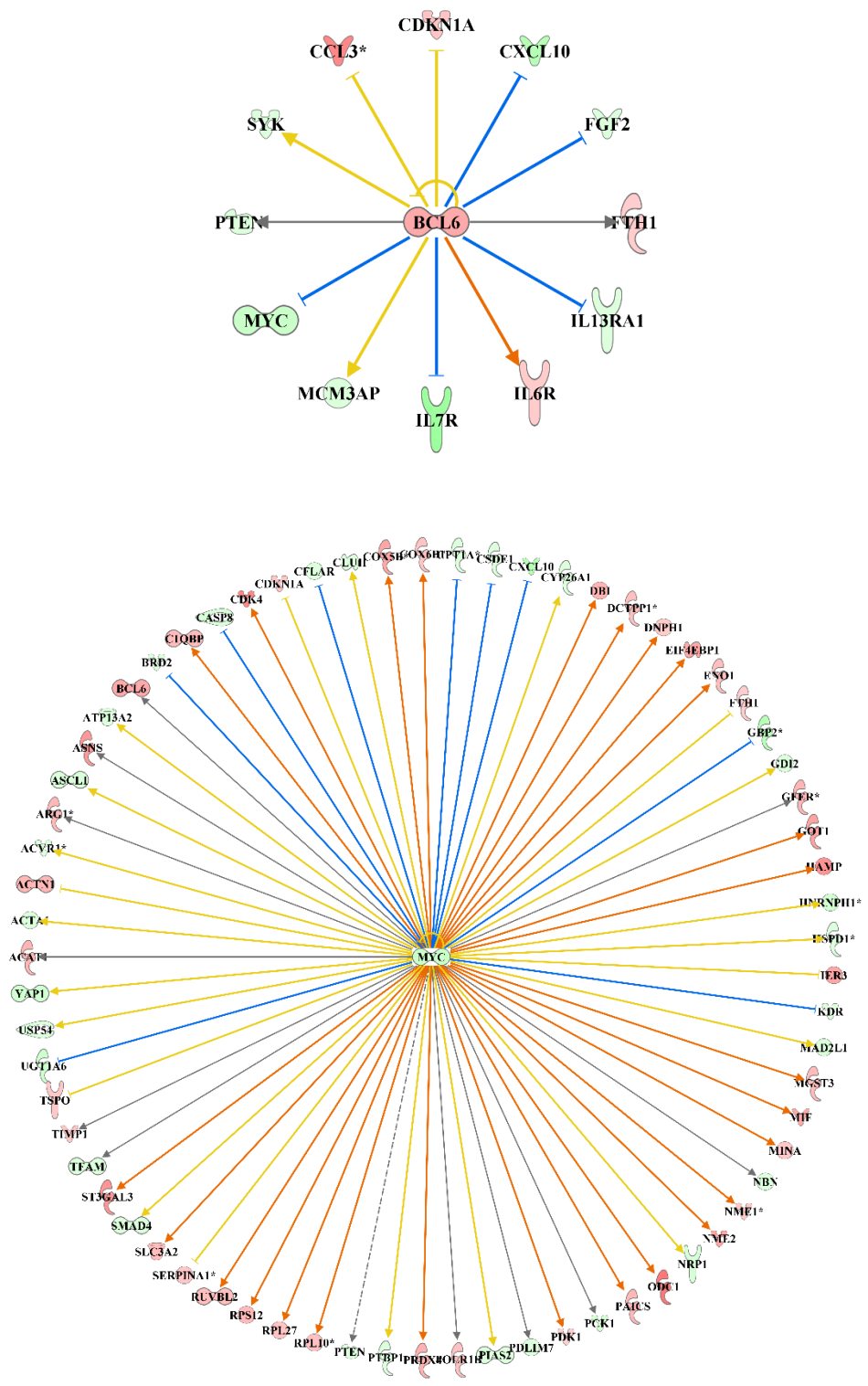

Figure 5.3: Transcription regulator network of BCL6 and MYC genes and their targets at 3 d during the SU vs. SP.

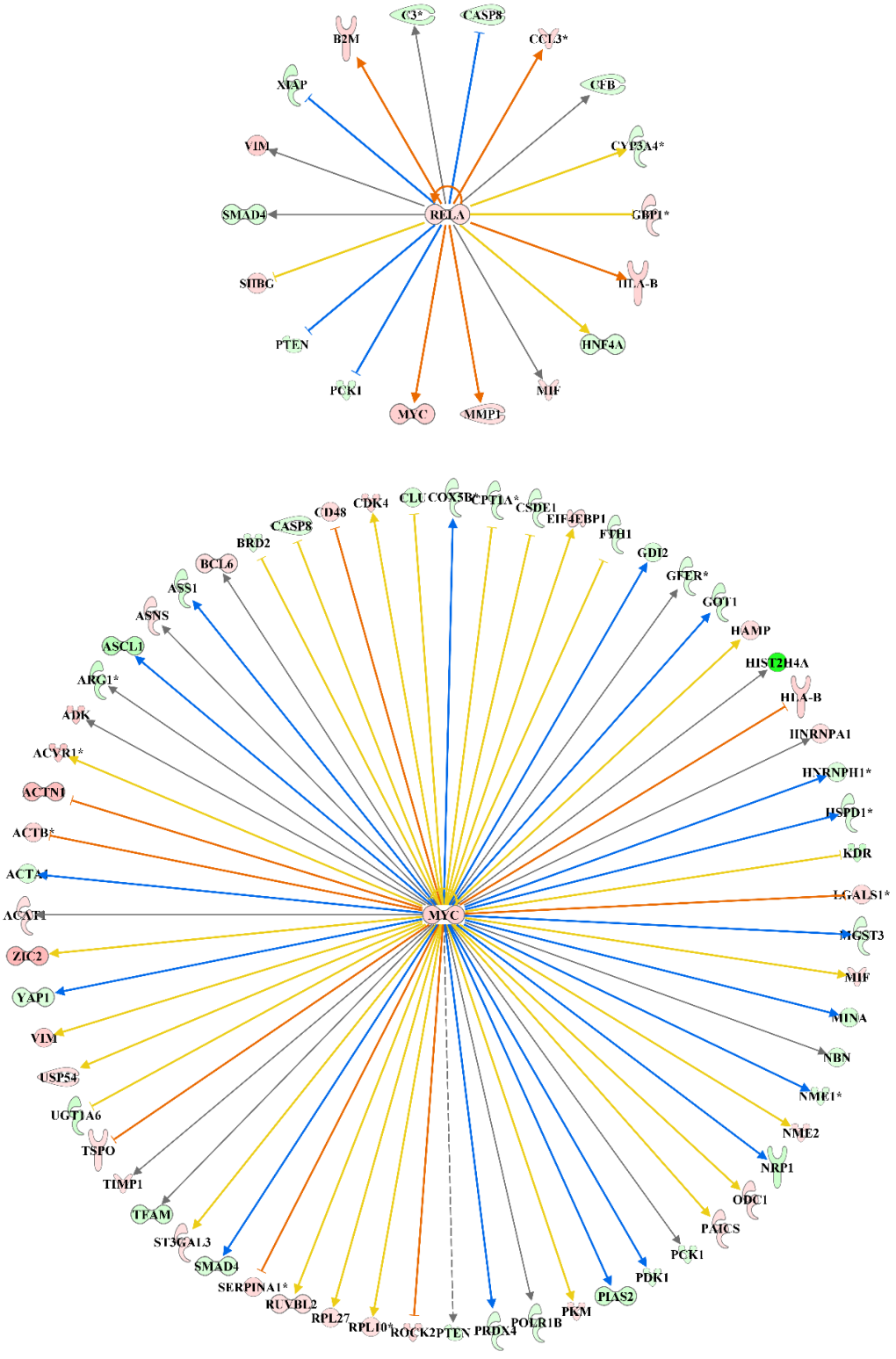

Figure 5.4: Transcription regulator network of **RELA** and **MYC** genes and their targets at 35 d during the SU vs. SP.

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CHAPTER # 6

Summary and conclusions

The overall focus of the study is to evaluate the hepatic gene expression under different physiological conditions by means of transcriptomics and metabolomics data using bioinformatics and systems biology approaches. Application of systems concepts to better understand physiologic and metabolic changes in dairy cows during the transition into lactation could enhance our understanding of the role of nutrients in helping meet the animal's requirements for optimal production and improved health. The transition period is marked by increased hormonal changes that lead to decreased dry matter intake, and increased energy demands for hepatic gluconeogenesis, fatty acid mobilization, and muscle degradation. These conditions may give rise to several metabolic disorders such as fatty liver, milk fever, mastitis, metritis and ketosis. Ketosis is one of the main metabolic disorders that arise from increased level of non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHBA) concentration. It is characterized into two different types clinical and sub-clinical based on the BHBA threshold levels in the blood. It has been shown that supplementation of methionine to maintain 3:1 ratio of lysine to methionine during the dry period may help to prevent ketosis development during early lactation. The onset of lactation is a critical step in regaining health of dairy cows postpartum. In the current research, four different studies were conducted to investigate the effects of methionine supplementation and the response of heat stress on the health of transition dairy cows using metabolomics and transcriptomics profiling techniques. The first three analyses dealt with supplementation of methionine to prevent clinical ketosis in high-genetic merit dairy cows. Four groups of cows were formed retrospectively based on clinical health evaluated at 1 week

postpartum: cows that remained healthy (OVE), cows that developed ketosis (K), and healthy cows supplemented with one of two commercial methionine products [Smartamine M (SM), or MetaSmart (MS)]. The analyses were performed in liver tissue harvested at -10 d relative to parturition from cows that were healthy on +7 d postpartum or were diagnosed with ketosis.

The first study deals with the comparison of the two groups of cows fed with moderate energy diet during the close-up dry period (-21 d to calving), and remained healthy (OVE, n=6) or developed ketosis (K, n=6) postpartum. 'Omics' and bioinformatics tools were used to identify the unique signatures characterizing the liver of cows with postpartal ketosis relative to healthy cows. The data was analyzed by MIXED procedure of SAS for both metabolomics and transcriptomics experiments. The metabolomics analysis resulted in 15 biochemical compounds ($p \leq 0.10$) out of 313 identified in the liver tissue, while the transcriptomics analysis resulted in 3,065 (2,091 up and 974 down) differentially expressed genes (DEG, $p \leq 0.05$ and FC $\geq |1.5|$) from 44 K Agilent bovine microarray for K vs. OVE. The functional analysis was performed using the Dynamic Impact Approach (DIA) and Ingenuity Pathway Analysis (IPA). The data revealed the involvement of several important pathways and biochemical compounds in the ketogenic process. In the ketotic group of cows compared with healthy group, we found the inhibition of several carbohydrate and lipid metabolism related pathways such as 'Glycolysis / Gluconeogenesis', 'Pentose phosphate pathway', Fatty acid biosynthesis', and 'Biosynthesis of unsaturated fatty acids'. However, the pathways related to amino acid metabolism were induced such as 'Glycine, serine and threonine metabolism' and 'Histidine metabolism'. The integration of metabolomics and transcriptomics results revealed the involvement of several non-metabolic pathways such as cell growth, proliferation, apoptosis, immune response, and insulin signaling.

The second study deals with the comparisons among different groups of cows fed moderate energy diet (OVE, n=6) and supplemented with Smartamine M (SM, n=6) and MetaSmart (MS, n=6) during the close up dry period. Supplements of methionine were top-dressed over the total mixed ration at a rate of 0.19 or 0.07% (DM) of feed for MS or SM. The liver tissue samples were used for metabolomics and transcriptomics analyses. The metabolomics study was conducted via LC-MS and GC-MS (Metabolon Inc.) and transcriptomics study was conducted using a whole-transcriptome bovine microarray (Agilent). From a total of 313 biochemical compounds identified, metabolomics analysis revealed 16, 26, and 36 compounds ($p \leq 0.10$) affected in SM vs. OVE, MS vs. OVE, and SM vs. MS, respectively. Comparing profiles in SM vs. OVE revealed that compounds up regulated belong to the gamma-glutamyl amino acid, purine metabolism, pentose, and sterol related pathways, while down regulated compounds belong to secondary bile acid, dipeptides, TCA cycle and eicosanoid metabolic pathways. In MS vs. OVE, the compounds up regulated belong to primary and secondary bile acid, purine metabolism, and lysolipids, while compounds down regulated were linked with glycolysis, gluconeogenesis, urea cycle, sphingolipid, and pyruvate metabolism. The transcriptome analysis of these groups resulted in 710 (SM vs. OVE), 786 (MS vs. OVE) and 601 (SM vs. MS) DEG ($p \leq 0.05$ and FC $\geq |1.5|$). Bioinformatics analysis using the DIA revealed that SM vs. OVE resulted in a marked impact and activation of 'Nitrogen metabolism', 'Glycosaminoglycan biocynthesis-chondroitin sulfate', 'Synthesis and degradation of ketone bodies' and 'Selenoamino acid metabolism'. In MS vs. OVE, however, among the top-10 most-impacted pathways there was a marked inhibition of 'Base excision repair', 'Cyanoamino acid metabolism', 'One carbon pool by folate' and related pathways, whereas the 'Riboflavin metabolism', and 'Vitamin digestion and absorption' were activated. Unique

responses in SM vs. MS included a marked activation of 'Intestinal immune network for IgA production', 'Antigen processing and presentation', and 'Notch signaling pathway'. The data interpretation suggests that MS and SM induce distinct changes on the metabolome and transcriptome phenotype of the prepartal liver.

The third study involves the comparison among the cows that developed ketosis (K, n=6) and the ones supplemented with SM (n=6) and MS (n=6), and remained healthy on +7 d postpartum. From a total of 313 identified biochemical compounds, metabolome analysis revealed 13 or 43 compounds ($p \leq 0.10$) in K vs. SM or K vs. MS. Among the up regulated compounds, mostly belong to primary bile acid, fatty acid, phenylalanine and tyrosine metabolism, urea cycle, arginine, and proline metabolism. Among the down regulated compounds, lysolipids, citrate cycle, and di-carboxylic acids along with components of purine and sphingolipid metabolism were identified as biomarkers. Citrate was markedly lower in the liver of K vs. SM. In K vs. MS, the up regulated compounds include intermediates of glycolysis/gluconeogenesis/pyruvate, histidine, and fatty acid metabolism. Among down regulated compounds, lysolipids, pentose metabolism (xylitol, ribulose, ribitol, and xylonate) and tryptophan metabolism were affected significantly. Analysis of transcriptomics data resulted in 1021 or 771 DEG ($p \leq 0.05$ and FC $\geq |1.5|$) in K vs. SM or K vs. MS. The analysis using DIA revealed deactivation of several pathways in K vs. SM such as 'Cynoamino acid metabolism', 'Other glycan degradation', 'Erb signaling' and 'Pentose phosphate pathway'. In K vs. MS, we found deactivation of 'Riboflavin metabolism', 'Pentose phosphate pathway', and 'Glycolysis/gluconeogenesis', whereas 'Nitrogen metabolism' was activated in this group. These results suggest that supplementation with SM or MS elicit distinct metabolomics and transcriptomics responses in the liver of transition dairy cows. The cows developing K

postpartum also had a distinct molecular phenotype compared with those supplemented with methionine. These findings indicate that 'omics' data integration could be helpful in better understanding the links between nutrition and incidence of metabolic disorders during early lactation, and to diagnose ketosis development using molecular signatures even before calving.

The fourth study deals with the effects of heat stress on the health and productivity of dairy cows. During this study, we used gene network analysis on transcriptome data to uncover transcription regulators and their downstream target genes. Twelve multiparous Holstein dairy cows were selected to harvest liver tissues at -30, +3, and +35 d relative to parturition during the spring (SP: March 28-April 30, n = 6) and summer (SU: June 15-July 02, n = 6). Mean temperature-humidity indexes for SP (day/night: below 72) and SU (day: 79.5±2.9, night: 70.1±4.7) were recorded. Transcriptomics was conducted using the 44K Agilent bovine microarrays. Statistical analysis with FDR ≤ 0.10 resulted in 618, 1,030 and 894 DEG for -30, +3 and +35 d respectively during SU vs. SP. Among molecular and cellular functions, IPA analysis identified cell death, survival, cellular growth and development as the most enriched functions. Carbohydrate metabolism appeared to be the most enriched at -30 and +3 d, whereas nucleic acid metabolism and cellular development were the most enriched at +3 and +35 d. A total of 6, 7 and 7 transcription regulators were identified at -30, +3 and +35 d. The IPA analysis uncovered HNF4A, and MYC (-30, 3 and 35 d), RELA and KAT2B (-30 and 35 d), and BCL6 (3) as important transcription regulators. Comparing SU vs. SP at -30 d revealed HNF4A and MYC (both triggered by RELA) as key transcription regulators linked with several downstream target genes. The up regulated target genes were involved in oxidation of xenobiotic compounds (CYP3A4), tryptophan catabolism (ACMSD1), arginine catabolism (ARG1), apoptosis regulation, and ER Calcium homeostasis (CFLAR, TMBIM6). In contrast, the down-regulated

target genes were involved in cellular proliferation, anti-apoptotic activities, immune related disorders (CDKN1, LGALS1, TSPO), and liver disease (SERPINA1, FTH1). At +3 d, both HNF4A and MYC were down regulated. The up regulation of BCL6 was directly linked with the IL-6 dependent immune response and cell growth. Additionally, the BCL6 was associated with down regulation of IL7R, IL13R1 and CXCL10-dependent immune responses. During lactation at +35 d, the up regulation of RELA was associated with target genes involved in activation of anti-inflammatory reactions (CCL3, B2M), extracellular matrix breakdown (MMP1), regulation of cell cycle (MYC, PTEN, CASP8) and gluconeogenesis (PCK1). The evaluation of these results suggest that calving during the summer vs. spring is in fact associated with molecular phenotypes of the liver.