EFFECT OF RUMEN PROTECTED VITAMIN B COMPLEX ON METABOLIC PARAMETERS, MILK PRODUCTION AND DAY 14 CONCEPTUS AND ENDOMETRIAL GENE EXPRESSION IN HOLSTEIN DAIRY COWS

by

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Abstract

High milk production is associated with sub-fertility in dairy cows due to metabolic demands placed by lactation on the body. The aim of this project was to determine the effects of a rumen-protected vitamin B complex supplementation (VB) compared with a control diet containing no supplementation (CON) on the endometrial gene expression on d 14 of pregnancy. The secondary aim was to look at effect of VB on milk production and components; concentrations of β-hydroxybutyric acid, haptoglobin and progesterone in blood; and ovarian dynamics. Fifty-one multiparous Holstein cows from the herd at The University of British Columbia Dairy Education and Research Centre were enrolled into the study three weeks prior to parturition and were randomly assigned to one of the two treatments. Biweekly blood samples, weekly milk samples and daily feed intake were collected. Cows were enrolled onto a double-ovsynch protocol at 33±3 days post-partum and inseminated by timed artificial insemination (AI). Ovarian structures were monitored and measured using *per rectum* ultra-sonography. The uterus was flushed on day 14 post AI for conceptus collection and endometrial samples were collected at the same time. Data was analyzed by analysis of variance (ANOVA) using the generalized linear model (GLM) procedure of SAS. Overall, 42 cows were flushed and 13 embryos were collected. VB supplementation had no effect on the size of the embryo, ovulatory follicle size or CL size at embryo collection. Milk production and milk fat values were also similar between the two groups. BHBA and haptoglobin levels between the two groups were also similar. Analysis of expression of genes showed that *OXTR*, *MUC5B*, *MUC1*, *IL1β*, *SPP*, *TRD*, *FZD8* and *FOLR1* genes were significantly upregulated in the VB group. *SELL*, *PLAU* and *MYH9* genes showed a tendency to be more upregulated in the endometrium of cows in the VB group compared to those

in the CON group. In conclusion, expression of genes related to embryo development, immune system, adhesion and regulation of folate transport were upregulated by supplementation. VB supplementation did not affect of production and health outcomes in lactating dairy cows.

Lay Summary

Lactating dairy cows have extensive embryonic loss, but the mechanisms behind this pregnancy failure are not clearly understood. It is suggested that cows experiencing pregnancy loss might have a marginal deficiency of nutrients such as B-vitamins, and their supplementation is positively associated with health and reproduction. The objectives of this project were to determine the effect of rumen-protected vitamin B complex supplementation compared with control diet on milk production, plasma levels of BHBA, haptoglobin and progesterone, ovarian dynamics and uterine expression of target genes on day 14 of pregnancy. Although no differences in milk production or metabolic parameters were observed, supplementation with B-vitamins upregulated the expression of 11 genes in the endometrium which are important in regulating immune system, adhesion, embryo growth and development and folate transport. These results help in increasing our understanding of the role of vitamin B complex in preventing embryonic loss in dairy cows.

Preface

The work presented in this thesis was conducted at The University of British Columbia (UBC) Dairy Education and Research Centre in Agassiz, BC. The project and associated methods within this thesis were approved by the University of British Columbia's Animal Care Ethics Committee [certificate # A150089]. The RNA extraction was performed at the UBC Dairy Education and Research Centre and nanostring analysis were performed at the Centre for Heart Lung Innovation at St. Paul's Hospital, Vancouver, BC.

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List of Abbreviations

ACP = Acyl carrier protein

AGPAT1 = 1-Acylglycerol-3-Phosphate O-Acyltransferase 1

AI = Artificial insemination

ANOVA = Analysis of variance

APC = Adenomatous polyposis coli

AXIN1 =Axin 1

AXIN2 = Axin 2

B3GAT1 = Beta-1,3-Glucuronyltransferase 1

BHBA = β-hydroxybutyrate

BMP15 = Bone morphogenetic protein 15

CADM3 = Cell adhesion molecule 3

CALB2 = Calbindin 2

CAPN6 = Calpain 6

CDH1 = Cadherin-1

CIDR = Controlled internal drug release

CL = Corpus luteum

CLD4 = Claudin 4

CoA = Coenzyme A

CTNNA2 = Catenin (cadherin-associated protein), alpha 2

CTNNB1 = Catenin beta 1 (also known as β-catenin)

CXCL10 = C-X-C motif ligand 10

CXXC4 = CXXC finger protein 4

CYP3A4 = Cytochrome P450 3A4

CYP4F2 = Cytochrome P450 Family 4 Subfamily F Member 2

CYP4X1 = Cytochrome P450, family 4, subfamily X, polypeptide 1

DAG = Diacylglycerol

DEFB1 = β-defensin

DGKA = Diacylglycerol kinase alpha

DHF = Dihydrofolate

DIM = Days in milk

DKK1 = Dickkopf homolog 1

DMI = Dry matter intake

dTMP = Deoxythymidylate monophosphate

dUMP = Deoxyuridine monophosphate

ECM = Extracellular matrix

EEF1A1 = Eukaryotic elongation factor 1-alpha-1

EMMPRIN = Extracellular matrix metalloproteinase inducer

ERα = Estrogen receptor alpha

ERβ = Estrogen receptor beta

FAD = Flavin adenine dinucleotide

FGCP = Folypoly γ-glutamyl carboxypeptidases

FMN = Flavin mononucleotide

FOLR1 = Folate receptor 1

FTH1 = Ferritin heavy chain polypeptide I

Fzd = Frizzled

FZD4 = Frizzled 4

FZD7 = Frizzled 7

FZD8 = Frizzled 8

GLM = Generalized linear model

GLYCAM1 = Glycosylation-dependent cell adhesion molecule-1

GnRH = Gonadotropin releasing hormone

GPX4 = Glutahione peroxidase-4

GSK-3 β = Glycogen synthase kinase 3β

HPGD = 15-alpha hydroxyprostaglandin dehydrogenase

HOXA10 = Homeobox A10

HOXB7 = Homeobox B7

IDO = Indoleamine 2,3-dioxygenase

IF = Intrinsic factor

IFNT = Interferon-tau

IGF1 = Insulin like growth factors 1

IGF2 = Insulin like growth factors 2

IGF1R =IGF1 receptor

IGFBP1 = IGF binding protein 1

IGFBP2 = IGF binding protein 2

IGFBP3 = IGF binding protein 3

IGHG1 = Immunoglobin heavy constant gamma 1

IGLL1 = Immunoglobulin lambda like polypeptide 1

IL-1β = Interleukin-1 beta

IL-6 = Interleukin-6

IL-8 = Interleukin-8

IL-10 = Interleukin-10

ISG = Interferon stimulated genes

ISG15 = Interferon-stimulated gene 15

LGALS3BP = Galectin-3-binding protein

LH = Leutinizing hormone

LIF = Leukemia inhibitory factor

LIFR = LIF receptor

LYZ2 = Lysozyme 2

MCT = Monocarboxylate transporter

MMP19 = Matrix metalloproteinase 19

MOGAT1 = Monoacylglycerol O-acyltransferase 1

MSH1 = Msh homeobox 1

MUC1 = Mucin 1

MUC4 = Mucin 4

MUC5B = Mucin5B

MX2 = Myxovirus resistance 2

MYH9 = Myosin heavy chain 9

MYH10 = Myosin heavy chain 10

MYL12A = Myosin light chain 12A

NAD = Nicotinamide adenine dinucleotide

NADP = Nicotinamide adenine dinucleotide phosphate

NEBAL = Negative energy balance

NEFA = Non-esterified fatty acids

NF-κB = Nuclear factor kappa B

NNMT = Nicotinamide N-methyltransferase

NRC = National research council

NR1I2 = Nuclear receptor subfamily 1 group I member 2

OXT = Oxytocin

OXTR = Oxytocin receptor

PABA = Para-aminobenzoic acid

PC = Phosphatidylcholine

PCFT = Proton-coupled folate receptor

PEMT = Phosphatidylethanolamine N-methyltransferase

PFKFB2 = 6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 2

PGE_2 = Prostaglandin E_2

PGF2α = Prostaglandin F2α

PGR = Progesterone receptor

PL = Pyridoxal

PLAU = urokinase-type plasminogen activator

PLP = Pyridoxal phosphate

PM = Pyridoxamine

PMP = Pyridoxamine phosphate

PN = Pyridoxine

PNP = Pyridoxine phosphate

PRKCG = Protein kinase C

PTGES = Prostaglandin E synthase

PTGS2 = Prostaglandin-endoperoxide synthase 2

PTX3 = Pentatraxin-related protein 3

PXR = Pregnane X receptor

RELN = Reelin

SAM = S-adenosyl methionine

SELL = L-selectin

SERPING1 = Serpin Family G Member 1

SGK1 = Serum/Glucocorticoid Regulated Kinase 1

SLC = Solute carrier

SLC2A5 = Solute Carrier Family 2 Member 5

SLC5A6 = Solute Carrier Family 5 Member 6

SLC7A10 = Solute Carrier Family 7 Member 10

SLC27A6 = Solute Carrier Family 27 Member 6

SLPI = Secretory leucocyte protease inhibitor

SMVT = Sodium dependent multivitamin transporter

SPP1 = Secreted phosphoprotein 1

TAG = Triacylglycerol

TC = Transcobalamin

TDP = Thiamin diphosphate

THF = Tetrahydrofolate

TIMP2 = Tissue inhibitor of metalloproteinases 2

TMP = Thiamin monophosphate

TNFα = Tumor necrosis factor alpha

TPP = Thiamin pyrophosphate

TRD = T cell receptor delta

TTP = Thiamin triphosphate

UHRF1 = Ubiquitin-like with PHD and RING finger domains 1

VEGFA = Vascular endothelial growth factor A

VLDL = Very low density lipoproteins

WISP2 = WNT1 inducible signaling pathway protein-2

Wnt = Wingless type

WNT2 = Wnt Family Member 2

WNT3 = Wnt Family Member 3

WNT3A = Wnt Family Member 3A

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Chapter 1: Introduction

1.1 Background Information

The historical decrease in fertility of the lactating dairy cow worldwide is affected by high demands of milk production, and genetic, physiologic, environmental and human factors. Lactating dairy cows have extensive embryonic loss, but the mechanisms that lead to this failure to maintain pregnancy are not clearly understood (Bauersachs et al., 2005). The association between sub-fertility and lactation are thought to be the result of metabolic changes post-calving, which are necessary to support milk production, and thus directly affect reproductive tissues (Santos et al., 2004).

Milk production in North America has nearly doubled over the last 50 years, from approximately 65 million tonnes in 1963 to nearly 100 million tonnes in 2013 (FAOSTAT, 2015). Not only has total milk production increased, but so has production per animal. Cows in the U.S. produced approximately 2,074 kg milk per year in 1944 but this has risen to 9,193 kg per year in 2007, when adjusted for a 14-month calving interval and a 60-day dry period (Capper et al., 2009).

Greater milk production has been associated with physiological changes that can reduce fertility such as accentuated negative energy balance in early postpartum cows (Walsh et al., 2011) and lower circulating concentrations of progesterone and estradiol (Wiltbank et al., 2006). Energy balance is defined as the difference of net energy intake minus net energy expenditure for maintenance and milk production. If energy expenditure is greater than energy intake, energy balance is negative (van Knegsel et al., 2005). Parturition causes an abrupt shift in the metabolic demands of dairy cows from storing nutrients to rapid mobilization of lipid and protein stores in order to support the sudden onset of high milk production (Butler, 2000). This rapid increase in

energy requirements at the onset of lactation results in a negative energy balance which begins a few days before calving and usually reaches its most negative level about 2 weeks post calving (Butler, 2003). In cows having prolonged negative energy balance during early lactation, resumption of estrous cycle has been shown to be delayed. Both duration and magnitude of negative energy balance have been associated with reduced hypothalamic GnRH pulse frequency as well as persistence of a negative feedback loop between estradiol concentration and required LH surge necessary for development of dominant follicle and ovulation (Walsh et al., 2007; Rutherford et al., 2016). Exacerbated negative energy balance also prolongs the period of anovulation and compromises subsequent fertility (Butler, 2000).

Studies have shown that specific nutritional supplements such as essential fatty acids (Thatcher et al., 2011), choline (Santos et al., 1998) and vitamin B complex molecules (Juchem et al., 2012) have a positive effect on negating effects of negative energy balance and improving reproductive success. It is suggested that the high-producing dairy cows are marginally deficient in these nutrients and supplementation may restore the optimal function of vital systems such as metabolic balance and reproduction (Girard and Matte, 1999). Because the success of every dairy farm depends on the health and production of their cows, recent research has focused on the effects of vitamin B supplementation on production and health of dairy cattle, given the role of B-vitamins in metabolism and energy production.

1.2 Vitamin B complex and ruminants

Vitamin B complex is a group of 8 distinct water soluble vitamins that are vital as cofactors to several metabolic processes, including fatty acid transport and milk protein synthesis. Ruminant B-vitamin research was quite prevalent during the mid 1940's to late 1950’s, with the majority of this research being conducted in sheep, calves, and steers. Ruminants are

characterised by a multicompartmental stomach consisting of rumen, reticulum, omasum and abomasum. Ruminal bacteria help in fermentation of cellulose, producing volatile fatty acids such as acetate, propionate and butyrate, which are the main energy source for the ruminants and are absorbed by the ruminal walls. The rest of the alimentary canal and digestion process is similar between monogastrics (such as pigs, dogs and humans) and ruminants (Baile and Forbes, 1974). Bechdel et al. (1928) showed that along with cellulose fermentation, the bacteria present in rumen are also able to synthesize most B vitamins in the body. Earlier research into B vitamins indicated that the amount synthesized was enough to meet the requirements of the body, when provided with a balanced ration (Hunt et al., 1941; Lardinois et al., 1944). This postulation was also supported by National Research Council (NRC, 2001). In fact, true clinical deficiency of these vitamins is very rare in ruminants with a healthy and functioning rumen (Girard and Duplessis, 2016). NRC (2001, p173) calculated an estimate of dairy cow vitamin B requirements and reported only folic acid and pantothenic acid to be the limiting B-vitamins in a 650 kg lactating cow. Based on results reported by Schwab et al. (2006) and Santschi et al. (2005), Sacadura et al. (2008) estimated a deficit for folic acid, pantothenic acid and pyridoxine in cows, which indicates that the demand for B-vitamins could have changed in modern dairy cows and ruminal synthesis alone might not be sufficient to meet the requirements.

Very limited amount of research has been done in the last few decades on vitamin B and there is inadequate and conflicting evidence on the ruminal synthesis, bioavailability and requirement of vitamin B molecules in modern dairy cattle (Santschi et al., 2005b). Attempts to measure ruminal synthesis of B vitamins have delivered conflicting results, given the effect of ration composition on ruminal synthesis. Different forage to concentrate ratios generally influences the kind and quantity of B vitamin being synthesized by ruminal bacteria (Santschi et

al., 2005b; Seck et al., 2017). Seck et al. (2017) found no changes in B_{12} synthesis with different forage to concentrate ratios, however Santschi et al. (2005b) reported a decrease in B_{12} with low forage diets and Schwab et al. (2006) reported an increase in synthesis with lower forage diets. Seck et al. (2017) suggested that the dietary ingredients and nutrient composition of forage and concentrates affect the ruminal synthesis more than forage to concentrate ratios. Even though these studies showed different results, the authors agreed that B vitamin requirements of dairy cows have changed and more studies are required to understand ruminal synthesis in modern cows, to come up with supplementation strategy.

To further support the postulation that cows can benefit from vitamin B complex supplementation, recent research has shown an increase in milk production and/or component yield when cows are supplemented with one or more vitamin B molecules (Sacadura et al., 2008; Chen et al., 2011; Evans and Mair, 2013). Similarly, a positive effect of supplementation on health has also been reported, especially in early lactation dairy cows (Graulet et al., 2007; Duplessis et al., 2014; Li et al., 2016), which is achieved via improvement in metabolic efficacy. The research focus of supplementation of individual or multiple B vitamins has been on improving milk production and component yield. Juchem et al. (2012) measured effect of B complex (biotin, pantothenic acid, folic acid, cyanocobalamin and pyridoxine) supplementation on reproductive performance and reported a significantly increased first service conception rate in supplemented cows, at d 42 after AI. This study demonstrated that effects of B complex supplementation extend beyond improved production and metabolic efficacy. The results seen in this study were attributed to improved oocytes quality due to the important role that B vitamins play in one carbon metabolism.

Results observed by Juchem et al. (2012) as well as the previous research on milk production highlight the complex interactions between B complex vitamins occurring in the body. Juchem et al. (2012) used a commercial vitamin B complex supplement for dairy cattle, Jefo Lactation VB, which contained biotin, pantothenic acid, pyridoxine, folic acid and B_{12}. In this project, along with Lactation VB, Jefo Transition VB was supplemented as well during the transition period, which contained choline, riboflavin, folic acid and B_{12}. The following section contains an overview of fate of supplemented B vitamins in the gastro-intestinal tract and their functions in the mammalian body.

1.3 Vitamin B Complex – An overview

Vitamins are organic substances that are essential in minute quantities to the nutrition of most animals and some plants, act especially as coenzymes and precursors of coenzymes in the regulation of metabolic processes but do not provide energy or serve as building units, and are present in natural foodstuffs or sometimes produced within the body. Vitamins are broadly classified based on their solubility as either fat-soluble or water-soluble and each group has certain characteristic properties. As the name suggests, fat-soluble vitamins are lipophilic and readily dissolve in fat. Their absorption, therefore, is associated with the absorption of lipids and requires bile salts. Fat-soluble vitamins are also stored in the body for longer periods of time. Vitamins A, D, E and K are the four fat-soluble vitamins. On the other hand, water-soluble vitamins (vitamin B and C) dissolve easily in water, and hence are directly absorbed into portal blood and they are excreted from the body rapidly.(Gropper and Smith, 2013).

Thiamin, riboflavin, niacin, pyridoxine, pantothenic acid, biotin, folic acid and B_{12} have very distinct chemical structures and generally different functions, although they are all involved, in metabolism. Another water soluble micronutrient choline is also grouped together with B

vitamins, due to functional interactions with some B complex vitamins. These 8 distinct vitamins and choline are grouped as a complex, also named vitamin B complex, because of the interdependency of their functions.

1.3.1 Riboflavin

Riboflavin or Vitamin B_2 was discovered in 1917 and was originally called Vitamin G in the United States (Gropper and Smith, 2013). The name riboflavin is derived from the ribose like side chain (ribitol) and its yellow color (*flavus* = "yellow" in Latin).

1.3.1.1 Digestion, Absorption, Transport and Storage

Riboflavin is found in food in its free form, bound to proteins, as riboflavin phosphate or in one of its two coenzyme forms - flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Since riboflavin is absorbed in its free form, protein-bound riboflavin is released by action of hydrochloric acid in the stomach as well as the gastric and intestinal enzymes (Gropper and Smith, 2013). In the intestine, the coenzyme form FAD is converted to FMN by FAD pyrophosphatase and FMN phosphatase converts FMN to riboflavin. Riboflavin phosphates are hydrolyzed by other intestinal phosphatases to release riboflavin. Histidine or cysteine bound riboflavin, however, is not digested and remains unavailable to the body (McDowell, 2008; Gropper and Smith, 2013).

Riboflavin is absorbed primarily in the proximal small intestine. The absorption into intestinal cells occurs actively via riboflavin transporter 2. In larger concentrations, absorption also occurs via diffusion (Gropper and Smith, 2013). Inside the intestinal cells, and other tissue cells as well, riboflavin undergoes the process of metabolic trapping, where it is converted to its coenzyme forms by flavokinase (riboflavin → FMN) and FAD synthetase (FMN → FAD) (McCormick, 1989; Gropper and Smith, 2013).

Since only free riboflavin can cross the cell membranes, FMN is converted back to riboflavin at the serosal membrane of small intestine for transportation. Riboflavin, FMN and FAD are transported in the blood by proteins such as albumins, globulins and fibrinogen (Gropper and Smith, 2013). Within tissues, riboflavin is primarily stored as FMN, followed by FAD and greatest concentrations are found in the liver, kidney and heart (McDowell, 2008; Gropper and Smith, 2013).

1.3.1.2 Functions

Riboflavin plays a major role in oxidation-reduction reactions in energy, carbohydrate, lipid and amino acid metabolism as coenzyme in the form of FMN and FAD (Depeint et al., 2006a; Henriques et al., 2010; Gropper and Smith, 2013). A primary deficiency of dietary riboflavin has wide implications for other Vitamins as well, since flavin coenzymes are involved in the metabolism of folates, pyridoxine, and niacin as well.

As part of the respiratory chain, both FMN and FAD play a major role in controlling the flow of electrons along complex I and II of the electron transport chain to oxygen (Gropper and Smith, 2013). In complex I, FMN transfers electrons between niacin containing coenzymes and cytochromes. Flavoenzymes also link complex II of the electron transport chain to Krebs' cycle. In Krebs' cycle, FAD is necessary for oxidation of succinate to fumarate in the presence of succinic dehydrogenase. FAD is reduced to $FADH_2$, which is utilized in complex II and oxidized back to FAD. The end results of these reactions are generation of energy via production of ATP. In Krebs' cycle, FAD is required as electron carrier in oxidative decarboxylation of pyruvate and α-ketoglutarate (Depeint et al., 2006a; Gropper and Smith, 2013).

Another important metabolic role of riboflavin is in fatty acid oxidation. Fatty acids are carboxylic acids with long straight or branched hydrocarbon chains and they are very important

in cells as they are needed for enzymes, hormones, cell membrane and as a source of energy. Fatty acids are broken down in the mitochondria by the process of beta oxidation, to produce acetyl-CoA, NADH and FADH, which will enter the Krebs' cycle and electron transfer chain, respectively (Depeint et al., 2006a; Henriques et al., 2010). This oxidation occurs in the presence of enzyme acyl-CoA dehydrogenase, which utilizes FAD as a co-factor (Gropper and Smith, 2013). In amino acid metabolism, riboflavin is required as part of amino acid oxidases to oxidize α-amino acid to corresponding amino acids and α-keto acids (Gropper and Smith, 2013).

Riboflavin shows extensive interdependency with other vitamins. The flavoenzymes affect the conversion of vitamin B_6 to its physiologically active form (McCormick, 1989). Synthesis of active form of folate, 5-methyl tetrahydrofolate, also requires riboflavin dependent enzymes, as do synthesis of niacin from amino acid tryptophan and choline metabolism. Another important role of FAD is in antioxidant metabolism and oxidant production by reducing oxidized form of glutathione (GSSG) into reduced form (GSH) (McDowell, 2008; Gropper and Smith, 2013).

1.3.1.3 Deficiency

Isolated riboflavin deficiency is rare and the deficiency generally occurs along with other nutrient deficiencies. In humans, a deficiency of riboflavin results in ariboflavinosis, which is characterized by glossitis, geographic tongue, dermatitis and cheilosis (Gropper and Smith, 2013).

In young ruminants, without rumen microflora, riboflavin deficiencies can be established which results in lesions of mouth, inflammation of oral mucosa, hair loss and reduced growth. In swine, riboflavin deficiency causes reproductive impairment resulting in fetus resorption and premature parturition, with piglets dying in a few days of birth (McDowell, 2008).

1.3.2 Pantothenic Acid

Pantothenic acid derives its name from the Greek word *pantos*, which means everywhere and this vitamin is also found widely in nature. It is found in virtually all plant and animal food sources, making its deficiency very unlikely. This vitamin is metabolically active as component of coenzyme A (CoA) and acyl carrier protein (ACP) (McDowell, 2008; Gropper and Smith, 2013).

1.3.2.1 Digestion, Absorption, Transport and Storage

Pantothenic acid occurs both in free form as well as bound to CoA and ACP. Bound vitamin is hydrolyzed in several steps to pantothenic acid in the intestine. Absorption occurs mainly in the jejunum through passive diffusion at higher concentrations and at low concentrations, via an active sodium dependent multivitamin transporter (SMVT), which it shares with biotin and lipoic acid (Gropper and Smith, 2013).

Pantothenic acid travels in free form in blood plasma and red blood cells. Tissue uptake is also through SMVT. In the cells, most of pantothenic acid is used for the synthesis of CoA and some for ACP (Depeint et al., 2006a; Gropper and Smith, 2013). This vitamin is not stored to that extent in the body. Highest concentrations are found in liver, kidney, adrenal glands, brain and heart, where it can be found in CoA form (McDowell, 2008; Gropper and Smith, 2013).

1.3.2.2 Functions

The major role of pantothenic acid is in nutrient metabolism, as a constituent of CoA and ACP (Depeint et al., 2006a; Gropper and Smith, 2013). One of the most crucial roles of CoA is in conversion of pyruvate to acetyl-CoA. Acetyl-CoA is crucial for energy transformation reactions as it is important for the metabolism of three energy producing nutrients –

carbohydrates, amino acids and fatty acids (Depeint et al., 2006a; Gropper and Smith, 2013). A brief summary of this process is given below:

1. Carbohydrate metabolism: During glycolysis, glucose is converted to pyruvate, which it undergoes oxidative decarboxylation to form acetyl-CoA in the presence of CoA. Acetyl-CoA then condenses with oxaloacetate to introduce acetate for oxidation in the Krebs' cycle (Depeint et al., 2006a; Gropper and Smith, 2013).
2. Lipid metabolism: The process by which fatty acids are used to make acetyl-CoA is called beta oxidation. The fatty acids first react with free CoA in cytosol to fatty acyl-carnitine, in a series of steps, which is shuttled inside mitochondrial membranes and converted to fatty acyl-CoA (McDowell, 2008; Gropper and Smith, 2013). Acyl-CoA is then converted to acetyl-CoA through the process of beta oxidation. This acetyl-CoA can either enter Krebs' cycle or condense with another acetyl-CoA molecule to form acetoacetyl-CoA. In the liver, acetoacetyl-CoA forms two ketone bodies, acetoacetate and β-hydroxybutyrate (BHBA). These ketone bodies act as a substitute for glucose, as they can be converted back into acetyl-CoA by cells and used as fuel for Krebs' cycle (Depeint et al., 2006a; Gropper and Smith, 2013). Acetoacetyl-CoA can also condense with acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA. This is a rate limiting step in the synthesis of cholesterol. Cholesterol is then utilized for the formation of steroid hormones, such as progesterone and estrogen, bile salts and Vitamin D (Tsuchiya et al., 2005; Gropper and Smith, 2013). Acetyl-CoA and CO_2 condensation results in the formation of malonyl-CoA, which is the first step in the formation of fatty acids. These fatty acids are then used for the synthesis of triglycerides, phospholipids and sphingolipids (McDowell, 2008; Gropper and Smith, 2013).

3. Protein metabolism: under low glucose levels, proteins can be used to produce acetyl-CoA as well. The amino acids are first converted into α-keto acids through the process of transamination, which are then further converted to acetyl-CoA in a series of reactions. CoA is also required for the process of gluconeogenesis, when amino acids are converted to glucose thorugh oxidative decarboxylation of α-ketoglutarate to succinyl-CoA (McDowell, 2008; Gropper and Smith, 2013).

Some other functions of pantothenic acid as CoA include posttranslation acetylation of proteins and sugars, which affects their function, activity and location. Acylation can help prolong the half-life of some proteins, such as proteins important for maintaining cell's cytoskeleton, histones and DNA binding proteins. Acylation can also play a role in activation/deactivation of some enzymes. Acylation of choline results in formation of neurotransmitter acetylcholine (McDowell, 2008; Gropper and Smith, 2013).

Another important function of pantothenic acid is as a part of ACP, which is a component of fatty acid synthase complex that catalyzes fatty acid synthesis. CoA is part of phosphopantetheine, a prosthetic group which is attached to ACP and help in the transfer of acyl chains during fatty acid synthesis (Depeint et al., 2006a; Gropper and Smith, 2013).

1.3.2.3 Deficiency

In humans, deficiency of pantothenic acid usually occurs in conjunction with other nutrient deficiencies. The deficiency of pantothenic acid results in burning feet syndrome which is characterized by burning sensation in feet and numbness of toes. Some other symptoms include irritability, vomiting, fatigue, weakness and restlessness (Depeint et al., 2006a; Gropper and Smith, 2013).

Clinical signs of this deficiency differ in different animal species. In younger ruminants, a pantothenic acid deficiency results in reduced growth, scaly dermatitis, rough hair, diarrhea and eventually death. Demyelination of peripheral nerves is also seen in cases of extreme prolonged deficiency. In pigs, the primary signs of this deficiency are severe locomotor disability, followed by paralysis. Demyelination in brachial nerves is seen as well (McDowell, 2008).

1.3.3 Biotin

Biotin was discovered as a result of investigations to study the cause of egg white injury. Consumption of egg whites resulted in hair loss, dermatitis and neuromuscular problems and Factor H was reported to reverse these symptoms. This factor H or 'anti-egg white injury factor' is now known as Biotin (McDowell, 2008; Gropper and Smith, 2013).

1.3.3.1 Digestion, Absorption, Transport and Storage

In food, biotin is primarily found bound to proteins or to lysine as biocytin. Bound biotin undergoes proteolytic digestion by pepsin and intestinal proteases to form free biotin, biotinyl peptides as well as biocytin. Biotinyl peptides are further hydrolyzed in small intestine by peptidases and biocytin is hydrolyzed by biotinidase to release free biotin (Gropper and Smith, 2013).

Free biotin is absorbed in proximal small intestine. At pharmacologic doses, given in cases of deficiencies, absorption occurs via passive diffusion. At physiological levels, absorption occurs via SMVT, which is also the carrier for pantothenic acid. However, SMVT expression is mediated by biotin, with lower expression levels at higher vitamin concentrations (Vadlapudi et al., 2012; Gropper and Smith, 2013). Some biocytin can also be absorbed by peptide carriers and then hydrolyzed by cellular peptidases to release free biotin. Absorption of biotin produced in the proximal colon by bacteria occurs via a sodium dependent carrier mediated system. Biotin

absorption is greatly affected when high amounts of raw egg whites are consumed. This is due to the presence of a glycoprotein, avidin, which irreversibly binds biotin and prevents its absorption (Gropper and Smith, 2013; Ferreira et al., 2015). Avidin is heat labile, so consumption of only raw egg whites affects biotin absorption (Gropper and Smith, 2013).

Biotin is found in plasma primarily in free form and some bound to albumin, globulins and biotinidase. Tissue uptake occurs through SMVT and monocarboxylate transporter (MCT) I. a small amount of biotin is stored in muscles, liver and brain (McDowell, 2008; Gropper and Smith, 2013).

1.3.3.2 Functions

Biotin functions in both coenzyme and non-coenzyme capacity. As a coenzyme, it plays an important role in metabolism of glucose, amino acids and fatty acids. As a coenzyme, biotin covalently binds to four carboxylases. Biotin is first converted to its activated form biotinyl adenosine monophosphate or activated biotin, in the presence of ATP and magnesium ions. The activated biotin then reacts with any of the four carboxylases in the presence of holocarboxylase synthetase to form activated holoenzyme carboxylase (also known as holocarboxylases and biotinylated carboxylases) (McDowell, 2008; Gropper and Smith, 2013).

The four biotin activated carboxylases are pyruvate carboxylase, acetyl-CoA carboxylase, Propionyl-CoA carboxylase and β-methylcrotonyl-CoA carboxylase. Pyruvate carboxylase catalyzes the formation of oxaloacetate from pyruvate. The oxaloacetate can either enter Krebs' cycle, if there is a deficiency of ATP in the cell, or it can be utilized in gluconeogenesis (Depeint et al., 2006a). Acetyl-CoA carboxylase is required for conversion of acetyl-CoA to malonyl-CoA, which initiates fatty acid synthesis. Propionyl-CoA carboxylase is important in metabolism of some amino acids and odd chain fatty acids. Catabolism of amino acids isoleucine, threonine

and methionine and odd chain fatty acids produces propionyl-CoA. The former reaction is catalyzed by propionyl-CoA carboxylase, as well as the metabolism of propionyl-CoA to D-methylmalonyl-CoA. D-methylmalonyl-CoA is further metabolized in several steps to succinyl-CoA, in presence of vitamin B_{12}, and enters the Krebs' cycle. The fourth biotin activated carboxylase is β-methylcrotonyl-CoA carboxylase, which is important for the catabolism of amino acid leucine. β-methylcrotonyl-CoA is formed during the catabolism of leucine and β-methylcrotonyl-CoA carboxylase further catabolises it in a series of reactions to yield acetyl-CoA and acetoacetate. Biotin is also a cofactor in the microbial enzyme methylmalonyl-CoA-carboxytransferase, which catalyzes a step in the synthesis of propionic acid in the rumen. This propionic acid then enters the Krebs' cycle for energy production (Depeint et al., 2006a; McDowell, 2008; Gropper and Smith, 2013).

The non-coenzyme roles of biotin are significant in cell proliferation and gene expression. One of the most important non-enzyme roles of biotin is in biotinylation of proteins (Gropper and Smith, 2013). These proteins are both histone and nonhistone proteins. Histone proteins are required in DNA "packing", a process in which DNA is tightly wrapped around histones. This tight wrapping prevents access to gene promoter sequences and can thus regulate expression levels of genes. Modification of histones due to biotin binding can create pores through which the transcription factors can reach the DNA. Some of these transcription factors are also regulated by biotinylation. Examples include Sp1, Sp3, nuclear factor-κB etc. biotin also plays a role in cell proliferation. This has been shown that cells that are actively dividing have greater number of biotinylated histones; while biotin deficiency results in cells getting arrested in G_1 phase (Kothapalli et al., 2005; Hassan and Zempleni, 2008; Gropper and Smith, 2013).

Biotin has been reported to be essential for two major events during horn production- keratin protein synthesis and formation of the intercellular cement. Supplementation improves quality of the intercellular cement, which improves cell to cell adhesion (Muelling, 2009). In dairy cows it was demonstrated that supplemented animals had a reduced susceptibility to claw diseases such as sole ulcers, dermatitis digitalis, and horn erosion (Weiss and Ferreira, 2006; McDowell, 2008; Muelling, 2009).

1.3.3.3 Deficiency

Since, some biotin is produced in intestines, true deficiency of biotin is very rare. But deficiency can occur in case of genetic disorders that affect biotin absorption or consumption of raw eggs in high amounts. The major symptoms of deficiency are neurological and cutaneous (Gropper and Smith, 2013). The neurological symptoms include lethargy, parasthesia in the extremities, hypotonia, depression and hallucinations, while cutaneous symptoms are characterized by red, scaly dermatitis around eyes, nose and mouth. Anorexia, hair loss, nausea and muscle pain is also experienced (McDowell, 2008; Gropper and Smith, 2013).

Biotin deficiency is not expected in ruminants, due to ruminal and intestinal synthesis. In calves, a diet low in both potassium and biotin reportedly causes paralysis in the hind legs and rapid death. In adult ruminants, even though biotin supplementation was deemed unnecessary due to bacterial synthesis, numerous studies have shown that biotin supplementation improves keratin production and subsequently hoof health (McDowell, 2008). Biotin supplementation has been used to treat lameness and is also used as a preventative measure for lameness in dairy cattle (Weiss and Ferreira, 2006; McDowell, 2008; Muelling, 2009).

1.3.4 Vitamin B_6

Vitamin B_6 is the name used for the vitamers pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM). These compounds are pyridine derivates and differ in their functional groups, which are alcohol, aldehydes and amine groups, respectively. In food, all B_6 vitamers are found primarily in their phosphorylated forms (McDowell, 2008; Gropper and Smith, 2013).

1.3.4.1 Digestion, Absorption, Transport and Storage

Vitamin B_6 is absorbed in its free forms. The phosphorylated are first hydrolyzed by zinc dependent alkaline phosphatase and other intestinal phosphatases to release the free vitamers. Absorption occurs mainly in jejunum and is via passive diffusion. At high concentrations, even some phosphorylated forms are absorbed by diffusion (McCormick, 1989; Gropper and Smith, 2013).

Absorbed PL, PM and PN are taken up by the liver and phosphorylated by an ATP-dependent kinase. PN, PL and PM are respectively phosphorylated to pyridoxine phosphate (PNP), pyridoxal phosphate (PLP) and pyridoxamine phosphate (PMP). PMP and PNP are then converted to PLP by FMN dependent PMP and PNP oxidases. Riboflavin status of the body regulates this step (McCormick, 1989; Gropper and Smith, 2013).

PLP stays bound to proteins, such as albumin, for transportation. For tissue uptake, PLP is hydrolyzed by alkaline phosphate, as only unphosphorylated forms can cross the cells membranes. Muscles are the primary reservoirs of PLP, with some amounts stored in liver as well. PL also undergoes metabolic trapping and is stored in muscles as PLP, bound to glycogen phosphorylase, which prevents the action of phosphatases on PLP (McDowell, 2008; Gropper and Smith, 2013).

1.3.4.2 Functions

PLP is the primary coenzyme form of the vitamin. The major role of B_6 as a coenzyme is in amino acid metabolism. Some of the major reactions that PLP plays a role in include transamination, deamination, decarboxylation, transsulfhydration, cleavage and racemization (McDowell, 2008; Gropper and Smith, 2013).

Transamination reactions occur between an amino acid and a keto acid, and results in the formation of new amino acids. This reaction occurs in two half reactions. In the first reaction, the amino group from amino acid is transferred to PLP and in the second reaction, the amino group is transferred to the keto acid to form a new amino acid. Transamination of alanine and aspartic acid results in the formation of pyruvate and oxaloacetate, respectively, which feed into the Krebs' cycle for energy production (McCormick, 1989; Depeint et al., 2006b; Gropper and Smith, 2013). Since this reaction is reversible, pyruvate and oxaloacetate could also be used for the production of their respective amino acids. In deamination reactions, PLP removes amino group from amino acids and is released as ammonium ion. An important example of this reaction is deamination of serine to release ammonia and water, resulting in formation of pyruvate, which is utilized in Krebs' cycle (Gropper and Smith, 2013).

Removal of carboxy group from amino acids also requires PLP, in decarboxylation reactions. Some examples of this reaction include formation of histidine from amino acid histamine and neurotransmitter GABA from glutamate. Transsulfhydration reaction is important for synthesis of cysteine from methionine and PLP is required by two enzymes that carry out this reaction (McCormick, 1989; Gropper and Smith, 2013). PLP is also important in a cleavage reaction involving removal and transfer of hydroxymethyl group from serine to tetrahydrofolate (THF) and formation of glycine. THF is converted to methylene-THF, which enters the folate

cycle (Depeint et al., 2006b). Some racemization reactions that convert D-amino acids into L-amino acids also require PLP as a coenzyme. Reacemization reactions and the levels of L- and D- amino acids are very important in pathways requiring amino acids (Gropper and Smith, 2013). Some other reactions in the body that require PLP dependent enzymes include synthesis of niacin from tryptophan, fatty acid oxidation, sphingoloipid synthesis and heme synthesis, as well as glycogen breakdown (McCormick, 1989; McDowell, 2008; Gropper and Smith, 2013).

Vitamin B_6 works primarily in amino acid metabolism as an enzymatic co factor in the form of PLP. However, it also plays an important non-enzymatic role in modulating gene expression, especially that of steroid hormone receptors (Oka, 2001). Elevation of PLP levels on the cells have been reported to affect the response to glucocorticoids, progesterone, estrogens and androgens. PLP specifically binds to the regulatory regions of DNA and prevents binding of transcriptional factors and thereby prevents mRNA synthesis (Bender, 1994; Oka, 2001).

1.3.4.3 Deficiency

Deficiency of vitamin B_6 is quite rare in human beings. However, consumption of high amounts of alcohol and certain drugs such as isoniazid, penicillamine, corticosteroids, anticonvulsants and oral contraceptives can interfere with vitamin activity and induce deficiency. Symptoms include seborrheic rash on face, neck and shoulders, weakness, fatigue, cheilosis, glositis, angular stomatitis and neurological issue such as confusion, convulsions and seizures (Depeint et al., 2006b; Gropper and Smith, 2013). Since PLP is an important coenzyme, a deficiency can also cause decreased synthesis of niacin in liver, microcytic anemia due to impaired heme synthesis and increased plasma total homocysteine levels, which is a risk factor for heart disease (Gropper and Smith, 2013).

In healthy adult ruminants, deficiency of vitamin B_6 is not seen generally. An induced deficiency in calves results in apathy, diarrhea, loss of appetite, and loss of coordination, followed by convulsions and death (McDowell, 2008).

1.3.5 Folate

Vitamin B_9 or "folate" refers to the reduced form of the vitamin present naturally in foods and biological tissues. "Folic acid" refers to the oxidized form of the vitamin that is found in supplements and fortified foods. The discovery of folate was the result of treatment methods for megaloblastic anemia, which was a major problem in the late 19^{th} century (McDowell, 2008; Gropper and Smith, 2013).

Chemically, the structure of folic acid consists of pteridine and para-aminobenzoic acid (PABA), known as pteroic acid. Pteroic acid is bound to glutamic acid to form folate, which is also known as pteroylglutamate. In the body, multiple glutamic acid residues are attached to pteroic acid to form pteroylpolyglutamate (Gropper and Smith, 2013).

In the body, folate exists in interconvertible coenzyme forms. Folic acid does not naturally occur and has to first be reduced to dihydrofolate (DHF) and then tetrahydrofolate (THF), which is further converted to other coenzyme forms, as per requirement, in folate cycle. The other coenzyme forms of folate are 5,10- methylene THF, 5- methyl THF and 10-formyl THF, and others. PLP (pyridoxine), riboflavin, niacin (as NADP) and vitamin B_{12} play important roles in the interconversion of folate forms (Gropper and Smith, 2013).

1.3.5.1 Digestion, Absorption, Transport and Storage

Folic acid does not require any digestion as it is present in monoglutamate form in fortified foods, however, the polyglutamate forms of ingested dietary folate requires hydrolysis to monoglutamate forms by folypoly-γ-glutamyl carboxypeptidases (FGCP) or conjugases. This

enzyme is present in both the pancreatic juices and bile as well as bound to brush border of the enterocytes, which is dependent on zinc and a zinc deficiency can severely impact folate digestion (McDowell, 2008; Gropper and Smith, 2013).

The monoglutamate forms of folate are absorbed primarily via proton-coupled folate receptor (PCFT) in the duodenum and upper jejunum. At pharmacological doses, absorption via diffusion also occurs. Folate is converted to its coenzyme forms in the intestinal cells; at higher doses in the liver. In circulation, the vitamin is found primarily as folate and 5-methyl THF, although other monoglutamate coenzyme forms are also found, either in free forms or bound to albumin and folate binding proteins. Tissue uptake occurs primarily via the reduced folate carrier. PCFT and three folate receptors (α, β, or γ) are also present on some tissues and mediate uptake (McDowell, 2008; Gropper and Smith, 2013).

Similar to riboflavin and vitamin B_6, folate forms undergo metabolic trapping in the cells with the addition of glutamate residues. During erythropoeisis, folate is incorporated in the red blood cells, which acts as an indicator of long-term folate status as mature red blood cells do not take up or release folate. Some storage of folate occurs in the body, with half of the reserves found in the liver (McDowell, 2008; Gropper and Smith, 2013).

1.3.5.2 Functions

One of the most important biochemical functions of folate and its coenzyme forms in mammals is to accept and release one-carbon units (Girard and Matte, 2005a; Depeint et al., 2006b). This role of folate is crucial for synthesis of purines and pyrimidines, *de novo* synthesis of methyl groups for synthesis of methionine and for methylation of DNA and proteins. Different folate coenzymes act as one-carbon donors in these pathways, resulting in regeneration of THF,

which can accept more one-carbon units and continue on the cycle (Depeint et al., 2006b; Gropper and Smith, 2013).

The amino acid serine is one of the major sources of one-carbon units in the folate cycle. Generation of glycine from serine occurs in the presence of the enzyme serine hydroxymethyltransferase and coenzyme PLP, with a one-carbon unit being transferred from serine to THF to generate glycine and 5,10-methylene THF, respectively. Folate is also required for synthesis of glycine from choline. Choline is metabolized to betaine in the liver, which acts as a donor of one-carbon units to THF. During this series of reactions, betaine is metabolized to dimethylglycine, sarcosine, and finally glycine (Depeint et al., 2006a; b; Pinotti et al., 2008; Gropper and Smith, 2013).

Another amino acid that utilizes folate in its metabolism is methionine. In the methionine cycle, methionine regeneration from homocysteine requires 5-methyl THF. Methionine is first converted to S-adenosyl methionine (SAM), which yields homocysteine after removal of a methyl and adenosyl groups in a series of steps. When SAM concentrations are low, methionine is regenerated from homocysteine in the presence of 5-methyl THF and vitamin B_{12} (as methylcobalamin). This reaction is quite important because of the role of SAM as a methyl donor in reactions such as DNA and RNA methylation, neural functions and catecholamine synthesis (Depeint et al., 2006b; Gropper and Smith, 2013; Pauwels et al., 2016).

Another important function of folate is synthesis of purines and pyrimidines, which are required for DNA synthesis and cell division. 5,10-methylene THF is required for the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidylate monophosphate (dTMP), which is the rate limiting step in DNA synthesis. Inadequate amounts of folate affect this reaction and increases amount of uracil in the cell, which can get incorporated in the DNA instead of

thymidine and increase the risk of DNA strand breakage, ultimately affecting normal cell cycle progression. In purine synthesis, 10-formyl THF supplies formate for adenine and guanine rings formation (Depeint et al., 2006b; Fenech, 2012; Gropper and Smith, 2013).

1.3.5.3 Deficiency

Megaloblastic macrocytic anemia is the major sign of folate deficiency. It is characterized by large, nucleated and immature red blood cells in circulation. These red blood cells are a result of defective cell division and differentiation. The symptoms include fatigue, weakness, headaches, irritability, palpitations and difficulty concentrating (Depeint et al., 2006b; Gropper and Smith, 2013). Megaloblastic anemia is a result of not only folate deficiency, but vitamin B_{12} deficiency as well, as both these vitamins play a role in DNA replication and cell division. Since folate plays an important role in cell division, tissues with a rapid cell growth rate such as the gastrointestinal tract and bone marrow are also affected. The intestinal villi have shortened height and the layers of the tract also thin out, which affects the nutrient absorption (McDowell, 2008; Gropper and Smith, 2013).

The need for folate increases during pregnancy and a deficiency of folate or impaired folate metabolism has been implicated in development of neural tube defects (Molloy et al., 1998; Zetterberg, 2004). Folate deficiency is also reported to play a role in development of some cancers, especially gastrointestinal cancers such as colon cancer; hyperhomocysteinemia, which increases the risk for vascular diseases and heart diseases; and cognitive dysfunction, increasing the risk of dementia (Blom and Smulders, 2011; Nazki et al., 2014; Necela et al., 2015).

Due to the synthesis of folate in the rumen, clinical deficiency is not seen in adult ruminants. Younger animals, receiving inadequate diet, can develop a deficiency which is characterized by leucopenia, diarrhea, pneumonia and death (McDowell, 2008). Even though

adult ruminants do not exhibit the signs of folate deficiency, a decrease in serum folate is reported during pregnancy and lactation (Girard et al., 1989; Girard and Matte, 1999). This decrease might prevent the cows from achieving maximal milk production, a claim supported by results showing increase in milk production when supplemented with folic acid (Li et al., 2016; Duplessis et al., 2017).

1.3.6 Vitamin B_{12}

Vitamin B_{12}, also known as cobalamin, was the last vitamin to be discovered in 1948. This vitamin is unique because in nature it is synthesized only by microorganisms and is not found in plant based diets. The trace element cobalt is also an integral part of its structure and a cobalt supply is necessary for synthesis of cobalamin by microorganisms. Vitamin B_{12} is the most potent of the B complex vitamins and very low doses are required to meet the daily requirements (McDowell, 2008; Gropper and Smith, 2013).

1.3.6.1 Digestion, Absorption, Transport and Storage

Major sources of vitamin B_{12} are animal based products. Vitamin B_{12} found in plant based diet is either due to contamination with manure and in legumes, because of nitrogen-fixing bacteria in the roots. In animal products, vitamin B_{12} is found as adenosyl-, methyl- and hydroxocobalamins. Supplements contain cyanocobalamin, methylcobalamin and hydroxocobalamin forms (McDowell, 2008; Gropper and Smith, 2013).

For digestion, the vitamin is released from the polypeptides to which it is linked in foods by pepsin and hydrochloric acid in the stomach. Next the vitamin binds to R protein in gastric juice, which protects it from bacterial use. In duodenum, vitamin B_{12} is released from the R protein by pancreatic proteases and the free vitamin B_{12} binds to intrinsic factor (IF) to form vitamin B_{12}-IF complex and travels from duodenum to ileum where it interacts with cubulin or

IF receptor. Cubilin forms a complex with proteins amnionless and megalin, which facilitate the attachement of cubilin to cells of ileum and subsequent absorption by endocytosis (McDowell, 2008; Gropper and Smith, 2013).

In the enterocytes, vitamin B_{12} is released from IF and is bound to protein transcobalamin II (TC II) for transportation as methylcobalamin. Some adenosylcobalamin is also found in circulation. Two other transcobalamins, TC I and III, also participate in transportation, with TC I functioning as a circulating storage form and TC III delivering vitamin B_{12} from peripheral tissues to liver (McDowell, 2008; Gropper and Smith, 2013).

Unlike other B-vitamins, vitamin B_{12} can be stored in the body for longer time periods. Hence, a deficiency can take up to 3-5 years to appear in adults. Most of the vitamin is stored in liver, making animal liver one of the richest sources of vitamin B_{12}. Some vitamin B_{12} is also found in circulating blood, spleen, muscles, heart, bones, kidneys and brain (Gropper and Smith, 2013).

1.3.6.2 Functions

Vitamin B_{12} plays a role in numerous basic metabolic functions as part of enzyme systems. Two important biochemical reactions in which vitamin B_{12} acts as a cofactor are conversion of homocysteine to methionine and succinyl-CoA synthesis from methylmalonyl-CoA (Depeint et al., 2006b; Gropper and Smith, 2013).

Conversion of homocysteine to methionine requires vitamin B_{12} as methylcobalamin, which is formed when cobalamin bound to the methionine picks up the methyl group from 5-methyl THF, forming methylcobalamin (bound to methionine synthase) and regeneration of THF, which enters folate cycle. Next, the methyl group is released from bound methylcobalamin and transferred to homocysteine resulting in formation of methionine and cobalamin (Girard and

Matte, 2005a; Depeint et al., 2006b; Gropper and Smith, 2013). This reaction is not only important for homocysteine metabolism but also for preventing the methyl-folate trap. Formation of 5-methyl THF is irreversible, unlike other coenzyme forms of folate which can be interconverted in folate cycle. Vitamin B_{12} is required as methyl group acceptor to release THF and a deficiency of vitamin B_{12} can also potentially cause a functional folate deficiency (Gropper and Smith, 2013).

The second biochemical reaction utilizes vitamin B_{12} as adenosylcobalamin to act as coenzyme for methylmalonyl-CoA mutase in the mitochondria. Oxidation of methionine, isleucine, threonine and odd-chain fatty acids result in formation of propionyl-CoA. Propinyl-CoA is converted to D-methylmalonyl-CoA in the presence of biotin, magnesium ions and ATP. L-methylmalonyl-CoA is an enantiomer of D-methylmalonyl-CoA and it is converted to succinyl-CoA by methylmalonyl-CoA mutase and two molecules of adenosylcobalamin. Succinyl-CoA is an intermediate in Krebs' cycle and is used for energy production (Girard and Matte, 2005a; Depeint et al., 2006b; Gropper and Smith, 2013). Vitamin B_{12} deficiency can result in accumulation of methylmalonic acid, which is a metabolite of methylmalonyl-CoA, which is used in diagnosis of deficiency (Gropper and Smith, 2013).

Due to interactions of vitamin B_{12} with folate, choline and methionine, vitamin B_{12} is important for one-carbon metabolism reactions, purine and pyrimidine synthesis, red blood cell synthesis and nervous system integrity. These functions deteriorate during B_{12} deficiency (Depeint et al., 2006b; Gropper and Smith, 2013).

1.3.6.3 Deficiency

A deficiency of vitamin B_{12} can develop for several reasons. Inadequate intake is a common cause of deficiency in people with strict vegetarian diet consumption. Impaired gastric

function leading to insufficient hydrochloric acid and IF production; impaired intestinal function due to celiac diseases; parasitic infections and impaired pancreatic exocrine secretions can all contribute to a vitamin B_{12} deficiency (Gropper and Smith, 2013).

Like that in folate, vitamin B_{12} deficiency also results in maegaloblastic macrocytic anemia. The condition occurs because in absence of vitamin B_{12}, folate coenzymes once reduced to 5-methyl THF can't be converted back to other coenzyme forms of folate and result in methyl-folate trap. Due to unavailability of 10-formyl THF and 5,10-methylene THF, purine and pyrimidine synthesis, and consequently cell division, is affected (Depeint et al., 2006b; Gropper and Smith, 2013). Since red blood cells have such a rapid turnover, their differentiation and maturation are negatively impacted and abnormally large and nucleated cells are released in blood, which result in megaloblastic anemia (Gropper and Smith, 2013).

Apart from anemia, vitamin B_{12} deficiency also affects the nervous system. The symptoms include numbness in extremities, abnormal gait and loss of coordination, loss of sense of relative position, memory loss, disorientation, psychosis and dementia. The cause of these symptoms is not clear, but is believed to be related to the lack of SAM for methylation reactions, which are required for myelin maintenance and neural functions (Gropper and Smith, 2013).

Vitamin B_{12}, like other B-vitamins is synthesized in rumen, and deficiency is more common in calves with underdeveloped rumen. However, since cobalt is an essential part of the structure of cobalamin and is required for biosynthesis of the vitamin by ruminal microbes, adult ruminants can experience a vitamin B_{12} if sufficient amounts of cobalt are lacking from their diets (Girard and Matte, 2005b; McDowell, 2008). In calves, the deficiency is characterized by poor growth, muscle weakness and demyelination of peripheral nerves. In pigs, a vitamin B_{12}

deficiency also causes a reduction in litter size and piglet survival as well as an increase in abortions and embryo loss (McDowell, 2008).

1.3.7 Choline

Choline is an essential nutrient that is commonly grouped with the B-vitamins, even though it does not satisfy the definitions of a vitamin (Weiss and Ferreira, 2006). Unlike other B-vitamins, choline can be synthesized in the liver, is required in the body in greater amounts (g or mg vs µg) and it functions as a structural component rather than a coenzyme (McDowell, 2008; Shahsavari et al., 2016).

Choline is naturally found in feed stuff mainly as phosphatidylcholine (PC), also known as lecithin, and some sphingomyelin and glycerophosphocholine. Biosynthesis of lecithin occurs from phosphatidylethanolamine in the presence of amino acid serine or glycine and a source of methyl units (SAM) (Gropper and Smith, 2013).

1.3.7.1 Digestion, Absorption, Transport and Storage

Choline is released from PC by hydrolysis in small intestine. Pancreatic enzyme phospholipase A_2 hydrolyses PC into lysoPC and fatty acid. Both free choline and lysoPC are absorbed in the enterocytes via sodium dependent carrier proteins and at high concentrations, by passive diffusion. Some choline can be degraded by gut bacteria to betaine and trimethylamine (Gropper and Smith, 2013).

Free choline enters the portal circulation and is delivered to various tissues, where it is absorbed by both facilitated diffusion and carrier mediated transport. LysoPC, on the other hand, is re-esterified in the enterocytes form PC. PC is incorporated into chylomicrons and enters the lymphatic circulation, where it is transported to tissues by lipoproteins. Choline is stored in the body primarily as PC, which is incorporated into cell membranes. Depending on the tissue,

choline is also stored in the form of acetylcholine (nervous system), betaine and glycerophosphocholine (kidneys) (McDowell, 2008; Gropper and Smith, 2013).

1.3.7.2 Functions

Choline and its metabolites play an important role in several biological pathways including, one-carbon metabolism, lipid metabolism, cellular signaling and neurotransmission (Depeint et al., 2006b; Gropper and Smith, 2013).

Choline demand is primarily determined by its utilization in the one-carbon metabolism pathways, where it is required for methionine regeneration from homocysteine. Choline is oxidized to betaine in the liver, which provides a methyl group to homocysteine leading to formation of methionine. Methionine is further converted to SAM, which is a methylating agent for numerous biochemical reactions. Betaine metabolism also results in glycine and 5,10-methylene THF formation (Depeint et al., 2006b; Weiss and Ferreira, 2006; Gropper and Smith, 2013).

Choline is the major constituent of biological membranes, as PC, and is important for maintaining cellular structural integrity. A choline deficiency can affect the lipid bilayer structure and increase membrane permeability, which leads to leakage of cellular enzymes (Baldi and Pinotti, 2006; Gropper and Smith, 2013). Choline is also precursor of messenger molecules, such as diacylglycerol and ceramide, which play a role in signal transduction as second messengers. PC is also a precursor for platelet-activating factor, a signaling molecule involved in platelet activation, blood pressure regulation and inflammation as well as production of diacylglycerol (McDowell, 2008; Gropper and Smith, 2013).

PC is also required for the synthesis of very low density lipoproteins (VLDL) in the liver via phosphatidylethanolamine N-methyltransferase (PEMT) pathway (Shahsavari et al., 2016).

VLDLs are required for transport of fatty acids (as triglycerides) and cholesterol to tissues. PC is required for packaging of trigylcerides in the golgi cisternae of the hepatocytes and forms the major phospholipid on the surface of VLDL (Yao and Vance, 1988; Gropper and Smith, 2013). This role of choline is very important in fat and cholesterol metabolism and choline deficiency can cause accumulation of triglycerides in the liver leading to fatty liver syndrome (Yao and Vance, 1988; Shahsavari et al., 2016).

Choline has an important role in neural signaling as well. Choline is a precursor for acetylcholine, a neurotransmitter synthesized by cholinergic neurons in brain and peripheral nervous system. These neurons are involved in muscle control, circadian rhythm and memory. Some non-neural tissues, such as immune cells and placenta also produce acetylcholine, where it acts as a signaling molecule (McDowell, 2008; Gropper and Smith, 2013).

1.3.7.3 Deficiency

Choline deficiency in humans is rare, but can be induced by consumption of diet severely restricted in choline or administration of a choline free TPN solution (Gropper and Smith, 2013). The primary symptoms of choline deficiency are fatty liver, liver damage and muscle damage. Liver damage can be attributed to leakage of reactive oxygen species from the mitochondria, due to disruption of membrane integrity, which can cause oxidative damage to the cells. Reintroduction of choline on timely manner can reverse these symptoms (Depeint et al., 2006b; Gropper and Smith, 2013).

Deficiency signs in animals are characterized by poor growth, fatty liver, hypertension and hemorrhagic tissues, especially kidneys. Adult ruminants can synthesize choline in the rumen, however extensive degradation of ingested choline is reported. Calves fed choline deficient diets can develop weakness and inability to stand and choline supplementation can

reverse these symptoms. In newborn pigs, choline deficiency causes spraddled-leg condition, which affects their mobility. Sows with a choline deficiency are also reported to have lower conception rates, lower farrowing rate, smaller litter size and fewer live pigs per litter (McDowell, 2008).

In dairy cows, fatty liver syndrome is quite common after calving. This is because the energy requirements of cows increase after calving, leading to a negative energy balance (NEBAL) state and to support lactation, adipose tissue is mobilized (Weiss and Ferreira, 2006; Shahsavari et al., 2016). There is an increased synthesis of triacylglycerol from non-esterified fatty acids (NEFA) in the liver, at time of calving (Baldi and Pinotti, 2006; McArt et al., 2013). Uptake of NEFA can overwhelm the capacity of liver to produce and secrete the triglycerides as VLDL, leading to fatty liver, which is accompanied by an increase in production and release of ketone bodies in circulation. Insufficient dietary choline can further prolong this metabolic state. Prolong exposure to NEFAs and other cytokines released at time of calving affects the hepatocyte integrity and function, leading to cell leakage and apoptosis (Shahsavari et al., 2016). Studies with post ruminal choline delivery have shown to facilitate triglyceride secretion from liver and thereby aiding in minimizing the duration and level of NEBAL, making choline an important nutrient for transition dairy cows (Baldi and Pinotti, 2006; McDowell, 2008; Shahsavari et al., 2016).

1.4 Interdependent functions of B-complex vitamins

In the previous section, a detailed overview of the B vitamins and their functions was presented. In this section, a brief summary of the interdependent roles of B vitamins is provided. The primary function of B vitamins is in energy metabolism, is the process of generating energy (ATP) from nutrients. Transformation of dietary energy sources (carbohydrates, fats and

proteins) requires vitamins of B complex as co-enzymes and cofactors (Figure 1.1). Thiamine (as TPP), riboflavin (as FAD and FMN), niacin (as NAD) and pantothenic acid (in CoA) are involved in Krebs' cycle (Depeint et al., 2006a; McDowell, 2008). In amino acid metabolism, the amino acids are broken down to enter Krebs' cycle via transamination, decarboxylation and dehydrogenation processes, which require different members of B complex family as coenzymes (Figures 1.1 and 1.2). For example, synthesis of glycine from serine requires folate coenzymes and pyridoxine (B_6). Similarly, lipid metabolism also requires pantothenic acid (in CoA) as well as riboflavin and niacin to form acetyl-CoA (via beta oxidation), which enters the Krebs' cycle to generate energy. The carboxylases required to carry out these reactions also require biotin as a coe-enzyme, making it an important part of glucose, amino acid as well as lipid metabolism (Depeint et al., 2006a; Gropper and Smith, 2013).

Another important role of B vitamins is in one-carbon metabolism (Figure 1.2). One-carbon metabolism is a group of biochemical reactions that transfer one-carbon units. They are involved in amino acid metabolism and also play roles in nucleotide metabolism. Folate, vitamin B_{12} and amino acid methionine are the major contributors to these reactions, with choline and pyridoxine also playing an important role in the process (Depeint et al., 2006b). The metabolites of one-carbon reactions participate in important biological processes, such as DNA synthesis, DNA and histone methylationand amino acid synthesis. Mammalian oocytes and preimplantation embryos express almost all enzymes that participate in one carbon metabolism, indicating the important role B complex vitamins play in reproductive physiology (Ikeda et al., 2012; Juchem et al., 2012).

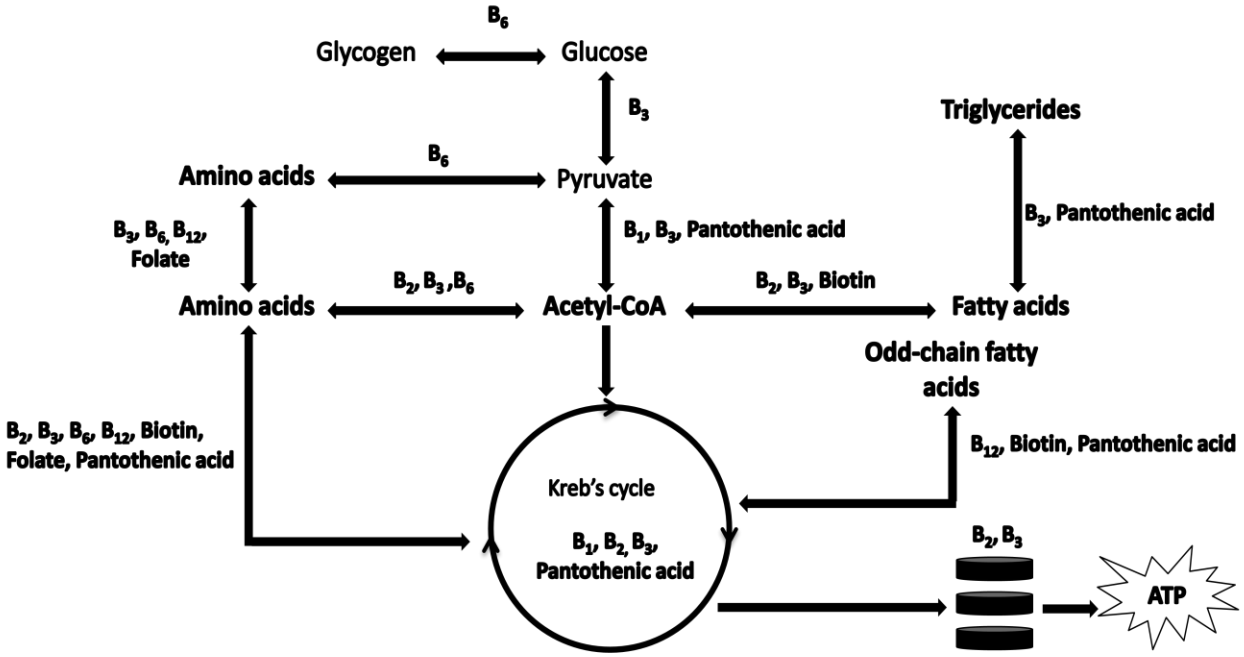

Figure 1.1 Role of B complex vitamins in energy metabolism

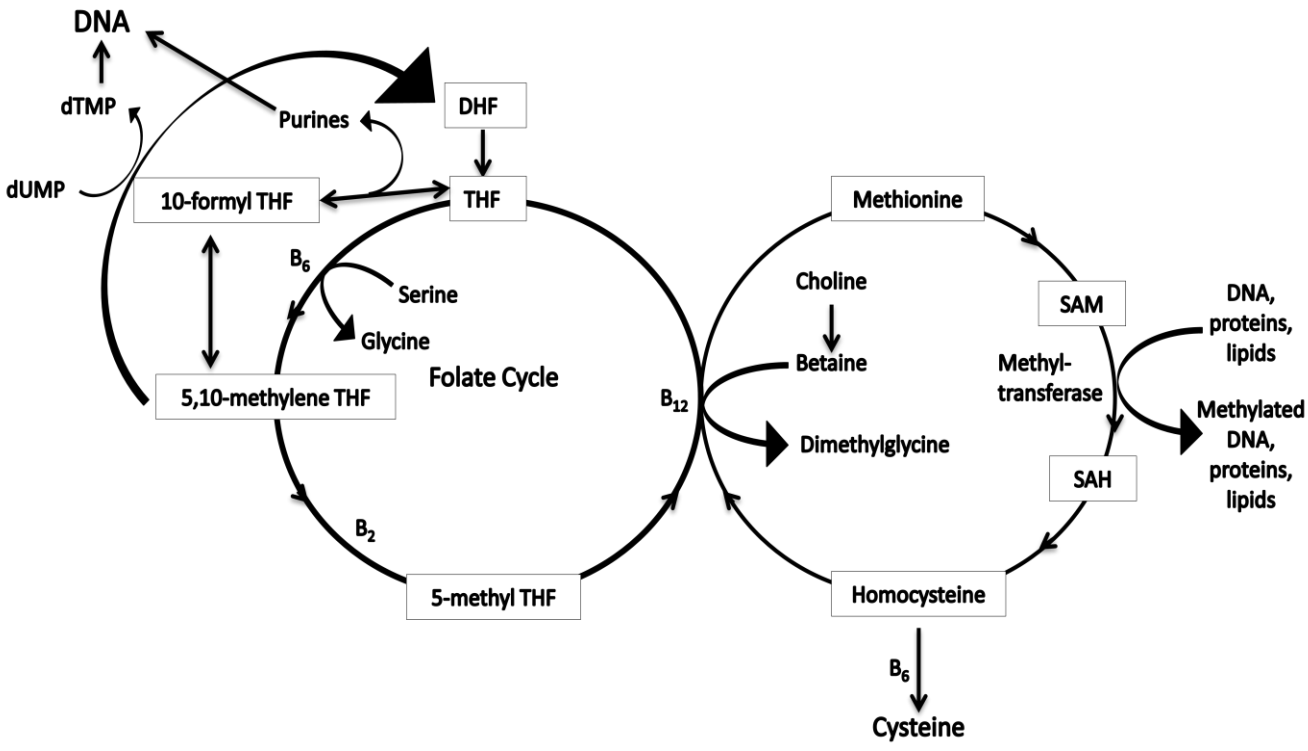

Figure 1.2 Role of B complex vitamins in one carbon metabolism

1.5 Early embryonic growth and endometrial changes in cattle

Dairy cattle have a synepitheliochorial cotyledonary placenta, where instead of having a single large area of contact between maternal and fetal vascular systems, these animals have multiple placentomes. Placentomes consist of fetal cotyledons interdigitated with complementary maternal caruncles and the chorionic epithelium (or trophoblast) is in direct microvillous contact with the uterine or caruncular epithelium (Zeiler et al., 2007).

In domestic animals such as dairy cattle, an extended pre-attachment period of about 8-15 days precedes implantation (Burghardt et al., 2002). The peri-implantation period in domestic animals is characterized by migration and spacing of embryos within the uterus. On d 4-6 of pregnancy, the morula enters the uterus and then forms blastocyst which contains an inner cell mass and a central cavity surrounded by a single layer of trophectoderm (Dorniak et al., 2013). The blastocyst hatches from the zona pellucida around d 8-9 and slowly grows into a tubular or ovoid form, at which point it is termed as conceptus. The ovoid conceptus begins to elongate on d 15 in cattle, and forms a filamentous conceptus which occupies the entire length of the uterine horn ipsilateral to the corpus luteum (CL) (Dorniak et al., 2013; Spencer et al., 2016), On d 19–20 of pregnancy, trophoblast attachment begins in the region of the embryonic disk, followed by implantation (MacIntyre et al., 2002).

Implantation is the first stage in development of the placenta and it is a dynamic process requiring complicated signaling interactions between maternal endometrium and fetal trophoblast cells, as well as remodeling of the endometrium to accommodate placental development. The chorionic epithelium consists of two types of trophoblast cells, namely uninucleate polarized trophoblast cells and nonpolarized, mostly binucleated trophoblast giant cells (Wooding et al., 1996). These binucleate cells are a prominent feature of bovine placenta

and constitute almost 20% of the trophoblast cells (Wooding et al., 1996) and they play an important role of releasing fetal hormonal products into the maternal compartment after leaving the fetal chorionic epithelium and migrating into the maternal epithelium, where they fuse with single epithelial cells to form trinuclear fetomaternal hybrid cells around d 24 (MacIntyre et al., 2002; Zeiler et al., 2007).

Structural changes and angiogenesis during implantation in cows require changes in extracellular matrix (ECM). However, there is limited knowledge about the nature of endometrial ECM associated with implantation in ruminants. It has been observed that the changes in ECM are influenced by integrins (Burghardt et al., 2002; MacIntyre et al., 2002). The integrins are a family of transmembrane glycoproteins that govern cellular interactions with the ECM. They have been implicated in the establishment of uterine receptivity and successful embryo implantation by modulation the endometrial epithelium (MacIntyre et al., 2002). Another important group of proteins observed to be involved bovine endometrial degradation and remodeling are matrix metallopeptidase (MMP), with increased expression of certain MMP enzymes seen at the site of implantation in cattle (Hashizume, 2007).

The upstream changes associated with endometrial remodeling begin with maternal recognition of pregnancy in cows during the peri-implantation period, which coincides with embryo elongation. However, it has been estimated that 60-70% of pregnancy losses in dairy cattle occur during the first three weeks of pregnancy (Diskin et al., 2006). Therefore, the peri-implantation period and endometrial changes occurring during this time period have been of a key interest to researchers.

1.6 Gene expression in peri-implantation endometrium

From fertilization to term, several molecules and systems work in synchrony to receive and maintain the conceptus. It is reasonable to hypothesize that the potential benefits from vitamin B complex strategic supplementation during the transition and peak lactation periods could come from direct influence of these molecules in the reproductive tract as well as indirect effects from better metabolic status and health. Evidence of improvement in pregnancy per AI in the first postpartum AI in cows supplemented with a combination of vitamin B molecules has been demonstrated by Juchem et al. (2012). It is unclear, however, the cellular or molecular mechanism by which this reproductive improvement was achieved. Recently, Richard et al. (2016) reported that the dietary supplementation of rumen-protected B-vitamins affected the differentiation of granulosa cells of dominant follicles toward an earlier LH response associated with genes expressed in conditions where oocyte developmental competence is improved. Considering that, the vitamin B complex could also potentially affect many genes or functional groups of interest in the endometrium. During the pre-attachment phase, the IFN-tau produced by the conceptus induces an array of changes in the uterus by promoting the expression of interferon stimulated genes (ISG) (Spencer et al., 2008), genes related to cell remodeling, adhesion and invasion, cell orientation and polarization, angiogenesis, and transporters of glucose and lipids are mostly upregulated by pregnancy and progesterone (Bauersachs et al., 2006; Forde et al., 2010) and could be potential target genes for vitamin B molecules. A brief literature overview of genes of interest that we studied is provided in the following sections.

1.6.1 Compounds of the Immune system and their importance in pregnancy

During pregnancy, maternal immune system is carefully regulated in the uterus to facilitate tolerance to the ‘foreign’ conceptus and to activate immune system to defend against

microbial and viral infections (Bauersachs and Wolf, 2013). The normal immunological interactions between mother and fetal tissues serve to promote the growth and development of the conceptus and any disturbance to this fine-tuned process could result in loss of pregnancy (Aagaard-Tillery et al., 2006). In this section, some important genes known to regulate maternal immune system during peri-implantation and implantation are briefly discussed.

Mucins are a family of large, heavily glycosylated proteins that form first line of defense of immune system by lubricating and protecting the lining of the airways, digestive system and reproductive system from microbial colonization. Mucin 1 is a transmembrane protein produced by gene *MUC1* and it exhibits both adhesive and anti-adhesive properties (Aplin et al., 1998). There is conflicting evidence regarding its expression at the time of implantation. Studies in several species showing that the downregulation of *MUC1* occurs at the site of embryo implantation (Lagow et al., 1999). However, more recent evidence indicates that *MUC1* is upregulated during implantation window in humans (Aplin et al., 1998; Lagow et al., 1999) and downregulated in women with repeated embryo implantation failures (Tapia-Pizarro et al., 2014), indicating that mucin 1 might play an adhesive, rather than anti-adhesive role in attachment.

SELL encodes a cell surface adhesion molecule, L-selectin, which is produced by neutrophils, and helps in leukocyte-endothelial cells adhesion. L-selectin has also been observed on the surface of human embryo trophoblasts prior to implantation into the uterus (Lagow et al., 1999; Genbacev et al., 2003). Similar to its function in lymphocytes, L-selectin acts as a receptor to facilitate adhesion of the embryo to the surface epithelium of the uterine endometrium, and thus is critical for establishing a pregnancy (Genbacev et al., 2003).

NR1I2 (Nuclear receptor subfamily 1 group I member 2) codes for pregnane X receptor (PXR), which is a nuclear receptor that plays a role in immune system by regulating CYP3A4 production, and binds endogenous and xenobiotic compounds. PXR is activated by a number of endogenous and exogenous chemicals, including steroids such as progesterone and pregnenolone (Lamba et al., 2004; Altmäe et al., 2010). Altmäe et al. (2010) reported a downregulation of this gene in the endometrium of women with unexplained infertility, during the implantation window. Its exact role in reproduction still remains to be elucidated, however it is proposed that PXR protects the mother and/or fetal cells from high levels of xenobiotics and endogenous compounds (Masuyama et al., 2001; di Masi et al., 2009)

IGLL1 encodes for immunoglobulin lambda like polypeptide 1, a protein expressed on pre-B1 cells during the development of B lymphocytes (Kioussis, 2007). *IGGL1* plays an important role in establishing pregnancy. Expression levels of this gene are higher in high fertility animals (Moran et al., 2015) and at day 7 of pregnancy in cows expressing estrus (Davoodi et al., 2016). Davoodi et al. (2016) also reported the influence of high progesterone levels on the expression levels of *IGGL1*.

CXCL10 encodes chemokine C-X-C motif ligand 10, which is a small cytokine produced in response to interferon tau (Bazer et al., 2012). *CXCL10* is involved in endometrium receptivity by attracting trophoblasts to the implantation site in ruminants (Spencer et al., 2008) and is upregulated in pregnant cows (Cerri et al., 2012; Davoodi et al., 2016).

Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine which activates a signalling pathway at feto-maternal interface resulting in expression of adhesion molecules (Rossi et al., 2005; Garlanda et al., 2008). *TNFα* expression has been reported in bovine

endometrium at d 8 of pregnancy and concentrations are tightly regulated during pregnancy (Correia-Álvarez et al., 2015).

Interleukines are another important group of cytokines, produced by leukocytes that are important in implantation. Interleukin-1 beta, encoded by *IL1β,* is a pro-inflammatory factor that plays a role in angiogenesis and tissue remodelling during embryo attachment and placenta formation (Rossi et al., 2005). *IL-1β* is known to be expressed in the porcine and bovine embryo and peri-implantation uterus (Davidson et al., 1995; Geisert et al., 2012) and its administration in the uterus has been reported to increase pregnancy rate following embryo transfer in cattle (Ideta et al., 2010). Interleukin-8 (IL-8) is a potent angiogenic factor and chemokine, playing an important role in implantation (Ideta et al., 2010). *Il-8* expression also increases around the time of implantation, along with *IL-1β* and *IL-6* (von Wolff et al., 2000). Interleukin-10 (IL-10) is an anti-inflammatory cytokine that plays an important role in implantation and pregnancy (Hanna et al., 2000). Endometrial γδ T cells are known to produce IL-10 (Fan et al., 2011; Cerri et al., 2012), where it suppresses trophoblast apoptosis and help in development of placenta (Fan et al., 2011).

Nuclear factor kappa B (NF-κB) is a protein complex involved in NF-κB inflammatory pathway, which is activated in response to stimuli such as cytokines (TNFα and IL-1β), stress and free radicals etc. (Lawrence, 2009). *NFKB*1 encodes one of the transcription factors for the protein complex and has been implicated in regulating the environment of uterus during estrous cycle (Bauersachs et al., 2006). In pregnant pigs, *NFKB1* expression increased between d 5 and d 17, with a 2 fold greater expression in pregnant animals compared to non-pregnant pigs and inverse relationship on d 17 of pregnancy/cycle (Ross et al., 2010).

In response to inflammatory signals such as IL-1β and TNFα, pentraxin-related protein 3 (PTX3) is released, which is coded by *PTX3*. Expression of *PTX3* has been reported in cumulus cells, where it plays a role in cumulus cell organization, and an increased expression at the time of implantation in uterus (Garlanda et al., 2008). Walker et al. (2012) reported downregulation of *PTX3* in the caruncular endometrium sub-fertile cows at d 17 of pregnancy, which likely contributes to poorer reproductive performance in these animals.

T cell receptor delta (TRD) locus protein contributes to the delta chain of gamma delta (γδ) T cells, which increase in number during the pregnancy and play a role in regulating maternal immune function in the uteri (Heyborne et al., 1992; Ditzian-Kadanoff et al., 1993). The upregulation of *TRD* animals is beneficial due to the important role of γδ T cells in enabling early embryonic implantation by inducing maternal immune tolerance to the fetus (Aagaard-Tillery et al., 2006; Fan et al., 2011).

Myxovirus resistance 2, coded by *MX2*, is an important ISG that has been reported in pregnant animals and a 659 fold change was observed at the site of placenta formation in cows by Mansouri-Attia et al. (2009). Expression levels are higher at d 14-18 compared to mid pregnancy (Shirozu et al., 2016) and Davoodi et al. (2016) also observed an effect of d 7 progesterone concentration on increasing *MX2* concentration on d 19 of pregnancy in beef cattle.

Indoleamine 2,3-dioxygenase (IDO) is a rate limiting enzyme in the catabolism of tryptophan and controls the growth of T cells, thereby playing an important role in regulating immune system prior to implantation (Aagaard-Tillery et al., 2006). Studies have shown that *IDO* expression is downregulated in women with repeated implantation failure (Tapia-Pizarro et al., 2014) and in sub-fertile cattle (Walker et al., 2012), indicating its importance in implantation.

Leukemia inhibitory factor (LIF) is a cytokine belonging to the IL-6 family and have been implicated in playing an important role in implantation in many species (Blitek et al., 2012; Hamid et al., 2012). LIF receptor (LIFR) has been detected in pre-implantation porcine embryos prior to implantation, where its interactions with endometrial LIF help in establishment of pregnancy (Blitek et al., 2012). *LIFR* expression bovine endometrium is controlled by estrogen (Shimizu et al., 2010) and abnormal expression of *LIF* and *LIFR* has been observed to affect the cell differentiation in in vitro bovine blastocysts (Eckert and Niemann, 1998).

Immunoglobin heavy constant gamma 1 (IGHG1) is an important immune system gene that has been reported to decrease expression levels in cows with severe negative energy balance (Wathes et al., 2009) and upregulated in the uterus of healthy lactating dairy cows (Cerri et al., 2012). Studies on effect of *IGHG1* expression on fertility have revealed mixed results with the gene being downregulated in cows with good fertility (Moran et al., 2015) as well as in women with recurrent implantation failures (Tapia et al., 2008). The authors did report the lack of knowledge about role of *IGHG1* in the uterus and implantation and thus, requires more research.

Secretory leucocyte protease inhibitor (SLPI) is an anti-inflammatory enzyme secreted by the reproductive tract to protect the tissues from serine proteases and microbial attack (Wira and Fahey, 2004). *SLPI* expression has been detected in the endometrium during early pregnancy in pigs and humans and has been observed to increase with gestation (King et al., 2000; Kim et al., 2015). Davoodi et al. (2016) saw an increased expression of *SPPI* in beef cattle endometrium in the presence of high progesterone and in pigs, a positive correlation with *SPP1* has also been reported (Kim et al., 2015).

Lysozyme 2 (LYZ2) is an important enzyme in the innate immune system that attacks the invading bacteria and is regulated by estrogen in the reproductive tract (Beltman et al., 2010;

Jeong et al., 2012). Beltman et al. (2010) reported an upregulation of this gene in 7 d old beef embryos with retarded development, suggesting the important role of immune system molecules in detecting and maintaining a viable pregnancy. Ubiquitin-like with PHD and RING finger domains 1 (UHRF1) was also upregulated in cattle with non-viable embryos (Beltman et al., 2010). UHRF1 labels proteins for proteasomal degradation and has been reported to be involved in epigenetic changes in the DNA (Mudbhary et al., 2014). There is still a lack of knowledge in regards to functions and importance of *LYZ2* and *UHRF1*in mammalian embryogenesis and pregnancy.

β-defensin, coded by *DEFB1*, is an antimicrobial peptide in the innate immune system. This gene has been reported to be upregulated in the endometrium of cows with severe negative energy balance (Wathes et al., 2009) and activation of inflammatory cytokines harmful for the embryos (Hansen, 1997). To further support this concept, uteri with viable embryos at d 7 of pregnancy had downregulated expression of *DEFB1* compared to non-viable embryo (Beltman et al., 2010). Similarly, pregnant sows also had a decreased expression of this gene at d 14 of pregnancy, compared to non-pregnant animals (Østrup et al., 2010).

Beta-1,3-Glucuronyltransferase 1 (B3GAT1) is an enzyme required for synthesis of human natural killer cells (Suzuki-Anekoji et al., 2012). Natural killer cells are known to adversely affect pregnancy outcomes in women (Vassiliadou and Bulmer, 1996), however the role of *B3GAT1* has not been studied much in relation with reproduction. One study done in women reported and upregulation of this enzyme in endometrium of women with infertility (Altmäe et al., 2010).

SERPING1 (Serpin Family G Member 1) codes for C1 inhibitor and is an important immune system regulator in the uterus. It has been reported to be upregulated in human and

bovine endometrium during the peri-implantation phase (Bauersachs et al., 2006; Labarta et al., 2011; Bauersachs and Wolf, 2013). Progesterone concentration have been shown to downregulate *SERPING1* expression on d 7 of pregnancy in heifers (Forde et al., 2012).

1.6.2 Role of adhesion molecules in early pregnancy

MUC4 and *MUC5B* produce mucin 4 and mucin5B, respectively, other anti-adhesive mucins. These genes are reportedly downregulated in the uterus during implantation window (Aplin and Kimber, 2004), with expression levels of *MUC5B* higher in cervix, where it aids in forming a mucus plug. Altmäe et al. (2010) found *MUC4* and *MUC5B* genes to be upregulated in women with unexplained infertility.

WNT1 inducible signaling pathway protein-2 (WISP2) is another signal transduction molecule associated with ECM, that is regulated by estradiol and progesterone (Dassen et al., 2007). Altmäe et al. (2010) detected an upregulation of *WISP2*in infertile women and more recently (Waters et al., 2014) reported a differential expression in cows supplemented with polyunsaturated fatty acids.

Matrix metalloproteinase 19 (MMP19) is important for regulation of embryo attachment in ruminants via remodeling of ECM (Bauersachs et al., 2008; Sponchiado et al., 2017). Low fertility heifers have lower expression of this genes in their endometrium on d 7 of pregnancy (Killeen et al., 2014) and Davoodi et al. (2016) also reported a 1.5 increase in expression of *MMP19* in the uterus of pregnant cows that expressed estrus, highlighting the importance of this gene in implantation.

Claudin 4 belongs to family of claudin, which are adhesion molecules present in tight junctions and are involved in intercellular sealing of epithelial cells. Upregulation of *CLD4* is reported in cows during the luteal phase of the cycle (Mitko et al., 2008). *CLD4* was found to

increase throughout the peri-implantation period in ewes (Satterfield et al., 2007) as well as sows (Samborski et al., 2013).

Glycosylation-dependent cell adhesion molecule-1 (GLYCAM1) adheres to L-selectin during inflammation and provides a scaffold for carbohydrates in milk (Dowbenko et al., 1993). *GLYCAM1* expression has been reported to increase between d 14-17 in pregnant ewe uterus (Spencer et al., 1999, 2003), indicating its important role at the time of implantation in ruminants.

Tissue inhibitor of metalloproteinases 2 (TIMP2) is a protein that inhibits *MMP2* and is required for maintaining tissue homeostasis. TIMP 2 has been detected in the histotroph of dairy heifers on d 13 of the cycle in high fertility cows (Mullen et al., 2012). It is believed to promote conceptus elongation (Lonergan and Forde, 2014) and has been reported to be differentially expressed in endometrium of cows with tubular vs filamentous conceptuses (Ribeiro et al., 2016a).

Secreted phosphoprotein 1 (SPP1) is an adhesion molecules involved in tissue remodelling at attachment site in the uterus, by affecting cell-cell communication, cell-ECM communication and increasing cell proliferation (Dunlap et al., 2008). *SPP1* expression increases in the uterus of pregnant pigs priori to implantation, where it promotes trophectoderm cell migration and attachment to luminal epithelial cells and playing a critical role in implantation (Kim et al., 2015). *SPP1* was also observed to be upregulated in bovine endometrium on d 18 of pregnancy (Bauersachs and Wolf, 2013).

Another important ISG, galectin-3-binding protein (LGALS3BP) has been shown to be an important candidate for cell-cell adhesion in early pregnancy in ruminants. Its expression was seen to increase in pregnant heifers and cows prior to implantation (Bauersachs et al., 2006;

Mitko et al., 2008; Okumu et al., 2011). It is believed to facilitate cellular adhesion by interacting with other galectins (Okumu et al., 2011).

Extracellular matrix metalloproteinase inducer (EMMPRIN) regulates the expression of MMPs and is important for ECM remodeling. *EMMPRIN* expression has been detected in implanting embryos (Xiao et al., 2002; Mishra et al., 2010). *EMMPRIN* expression has been detected in during the luteal endometrium, but its expression starts increasing significantly after d 30 in pregnant bovine uterus (Mishra et al., 2012).

Cadherin-1 (CDH1), also known as E-cadherin is a cellular adhesion glycoprotein most expressed in epithelial tissues. *CDH1* expression increased in the endometrium of pregnant ewes and trophectoderm of conceptuses after d 14 of pregnancy (Hayashi et al., 2007; Satterfield et al., 2007). In ovariectomized cows, its expression was observed to be regulated by progesterone (Shimizu et al., 2010).

Myosin heavy chain 9 and 10 (MYH9 and MYH 10) have been reported to be involved in cell polarity, adhesion and division. *MYH 9* has been detected in the oviduct epithelium during early pregnancy and is believed to be involved in embryo-maternal crosstalk (Almiñana et al., 2015). Tapia-Pizarro et al. (2014) reported downregulation of *MYH9* in women with repeated implantation failures and upregulation of *MYH10* on d 7 after insemination. In contrast, MYH10 was seen to have increased expression in the elongating bovine conceptus on d 15 of pregnancy (Ribeiro et al., 2016a) and in the macrophages of pregnant cows as well (Oliveira et al., 2010). Myosin light chain 12A (MYL12A) is another member of the myosin family, whose expression was reported to be upregulated in d 19 endometrium of pregnant cows that expressed estrus as well as in cows having higher d 7 progesterone concentration (Davoodi et al., 2016).

Cell adhesion molecule 3 (CADM3) is a protein involved in cell-cell adhesion. *CADM3* has higher expression in bovine blastocysts, compared to somatic cells (Chitwood et al., 2013). A knowledge gap exists in regards to its role in embryogenesis and implantation. A recent study reported no difference in expression levels of *CADM3* in retarded vs viable embryos by Beltman et al. (2013). Further research is required to understand its exact function in a pregnant endometrium.

Catenin (cadherin-associated protein), alpha 2 (CTNNA2) is a cell adhesion molecule involved in calcium-dependent cell-to-cell adhesion mechanisms and it has a role in leukocyte trans-endothelial migration. It has not been studied in terms of bovine reproduction, but recent studies in human endometrial receptivity have pointed towards *CTNNA2* being a potential gene of interest. *CTNNA2* expression is downregulated during window of implantation in a natural endometrial cycle in women (Labarta et al., 2011). However, women with unexplained infertility express higher levels if this gene in their endometrium on d 7 after LH surge, which coincides with the time of implantation in humans (Altmäe et al., 2010).

Protein kinase C (PRKCG) phosphorylates a variety of protein targets and plays a role in cellular signalling pathways involving focal adhesion, gap junctions, tight junctions and calcium signalling. It was observed that women with unexplained infertility express higher levels if this gene in their endometrium on d 7 after LH surge (Altmäe et al., 2010). In bovine endometrial cells, increase in protein kinase C activity was observed to be required for PGF2α production in the endometrial cells (Kim and Fortier, 1995), which eventually initiates luteolysis.

Calpain 6 (CAPN6) is a calcium- dependent intracellular protease believed to be involved in cell migration and cell adhesion (Altmäe et al., 2010). It is regulated by estradiol and progesterone in human and bovine endometrium (Dassen et al., 2007; Shimizu et al., 2010) and

was found to be dysregulated in infertile women around time of implantation (Altmäe et al., 2010).

Homeobox A10 (HOXA10) belongs to a family of enzyme that codes for transcription factors, and has been implicated in playing an important role in endometrial receptivity (Aplin and Kimber, 2004). *HOXA10* known to be regulated by estrogen and progesterone (Shimizu et al., 2010) and has been reported to be upregulated in the endometrium of pregnant sows during the peri-implantation period (d 12-15) of pregnancy (Blitek et al., 2010).

1.6.3 Compounds important for regulation of implantation and growth and development of conceptus

Members of wingless type (W) family have been known to play an important role in embryogenesis by affecting the differentiation and proliferation of cells (Tepekoy et al., 2015; Nayeem et al., 2016). WNT 2, 3 and 3A are the ligands in Wnt signalling pathways and have been detected in both endometrium and early embryos (Tepekoy et al., 2015). *WNT2* is expressed more in the conceptus compared to endometrium in both ewes (Hayashi et al., 2007) and cows (Lu et al., 2013). *WNT3* is expressed in early mouse gastrulas (Tepekoy et al., 2015) and is thought to regulate trophoblast function in a paracrine way during early pregnancy (Li et al., 2017). *WNT3* downregulation was observed in deciduas of women with spontaneous abortions (Li et al., 2017). *WNT3A* is another important conceptus gene detected in bovine morula (Hansen et al., 2014), which is involved in migration and invasion of trophoblasts (Tepekoy et al., 2015; Nayeem et al., 2016). Abnormal activation of this gene could lead to hyperplasia, resulting in infertility (Nayeem et al., 2016) as *WNT3A* expression was reported to be significantly higher in infertile women (Altmäe et al., 2010).

Wnt ligands bind to frizzled (Fzd) receptors, leading to cellular accumulation and nuclear translocation of β-catenin (Lu et al., 2013). *Fzd4* has been reported to be highly expressed in bovine caruncles (maternal side of placenta) (Lu et al., 2013) and is supposed to play an important role in placental development. Other receptors to having high expression levels in the placental and embryonic cells are FZD7 and 8 (Hansen, 1997; Hayashi et al., 2007; Tepekoy et al., 2015).

Catenin beta 1 (CTNNB1), or β-catenin, is an important part of Wnt canonical signalling pathway as well as cellular adhesion systems (Hayashi et al., 2007; Tepekoy et al., 2015). *CTNNB1*expression is known to increase after d 14 of pregnancy in conceptus and endometrium of ewes (Hayashi et al., 2007; Satterfield et al., 2007) and cows (Biase et al., 2013). Wnt molecules and pathways are tightly regulated in pregnant animals and any disruptions in Wnt signalling could adversely impact fetal-maternal recognition and pregnancy in cattle (Hayashi et al., 2007; Lu et al., 2013).

Axin is a scaffold protein two homologues *AXIN1* and *AXIN2* (also known as AXIL) and it is a part of Axin-GSK-APC complex. This complex acts as a negative effector of Wnt signalling pathway by degrading β-catenin (Nakamura et al., 1998; Kikuchi, 1999; Lu et al., 2013). During early bovine pregnancy, *AXIN1* was downregulated in fertile cows, compared to sub-fertile animals, indicating its important role in implantation via modulation of Wnt pathway (Walker et al., 2013). *AXIN2* was seen to be upregulated in women treated with an anti-progestin that causes endometrial breakdown at the end of luteal phase (Nayeem et al., 2016). Another negative effector in the Wnt regulating complex is adenomatous polyposis coli (APC) protein. In association with Axin, it degrades β-catenin and is known to suppress tumors by obstructing cell cycle (Baeg et al., 1995; Nakamura et al., 1998). *APC* mutation has been identified in cancerous

cells (Baeg et al., 1995) and due to its role in Wnt regulation and cell cycle, it would be interesting to study this gene in early pregnancy, when tissue remodelling and cell growth is occurring. Glycogen synthase kinase 3β (GSK-3β) is the third negative regulator in the Wnt regulating complex and its phoshorylation induces the β-catenin degradation cascade (Nakamura et al., 1998). Progesterone has been shown to downregulate GSK3 levels in rat uteri (Rider et al., 2006) and *GSK-3β* was also seen to be differentially expressed in ovoid bovine embryos at d 15 of pregnancy (Ribeiro et al., 2016a).

Dickkopf homolog 1 (DKK1) is another inhibitor of Wnt signalling which have been shown to play an important role in murine trophoblast cell invasion (Peng et al., 2008). *DKK1* expression is upregulated in bovine tubular embryos, compared to ovoid embryos, on d 15 of pregnancy (Ribeiro et al., 2016a) as well as in normal bovine placentomes (Ledgard et al., 2009). Any irregularities in the equilibrium between Wnt signalling and its inhibition could lead to improper plancentome formation in cows (Ledgard et al., 2009).

Msh homeobox 1 (MSH1) is involved in downregulation of Wnt ligands and plays a role in implantation (Nayeem et al., 2016). *MSX1* levels are observed to be lower in porcine, bovine, ovine and human uteri and embryos at the time of implantation (El-Sayed et al., 2006; Bauersachs and Wolf, 2013; Samborski et al., 2013).

Insulin like growth factors 1 and 2 (IGF1 and IGF2) are peptide hormones produced in the liver that share a structural similarity to insulin and are stimulated by growth hormone. Both IGF1 and IGF2 bind to IGF1 receptor (IGF1R) to exert their effects. IGF binding proteins 1-3 (IGFBP1-3) binding proteins bind to IGF1 and IGFBP3 binds IGF2 and transport these hormones in the blood and enhances their half-life. The role of IGF system in reproduction has been extensively studied and has been positively implicated in follicular and early embryonic

development (Velazquez et al., 2008; Steegers-Theunissen et al., 2013; Carvalho et al., 2014). Greater concentrations of *IGF1* has been reported in high fertility cows (Moran et al., 2017) and in cows with ovulatory dominating follicles (Butler, 2003). *IGF2* expression in the endometrium of cows during summer is reportedly lower compared to autumn (Sakumoto and Hayashi, 2015) and is known to increase in the bovine endometrium during pre-implantation period (Geisert et al., 1991; Carvalho et al., 2014) and appears to be primary regulator of placental growth (Velazquez et al., 2008). *IFG1R* has been also been detected in the bovine endometrium and is known to increase as the pregnancy progresses (Mccarthy et al., 2012) *IGFBP1* increases during pregnancy in porcine and bovine endometrium (Geisert et al., 1991; Kim et al., 2015) and its expression has been shown to be influenced by progesterone concentrations in ruminants (Spencer et al., 2016), where it is believed to help in conceptus elongation (Lonergan and Forde, 2014). *IGFBP2* expression increases after d 7 of pregnancy and luteal phase in cows (Geisert et al., 1991; Thatcher et al., 2003; Mccarthy et al., 2012) and its expression is stimulated by progesterone as well (Geisert et al., 1991). *IGFBP3* is also regulated by progesterone and is one of the predominant IGFBPs in the endometrium during early pregnancy in both ewes and cows (Satterfield et al., 2008; Labarta et al., 2011). Together these binding proteins regulated the availability and actions of the IGFs to regulate embryo growth and development in early pregnancy.

Reelin (RELN) protein is involved in migration of neurons and in brain development in early embryos (Cooper and Howell, 1999; Nichols and Olson, 2010). Cerri et al. (2012) observed an effect of lactation on *RELN* expression, with pregnant lactating cows having a downregulated expression on d 17 of pregnancy, compared to non-lactating pregnant and lactating cyclic animals. Another important protein found in nervous tissue is, Calbindin 2 (CALB2) which is a

calcium binding protein involved in intracellular signaling. *CALB2*expression levels are decreased during the luteal phase of the cycle, around time of implantation (Chan et al., 2013) and there have been evidence of decreased protein production in the uterine glands of women with repeated reproductive failures (Russell et al., 2014).

1-Acylglycerol-3-Phosphate O-Acyltransferase 1 (AGPAT1) plays a role in esterification of hepatic fatty acids and is elevated in first 10 d after calving (Shahsavari et al., 2016). *AGPAT1*was observed to be lower in endometrium of heifers with lower progesterone levels on d 7 of pregnancy (Forde et al., 2010). Monoacylglycerol O-acyltransferase 1 (MOGAT1) is involved in lipid metabolism and catalyses the synthesis of diacylglycerol (DAG) from monoacylglycerol. Beltman et al. (2010) reported lower expression of this gene in endometrium of heifers producing a viable embryo at d 7 of pregnancy. However, the expression levels of *MOGAT1* increase around the time of conceptus elongation from ovoid to filamentous, as has been shown in in vitro (d 13) and in vivo (d15) bovine studies (Mamo et al., 2012; Ribeiro et al., 2016b). Diacylglycerol kinase alpha (DGKA) encodes an enzyme that serves as a key attenuator of DAG during intracellular signaling functions. In non-viable embryos, *DGKA* expression was significantly upregulated, leading to decreased availability of DAGs and triacylglycerides, important for early embryo growth (Beltman et al., 2010).

6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 (PFKFB2) is one of the signaling molecules of the glycolysis pathway, in which glucose is metabolized to form pyruvate and ATP. Its expression is regulated by estrogen and progesterone in the bovine endometrium (Shimizu et al., 2010) and on d 7 of pregnancy, heifers with non-viable embryos had an increased expression of *PFKFB2* in the endometrium (Beltman et al., 2010).

Vascular endothelial growth factor A (VEGFA) is a principal growth factor that controls angiogenesis and is a potent simulator of endothelial cell proliferation and migration (Robinson et al., 2008). It has been to shown to increase during placenta formation, with higher *VEGFA* expression on d 42 compared to 28 in bovine endometrium (Moore et al., 2017) and is shown to modulate in vitro steroid synthesis in bovine placenta (Sousa et al., 2012). *VEGFA* expression is upregulated at the onset of conceptus elongation and is expressed differentially in ovoid embryos compared to tubular and filamentous bovine embryos on d 15 of pregnancy (Ribeiro et al., 2016a).

CXXC finger protein 4 (CXXC4) is an antagonist of canonical Wnt signaling pathway. Altmäe et al. (2010) observed an upregulation of this gene in infertile women during implantation phase, indicating its potential role in affecting the implantation process.

1.6.4 Steroid biosynthesis and regulation

Oxytocin (OXT) and oxytocin receptor (OXTR) are involved in the control of luteolytic response in the uterus. OXT is a neuropeptide released from the posterior pituitary that activates OXTR and induces release of Prostaglandin F2α (PGF2α) from uterus to luteolize the CL (Thatcher et al., 2001). *OXTR* expression is downregulated in ruminant uteri at the time of implantation to prevent luteolysis (Jenner et al., 1991). Subfertile animals have reportedly higher expression of this gene on d 16 of the cycle (Walker et al., 2012), similar to cyclic animals (Mansouri-Attia et al., 2009), which could be influencing the negative pregnancy outcomes.

Prostaglandin E synthase (PTGES) is responsible for production of prostaglandin E_2 (PGE_2) in the bovine uterus, with higher expression reported during implantation window (Arosh et al., 2002), and has been suggested as a candidate for rescuing CL at the time of maternal recognition of pregnancy (Arosh et al., 2004). *PTGES* expression has been positively correlated

with more elongated embryos on d 15 (Ribeiro et al., 2016a) and downregulation reported in cows with a non-viable embryo on d 7 of pregnancy (Beltman et al., 2010). In the bone marrow, PGE_2 has also been reported to increase the expression of *IDO*, an important gene that regulates immune system (Wang et al., 2015), thus indicating that PGE_2 is also potentially regulating the immune system in the uterus at the time of implantation. On the other hand, 15-alpha hydroxyprostaglandin dehydrogenase (HPGD) converts prostaglandins to their inactive forms (Bazer, 2013). In pregnant bovine and porcine endometrium, it is observed to be downregulated (Østrup et al., 2010; Sponchiado et al., 2017). It has been reported to be downregulated in endometrium of heifers yielding non-viable embryo (Beltman et al., 2010).

Prostaglandin-endoperoxide synthase 2 (PTGS2) is an enzyme required for the synthesis of prostaglandins and is also implicated in inflammation (Killeen et al., 2016). On d 7 of pregnancy, *PTGS2* expression was found to be higher in heifers that delivered a live calf (Salilew-Wondim et al., 2010), thereby increasing PGE_2 production. However, the expression levels decrease by d 14 in both bovine and porcine endometrium, to prevent the production of luteolytic prostaglandins (Salilew-Wondim et al., 2010; Dorniak et al., 2013; Samborski et al., 2013).

Members of cytochrome P450 family, such as CYP3A4, are involved in the xenobiotic and steroid metabolism (Tsuchiya et al., 2005; Wiltbank et al., 2012). *CYP3A4* expression is regulated by PXR and has been reported to be downregulated in endometrium women with unexplained infertility (Altmäe et al., 2010). In the same study Altmäe et al. (2010) saw an upregulation of *CYP4X1* (cytochrome P450, family 4, subfamily X, polypeptide 1**)** in women with infertility. *CYP4X1* is important in drug metabolism and synthesis of cholesterol, steroids and lipids. Cytochrome P450 Family 4 Subfamily F Member 2 (CYP4F2) is another member of

cytochrome P450 family and is also involved in synthesis of cholesterol, steroids and lipids as well as drug metabolism. It was observed to be upregulated in low fertility heifers, possible affecting the fatty acid metabolism pathways (Killeen et al., 2016).

Expression of nuclear progesterone receptor (PGR), which binds to progesterone, is known to play a very important role during early pregnancy in both humans and ruminants. In bovine pregnant endometrium, its expression starts decreasing around d 13-14 after sufficient exposure to progesterone, coinciding with conceptus elongation (Lonergan and Forde, 2014; Spencer et al., 2016). *PGR* have also been detected on bovine embryos, indicating a direct effect of progesterone on the embryo, which was observed with an increased conceptus length on d 14 when progesterone is elevated during early pregnancy (Clemente et al., 2009). An effect of polyunsaturated fatty acid supplementation on endometrial *PGR* expression was also observed in cows (Killeen et al., 2016), indicating a biochemical modulation of genes via nutrition supplements.

Nuclear estrogen receptors alpha and beta (ERα and ERβ) bind to 17-β-estradiol and related ligands to interact with DNA, inducing transcription. *ERα* levels are lower in endometrium of pregnant cows at d 17 of pregnancy as well as in pregnant sheep (Thatcher et al., 2003; Spencer et al., 2016), which is important for inhibition of luteolysis and maintenance of pregnancy (Bauersachs and Wolf, 2013). *ERβ* has anti-proliferative effects and counters the effect of *ERα*. *ERβ* has higher expression levels prior to and during conceptus elongation and then decrease after trophoblast elongation (Geisert et al., 2012). These results were observed in cows, with an increased expression of *ERβ* after d 7 of pregnancy in endometrium, and a downregulation of *ERβ* after d 13 in response to higher than normal progesterone levels (Okumu

et al., 2011). These results clearly show a complex interaction between the estradiol and progesterone and their receptors in uterus to regulate pregnancy.

1.6.5 Nutrient transporters

Nutrient transport from maternal tissue to the fetal tissues is very important for the embryonic and fetal growth. Solute carrier (SLC) group is an important group of transport proteins on the cell membranes for transport of nutrients across cell membranes. SLC2A5 (also known as GLUT5) belongs to SLC2A family of glucose transporters and is important for fructose transport (Steinhauser et al., 2016). Fructose is believed to be important for growth and development of porcine and bovine fetuses and is reportedly upregulated in presence of progesterone in pigs (Steinhauser et al., 2016) and prostaglandins in sheep (Minamizaki et al., 2009). *SLC5A6* codes for SMVT and is responsible for transport of pantothenic acid, biotin and lipoic acid across the cell membranes. This gene is believed to be regulated by progesterone as well, as pregnant heifers having lower progesterone at d 7 had downregulated *SLC5A6* in the endometrium (Forde et al., 2012). *SLC7A10* is required for transport of neutral amino acids, such as alanine, serine and cysteine (Gao et al., 2009), and in vitro studies have shown that supplementation with alanine (Moore and Bondioli, 1993) and the 20 amino acids (Takahashi and First, 1992) enhance the development of bovine embryos. Uptake of long-chain fatty acids is controlled by *SLC27A6*, which was seen to be upregulated in larger embryos on d 15 of pregnancy in cattle (Ribeiro et al., 2016a).

The role of folate and vitamin B_{12} deficiency in fertility and abortions has been shown in numerous studies (Zetterberg, 2004; Pront et al., 2009). Transcobalamin (TC) binds vitamin B_{12} for transport into cells. Folate receptor 1 (FOLR1) binds to folate and its derivatives to transport them into cells and is reportedly stimulated in the endometrium by actions of progesterone and

estrogen (Shimizu et al., 2010; Labarta et al., 2011). Horcajadas et al. (2005) saw a 27.3 fold increase in *TCI* and a 9.3 fold increase in *FOLR1* in women at the time of implantation.

Serum/Glucocorticoid Regulated Kinase 1 (SGK1) plays an important role in ion and solute transport processes as well as cellular stress response. Around the time of implantation, *SGK1* expression decreases in human , mice and bovine endometrium and embryo (Salilew-Wondim et al., 2010; Karger et al., 2016). In vitro and in vivo results showed implantation failure when higher *SGK1* levels were observed in mice (Karger et al., 2016).

1.6.6 Nutrient metabolism

Nicotinamide N-methyltransferase (NNMT) is an enzyme that catalyzes the N-methylation of nicotinamide (a co-enzyme form of niacin) and helps process drugs and xenobiotic compounds. *NNMT* expression is upregulated during the window of implantation in women and has been observed to be downregulated in infertile women as well as in women with repeated embryo implantation failure (Tapia et al., 2008; Tapia-Pizarro et al., 2014).

1.6.7 Morphogenesis

Trophoblast cells express urokinase-type plasminogen activator (PLAU), which helps in endometrial invasion and tissue remodelling in the uterus (Martínez-Hernández et al., 2011) by regulating the activity of matrix metallopeptidases (Wathes et al., 2009).

Interleukin-6 (IL-6) is a pro-inflammatory cytokine expressed in endometrium and placenta during early pregnancy. *IL-6* expression increases during early pregnancy in pigs (Blitek et al., 2012) and has also been detected in peripheral blood mononuclear cells of d 30 pregnant cows (Yang et al., 2016). Women with recurrent abortions have lower *IL-6* levels in endometrium, which further supports its importance in embryo implantation in different mammalian species (von Wolff et al., 2000; Yang et al., 2016). It is believed to play an important

but non essential role in implantation as mice lacking *IL-6* had normal implantation but the growth and development of the conceptus is compromised (Salamonsen et al., 2000; Hamid et al., 2012).

HOXB7 encodes homeobox B7, which plays a role in cell proliferation and angiogenesis (Murthi et al., 2008). *HOXB7* has been detected in human placental tissues (Meccia et al., 2003; Murthi et al., 2008) and cattle embryos at both morula and blastocyst stages (Ponsuksili et al., 2001). However, the exact role of this transcript during pregnancy is still relatively unknown, but its importance is derived from the downstream effect on target genes involved in angiogenesis (Murthi et al., 2014).

Bone morphogenetic protein 15 (BMP15) is a protein primarily expressed in oocytes, with roles in maturation of the follicles and regulating granulosa sensitivity to follicle stimulating hormone (Li et al., 2014). Davoodi et al. (2016) reported greater expression of *BMP15* in endometrium of cows with smaller embryos and a downregulation of the transcript in larger embryos on d 19 of gestation. Similar results were reported by Pennetier et al. (2004), where they saw a decrease in expression of BMP15 after blastocyst stage in bovine embryos.

Glutathione peroxidase-4 (GPX4) belongs to a class of enzymes that plays a role in protecting cells from oxidative stress (Ufer and Wang, 2011). GPX4, specifically is involved in preventing lipid peroxidation and its activity plays an important role in gamete development, fertilization and pregnancy, with irregularities in *GPX4* expression being fatal for embryos (Al-Gubory et al., 2010; Ufer and Wang, 2011). *GPX4* was one of the genes identified by Forde et al. (2012) as having an altered response in cows with low progesterone on d 13 of pregnancy and they suggested that it could potentially affect the conceptus elongation process.

Ferritin heavy chain polypeptide I (FTH1) is an enzyme that plays a role in iron uptake and intracellular storage and it has been known to play an important role in embryo development, with *FTH1* null mice dying during early developmental stages in mice (Goossens et al., 2007; Lonergan and Forde, 2014). Cagnone and Sirard (2016) reported differential expression of *FTH1* in in vitro embryos due to metabolic stress, which disrupts cellular function and affects viability of embryo.

Eukaryotic elongation factor 1-alpha-1 (EEF1A1) is important in protein synthesis as it delivers aminoacyl tRNAs to ribosomes. *EEF1A1* expression decreases with growth of embryo and it was found to be higher in earlier embryonic stages compared to blastocyst stage in bovine embryos (Goossens et al., 2007) and bovine blastocysts resulting in no pregnancies expressed higher levels of this gene in two different studies (El-Sayed et al., 2006; Salilew-Wondim et al., 2010).

1.6.8 Maternal recognition of pregnancy

IFNT encodes for interferon-tau (IFNT), a biochemical signal secreted by the growing conceptus, that acts as maternal recognition of pregnancy in cattle and prevent the luteolysis of corpus luteum (Spencer et al., 2004b; Roberts, 2007). IFNT is known to stimulate many endometrial genes that help regulate pregnancy, including *ISG15* and *MX2* (Bauersachs et al., 2006; Matsuyama et al., 2012) and delayed growth of embryo has been implicated in late embryonic losses due to insufficient IFNT production (Matsuyama et al., 2012).

ISG15 encodes interferon-stimulated gene 15, which plays a role in ubiquitination, a post-translational modification, of proteins (Bazer et al., 2012). *ISG15* expression is induced by IFNT and progesterone (Bauersachs et al., 2006) and non-pregnant animals have lower ISG15 mRNA in blood from d 17 to d25 (Han et al., 2006).

There is a renewed interest in bovine vitamin B complex and research because of improved production and health benefits observed after supplementation. However, knowledge of impact of vitamin B complex supplementation on reproductive performance of cows is lacking. An improvement in first service conception rate was seen after oral supplementation with rumen protected vitamin B complex (Juchem et al., 2012), which suggested the potential role vitamins of B complex can play in establishment of pregnancy in early lactation. Most of these embryonic losses in dairy cows occur prior to maternal recognition of pregnancy, which occurs around d 16 of pregnancy (Thatcher et al., 2003). Unfavorable uterine environment is believed to play a major role in these early pregnancy failures. Given to important role of B complex vitamins in cellular growth, DNA synthesis and regulation as well as metabolism, we decided to investigate the effect of supplementation on endometrial gene expression around the time of conceptus elongation and inception of maternal recognition of pregnancy in cows during early lactation.

Chapter 2: Body of Thesis

2.1 Objectives and Hypothesis

The main objectives of the project were to determine the effect of a rumen-protected vitamin B complex supplementation, which released the supplement post-ruminally, compared with a control diet containing no supplement, fed to dairy cows on:

1) Uterine gene expression of target transcripts related to embryo development, immune function and cellular adhesion on day 14 of pregnancy

2) Milk production and composition, and blood beta-hydroxybutyrate (**BHBA**) and haptoglobin concentrations in the early post-partum period

3) Ovarian follicle growth and plasma levels of progesterone during the key points of reproductive cycle in the experiment.

We hypothesized that supplementing dairy cows with the vitamin B complex would improve follicle growth and ovarian steroid synthesis. Milk production was expected to increase, but with no deleterious effect on the early postpartum metabolic profile. Furthermore, we expected that the vitamin B supplementation would improve the expression of key transcripts pivotal to pre-attachment embryonic development.

2.2 Materials and methods

This study was conducted at the University of British Columbia Dairy Education and Research Center, Agassiz BC, from March to December 2015. The practices recommended by the Canadian Animal Care Council (CACC, 2009) were followed and the local Institutional Animal Care Committee approved the study.

2.2.1 **Animals and Experimental Design:**

Fifty-one multiparous Holstein cows were enrolled 3 weeks before expected calving date and assigned randomly to either Vitamin B (VB) or Control (CON) groups. The two treatment groups were balanced for previous lactation milk production. The VB group was supplemented with 100g/cow/d of a vitamin B complex for transition cows containing choline, riboflavin, folic acid and B_{12} (Transition VB, Jefo Nutrition Inc., St. Hyacinthe, QC) from 3weeks prepartum to 14 DIM and with 40g of a vitamin B complex for lactating cows containing pyridoxine, pantothenic acid, biotin, folic acid and B_{12} (Lactation VB, Jefo Nutrition Inc., St. Hyacinthe, QC), from 15 DIM until endometrial biopsy. Composition of Transition VB and Lactation VB is provided in Table 2.1. The vitamin B supplement comes mixed on a saturated fat product (a palmitic acid source) and for this reason the CON treatment was also offered with the same amount of supplemental fat. The saturated fat coating protected the vitamin from utilization and degradation by the ruminal microbes and increased the efficacy of supplemented molecules by releasing them post ruminally (Sacadura et al., 2008).

Cows were housed in free stall barns and fed a total mixed ration (Table 2.1) to meet or exceed the requirements of a 750 kg pregnant Holstein cow around 270 d of pregnancy and 620 kg Holstein cow producing 40 kg/day of 3.5% fat corrected milk, during close up period before calving and lactation period post calving, respectively (NRC, 2001). The pens were equipped with an automated electronic feed and water intake system (Insentec, Marknesse, Holland). The cows were fed twice a day and all cows had free access to feed and water. The VB and CON supplements were mixed with distiller's grain and provided to cows in their individual feeding bin prior to afternoon feeding. To be identified by the electronic system, each cow was tagged with their own passive transponder to their ear tag. A panel fitted above each bin read a cow's

transponder and thereby allowed it to access feed from her individually assigned bin. The system recorded feed intake of individual cows.

2.2.2 Milk Sample Collection and Analysis:

The cows were milked twice a day and milk production of experimental cows was recorded daily and information was collected from Dairy Comp 305 (Valley Software, Tulare, CA). Milk samples were collected weekly starting from the first wk postpartum until the end of the study. Briefly, evening (pm) and morning (am) milk samples were collected into a plastic container containing a dissolvable milk fat preservative (BroTab10, Systems Plus Ltd., Ontario, CA) during the normal milking time. The weekly milk samples were analyzed for milk fat%, protein% and SCC at a commercial milk testing lab (Canwest DHI, Chillwack, BC, CA).

2.2.3 Ultrasonography:

Real time *per rectum* ultrasonography, using a portable ultrasound scanner with 7.5-MHz linear-array transducer (Ibex Pro; E.I. Medical Imaging, Loveland, CO), was used to monitor ovarian and uterine structures. With the onset of Ovsynch treatment, ultrasonography was carried out on the days of GnRH and PGF 2α injections in order to determine the ovulation response, follicle number and diameter, and corpus luteum (**CL**) diameter on both ovaries. During the study, the uterus was assessed for state of involution and any other abnormalities and the ovaries for follicles and CL.

2.2.4 Synchronization of Ovulation Protocol and Artificial Insemination:

All cows were placed on a presynchronization-synchronization reproductive management protocol and assigned to be bred by timed AI (TAI). Presynch was initiated with an i.m. injection of GnRH (100 µg; Cystorelin, Merial Ltd., Duluth, GA) at 33 ± 3 d after parturition, followed 7 d later by an injection of PGF2α (25 mg; Lutalyse, Pfizer Animal Health, New York, NY) and

another GnRH injection two d after the PGF2α injection. At 7d after PGF2α, the ovulation synchronization protocol was initiated by administering 100 µg of GnRH and inserting an intravaginal progesterone insert (CIDR; Easi-Breed, Pfizer Animal Health, New York, NY) for 7 d. Cows received an injection of PGF2α at the time of CIDR removal and another one 12 h later. A final GnRH (100 µg) was given 56 h after the first PGF2α injection and TAI was at the time of the last GnRH injection and 12 h later. Artificial insemination followed the UBC farm guidelines.

2.2.5 Blood Samples

Blood samples from the median coccygeal vein or artery were collected in two 10 mL tubes containing EDTA and clot activator, respectively. The blood samples were collected bi-weekly during the transition period, prior to administering the ovsynch injections and at days 2, 5, 7, 12 and 14 post AI. The samples were centrifuged at 2,000 x g for 15 min to obtain plasma and serum and were stored at -80ºC.

The serum haptoglobin was measured by **ELISA**, based on formation of hemoglobin-haptoglobin complexion, estimating the difference of activity of peroxidase in the plasma (Makimura e Suzuki, 1982). An ELISA reader (Gemini™ EM; Molecular Devices, Sunnyvale, CA, USA) was configurated for 450nm wave length, to evaluate the colorimetric reaction. Serum progesterone concentrations were determined using a commercial ELISA kit (Ovucheck® Plasma; Biovet, St. Hyacinthe, Quebec) according to the manufacturer's instructions. Whole blood BHBA levels were measured using a handheld meter (Precision Xtra BHBA test, Abbott Laboratories) at cow side.

2.2.6 Feed Sampling and Analysis

Feed and supplement samples were collected once a week for the duration of the study and pooled together into monthly samples. Then samples were dried at 55º C for 48 hours to determine the DM content, and subsequently ground. Pooled monthly samples were sent to a commercial laboratory (Cumberland Valley Analytical Services, Maryland, USA) for nutrient composition analysis (Table 2.2).

2.2.7 Conceptus Collection and Endometrial Biopsy

Forty-two cows had their uteri flushed on day 14 after AI for embryo collection, followed by endometrial biopsy. The day before biopsy, animals were scanned to confirm the presence and side of corpus luteum (CL) and.. On the day of flushing, the cows were administered epidural anesthesia, using 2 mL of 2% lidocaine (Bimeda-MTC Animal Health Inc., Cambridge, ON), followed by external genitalia cleaning.

For uterine flushing and embryo collection, the cervix was transpassed by a foley cateter (Fr=20), and commercial flushing solution (Bioniche Animal Health Canada Inc., Belleville, ON) was used. The first 20 mL of uterine flush recovered, was collected in a 50 mL tube (Fisherbrand™, UK), for later INT-t quantification. The embryos recovered were measured and frozen in RNAlater (QIAGEN, Ambion, TX, USA) in DNAase and RNAase free cryo-vials and stored at -80°C until further processing.

Uterine biopsy was performed immediately after uterine flushing, through an endometrial biopsy forceps (Kevorkian-Younge, Fine Surgicals, and 3.5 X 8 mm bite and 50.8 cm shaft), driving it to the ipsilateral uterine horn. Around 100-200 mg of endometrial tissue was collected from each cow. The samples were immediately after transferred to a 2 mL DNAase and RNAase freetube, filled by RNAlater (QIAGEN, Ambion, TX, USA), and stored at -80ºC.

2.2.8 RNA Extraction:

The RNA extraction of frozen endometrial and conceptus tissues was performed using total RNA isolation solution, Tri Reagent (Invitrogen, Carlsbad, CA) and a commercial kit (PureLink - Invitrogen, Carlsbad, CA). The qualitative and quantitative RNA analysis was performed by spectrophotometry (Nano drop 2000, Thermo scientific, Wilmington, DE).

2.2.9 Analysis of gene expression:

Expression levels of 100 key genes (Table 2.3) in endometrial biopsy and embryo samples were measured on the NanoString nCounter Analysis System (NanoString Technologies, Seattle, WA, USA). The nCounter assay is based on direct imaging of mRNA molecules of interest that are detected using target-specific, color-coded probe pairs, called the reporter and capture probes, which were custom designed for 96 target and 4 reference genes. The target mRNA was mixed in solution with a large excess of the reporter and capture probe pairs, so that each targeted transcript found its corresponding probe pair. After hybridization, excess unbound probes were washed away and the tripartite complexes, comprising target mRNA bound to specific reporter-capture probe pairs, were isolated. The biotin label at the 3′ end of the capture probes was used to attach the complexes to streptavidin-coated slides. An electric field was applied to orient and extend the tripartite complexes on the surface of the slide to facilitate imaging and detection of the color-coded molecules. A microscope objective and a CCD camera were then used to image the immobilized complexes using four different excitation wavelengths (480, 545, 580, and 622 nm) corresponding to the four fluorescent dyes. The different combinations of the four distinct colors allows for a large diversity of color-based barcodes, each designating a different gene transcript. The expression level of a gene is measured by counting the number of times the specific barcode is detected, and the barcode counts are

tabulated in a comma-separated value (CSV) format. The protocol was performed from start to finish, including hybridization, post-hybridization processing and digital data acquisition, on the nCounter System.

2.2.10 Statistical Analysis:

The data was analyzed for normality using the proc UNIVARIATE from SAS (version 9.4; SAS Institute Inc., Cary, NC) and log transformed when appropriate. An ANOVA for repeated measures using proc MIXED of SAS was used for treatment differences when analyzing for milk production and components and plasma concentrations of BHBA, haptoglobin and progesterone. Proc MIXED was also used to analyze the gene expression data and Tukey's test was used for post hoc analysis to correct for family wise error rate. Dichotomous outcomes (pregnancy outcome and disease incidence) were evaluated by logistic regression using the LOGISTIC procedure of SAS software ver. 9.4 (SAS Institute Inc., Cary, NC).

2.3 Results

2.3.1 Descriptive statistics

Fifty-one cows were enrolled in the study and nine cows were removed from the study because of various reasons (six cows because of postpartum disorders, one cow removed because of leg injury and two cows removed from the gene expression analysis because of failure to respond to ovsynch protocol). Uterine gene expression data was analyzed on 13 cows from which an embryo was recovered during flushing. Two embryos were removed from the gene expression analysis as there was insufficient mRNA to detect gene expression and embryo gene expression analysis was performed on 11 embryos. Progesterone concentrations in plasma were analyzed for 42 cows that were flushed. Milk production and component analysis as well as plasma concentrations of BHBA and haptoglobin analysis were performed for 44 cows.

2.3.2 Endometrial and embryo gene expression

Results from the endometrial gene expression analysis from 13 pregnant cows (CON = 7; VB = 6) showed that 11 genes that play a role in immune system, adhesion, steroid hormone regulation and nutrient transportation were different between cows that became pregnant in the VB and Con group (Table 2.4; Fig. 2.1). *MUC5B* and *SPP1* were upregulated significantly in VB group with a 3.32 (P = 0.05) and 2.02 (P = 0.03) fold increase, respectively, while *MYH9* showed a tendency to be upregulated in the VB animals with a fold increase of 1.09 (P = 0.10). These genes are involved in the regulation of adhesion proteins in the endometrium. Endometrial immune system regulators *TRD, IL1β* and *MUC1* were significantly ($P \leq 0.05$) upregulated, whereas *SELL* showed a tendency for increased expression in VB animals. The fold change differences observed in this group were 1.84 (P = 0.03), 2.01 (P = 0.05), 2.46 (P = 0.02) and 1.52 (P = 0.10), respectively. Other genes upregulated significantly in the VB group were *FOLR1* (fold change = 1.54; P = 0.05), *OXTR* (fold change = 2.02; P = 0.04) and *FZD8* (fold change = 1.18; P = 0.05) which are involved in folate transportation, oxytocin action regulation and Wnt signaling. *PLAU*, which plays a role in embryo morphogenesis, also showed a tendency to be upregulated in VB animals with a fold difference of 1.49 (P = 0.1). Expression levels of 100 genes studied in this project were similar in the embryos collected from the two groups.

2.3.3 Metabolites in plasma, milk production and components and feed intake

Supplementation with rumen protected Vitamin B complex did not impact concentrations of BHBA (Fig. 2.2) and haptoglobin (P = 0.3; Fig. 2.3) in plasma during the transition period (P = 0.84, P = 0.92). Milk production (P = 0.90, Fig. 2.4), milk fat (P = 0.86, Fig. 2.5) and milk protein (P = 0.37. Fig. 2.5) contents were also similar between the VB and CON groups. Feed intake was also similar between the two groups (P = 0.94, Fig 2.8). However, pregnant animals

had a significantly higher feed intake compared to non-pregnant cows, irrespective of the treatments (20.19 ± 0.31 vs 19.33 ± 0.21 kg/day; $P = 0.02$).

2.3.4 Ovarian dynamics and concentration of progesterone in plasma

Vitamin B supplementation had no effect on the size of the embryo ($P = 0.49$; Fig. 2.6), ovulatory follicle (18.4±0.7 vs 19.1±0.7 mm; $P = 0.51$) or CL diameter at embryo collection (29.3±1.2 vs 30.5±1.3; $P = 0.51$for CON vs. VB respectively). There was a parity effect seen on size of embryo, with cows with 3rd or higher parity having larger embryos compared to cows with 2nd parity (9.39 ± 1.44 vs 1.73 ± 1.76 mm; $P = 0.03$). Concentration of progesterone in plasma measured during key points of ovsynch protocol and on days 2, 5, 7, 12 and 14 were also similar between the two groups ($P = 0.80$, Fig. 2.7).

2.4 Discussion

In this study, we observed 11 genes that were upregulated that could cause changes in molecular mechanisms in the uterus able to promote a favorable uterine environment for the establishment of the pre-implanted embryo. The differential genes were grouped according to their role in regulating immune system, adhesion molecules, Wnt signaling pathway, morphogenesis of embryo, oxytocin regulation and folic acid transport. The importance of regulation of these functional transcripts still need further investigations, but it is indicative that dietary supplementation of the vitamin B complex, as offered in the current study, could potentially improve fertility by a direct effect in key molecules related to the early embryonic development.

2.4.1 Blood parameters and milk production

Supplementation with Vitamin B molecules, either individually or in a complex, has delivered variable results on improving milk production and health in previous studies (Graulet

et al., 2007; Sacadura et al., 2008; Juchem et al., 2012). All B-vitamins, as enzymatic co-factor or metabolic constituent, are crucial for proper functioning of metabolic processes such as Krebs' cycle, gluconeogenesis as well as metabolism of carbohydrates, fatty acids and proteins (Depeint et al., 2006a; b). The B-vitamins during the transition period can potentially become a limiting nutrient in modern cows, therefore, justifying additional supplementation during key periods of the lactation cycle. This role of B-vitamins in metabolic pathways could lead to an indirect downstream affect on the endometrial gene expression changes as well.

However, in this study, no differences in milk production or components between the two groups over a period of 10 wks post calving was observed. Similarly, no differences in the concentrations of BHBA and haptoglobin in plasma postpartum was observed, which could be interpreted as indicating a similar metabolic and inflammatory status for both groups in the transition period. The availability and requirement of these nutrients is dependent on environmental, nutritional, metabolic and production factors; some or all of which could have contributed to the lack of difference between the two groups.

We also noticed a low embryo collection rate in this study (31%). This was primarily due to unexpectedly high temperatures in the region from mid-July to mid-September. Including just the animals flushed during cooler months, the collection rate (46%) was comparable to some of our previous studies, with twelve successful collections from 26 cows.

2.4.2 Endometrial gene expression

Previous research on B vitamin supplementation in cows has been focused on effects on milk production and health with very little focus on reproduction. There is a lot of evidence supporting the positive effects of Vitamin B complex supplementation during pregnancy in humans (Steegers-Theunissen et al., 2013). However, similar knowledge and understanding of

effects of Vitamin B supplementation on bovine fertility is lacking. The discussion below focuses on the individual transcripts and functional groups with potential to improve the conceptus-endometrium communication in early pregnancy in dairy cattle.

2.4.2.1 Immune System

The local uterine immune system is intensively regulated during the pre-implantation and implantation phases of pregnancy (Bauersachs et al., 2006; Beltman et al., 2010). Inflammation around the site of cell division and differentiation, as it happens during embryogenesis and placentation, is partly regulated by the paracrine action of cytokines (Ben-Rafael and Orvieto, 1992). In VB cows, we saw an upregulation of *IL-1β* which is believed to play a role in tissue remodelling, embryo attachment and placentation process and has been reported to be downregulated in women experiencing recurrent miscarriages (Rossi et al., 2005). Addition of IL-1β to bovine embryos in vitro had a positive effect on growth of the blastocysts (Paula-Lopes et al., 1998). Similarily, Ideta et al. (2010) reported a significantly higher pregnancy rate in heifers that had peripheral blood mononuclear cells, that had high *IL-1β* expression, administered in the uterine horns. In pigs, IL-1β is reported to be involved in regulation of prostaglandins, with an increased *IL-1β* expression around the time of maternal recognition of pregnancy causing an increase in prostaglandin E_2 (PGE_2) secretion, which blocks luteolysis (Franczak et al., 2010). The potential benefits of upregulation of this gene in VB cows could be due to its role in preparing the uterus for implantation via pro-inflammatory mechanisms as well as providing immunotolerance via downstream effects on cytokines and growth factors in the uterus (Rossi et al., 2005; Geisert et al., 2012).

Another upregulated gene of interest was *TRD*, which encodes a T-cell receptor delta locus and plays a role in modulating the local maternal immune function. Women with recurrent

pregnancy losses had a significantly decreased concentration of these cells in their uterus, compared with women carrying normal gestations (Ditzian-Kadanoff et al., 1993). Greater number of γδ T cells is the uterus of pregnant mice has also been reported, compared with non-pregnant ones (Heyborne et al., 1992). The upregulation of *TRD* animals can be beneficial to embryo survival because of the important role of γδ T cells in enabling early embryonic implantation and survival by inducing local intrauterine tolerance to embryo through suppression of anti-fetal immune response in the uterus (Suzuki et al., 1995; Fan et al., 2011)**.**

We also observed an upregulation of *MUC1* in VB animals. *MUC1* produces the protein mucin 1, an anti-adhesion molecule, which acts as the first line of immune defense by lining the respiratory and reproductive tracts. Studies have shown that the downregulation of *MUC1* gene occurs at the site of embryo implantation in rodents, sheep and pigs (Johnson et al., 2001; Aplin and Kimber, 2004). Hashizume (2007) saw an increased expression of mucins in bovine endometrium around d 14 of pregnancy, which is similar to our observation, and right before implantation on d 21, the *MUC1* expression was downregulated in pregnant uterus. Studies in humans have reported an upregulation of certain isoforms of *MUC1* in the uterus during the receptive phase of the cycle, a small window during luteal phase when the uterus is ready for implantation. Aplin et al. (1998) suggested that surface modification of epitopes on *MUC1*, by decreased sulphation can promote adhesion by reducing repulsion between the embryo and uterine epithelium. *MUC1* also interacts with L-selectin, further supporting this proposal (Lagow et al., 1999). However, more evidence is needed to establish the role and regulation of *MUC1* in bovine during peri-implantation period.

L-selectin is a cell surface adhesion molecule which is produced by neutrophils' expression of *SELL*. L-selectin has been observed on the surface of human embryo trophoblasts

as well as endometrium prior to implantation into the uterus (Aplin and Kimber, 2004). Østrup et al. (2010) also reported a significantly increased expression of *SELL* in the endometrium of pregnant sows on d 14 of pregnancy. Similar to its function in lymphocytes, L-selectin acts as a receptor to facilitate adhesion of the embryo to the surface epithelium of the uterine endometrium (Genbacev et al., 2003). The increased expression and interactions of these molecules with the embryo in the peri-implantation and implantation phase would aid in the apposition as well as implantation process.

2.4.2.2 Adhesion molecules

In the adhesion molecules, we observed an upregulation of *SPP1*, *MUC5B* and *MYH9* (in addition to *MUC1* and *SELL*, which can also be part of an immune response). *SPP1* regulates production of secreted phosphoprotein 1, which is one of the most upregulated adhesion molecules in humans, during the receptive phase of cycle, when the uterus is ready to accept the embryo. Dunlap et al. (2008) suggested the role of *SPP1* in tissue remodelling at the uterine attachment site by affecting cell-cell communication, cell-extracellular matrix (ECM) communication and increasing cell proliferation. White et al. (2006) reported that *SPP1* present on the apical surface of luminal epithelium of the uterus helps to bridge the embryo to the endometrium during the implantation phase. An increased expression of *SPP1* has been reported in the endometrium of both pigs and sheep during early pregnancy (Bazer, 2013; Samborski et al., 2013; Kim et al., 2015). Kim et al. (2015) suggested that, similar to humans, upregulation of *SPP1* could be critical for cell-cell and cell-ECM adhesion in non-invasive placentation that is seen in pigs, as well as in ruminants such as cows.

MUC5B encodes mucin 5B, which is one of the major gel-forming mucin in mucus. It is particularly important in aiding the transport of sperm to the uterus and later on in formation of

cervical plug during pregnancy. However, not much has been reported on the role of *MUC5B* in the endometrium. Recently, Altmäe et al. (2010) reported an upregulation of *MUC5B* in women with unexplained infertility at the time of embryo implantation. The current result are in contrast with Altmäe et al. (2010), where *MUC5B* was upregulated in the pre-implantation phase of pregnancy. Further research on *MUC5B* expression in the endometrium, before and during the implantation, is required to determine its role in the pregnant uterus.

Another differential gene upregulated in VB animals that plays a role in cell adhesion was *MYH9. MYH9* encodes a non-muscle Heavy Chain 9 myosin, which has been reported to play a role in cytokinesis, cell shape and maintenance of focal contacts (Katono et al., 2015). *MYH9* has been reported to be downregulated during the receptive phase in women with repeated implantation failures (Tapia-Pizarro et al., 2014). MYH9 has also been detected in exosomes derived from bovine oviductal cells, indicating that it plays a role in embryo maternal cross-talk from early embryonic stages (Almiñana et al., 2015). The benefits of greater expression of this gene would be derived from its role in cell migration and adhesion in the endometrium.

2.4.2.3 Wnt signaling

The importance of Wnt signaling in embryogenesis is well established. Wnt signaling pathways are made up of Wnt ligands, transmembrane frizzled receptors and low density lipo-protein receptor related protein co-receptors (Tepekoy et al., 2015). We observed an upregulation of Wnt receptor frizzled-8 (*FZD8),* FZD8 expression has been observed in d 7 bovine blastocysts (Denicol et al., 2013) and similar to our results, Hayashi et al. (2007) also reported detection of *FZD8* expression in uterus of ewes and conceptus trophectoderm, prior to implantation. The current findings further support the important role played by this receptor of Wnt pathways in the implantation process.

2.4.2.4 Oxytocin regulation

The *OXTR* gene, which regulates oxytocin receptor expression has been reported to be downregulated in pregnant animals (Spencer et al., 2004a; Bazer et al., 2012). The expression of this gene is regulated by action of progesterone as well as interferon-tau. Even though *OXTR* and *OXT* genes are reportedly downregulated in pregnant animals, we observed greater expression of *OXTR* in VB pregnant animals. Similar results were reported by Jenner et al. (1991), who observed an increased *OXTR* (Jenner et al., 1991)on d 12 after estrus in pregnant as well as non-pregnant cows. It is still unclear based on the current results whether an increase in gene expression for *OXTR* would result in greater local synthesis of prostaglandin F2α that could, at the appropriate time, aid the placentation process.

2.4.2.5 Morphogenesis

The *PLAU* codes for urokinase-type plasminogen activator which has been reported to be expressed on mouse embryos and plays a role during the implantation process (Martínez-Hernández et al., 2011). The urokinase-type plasminogen converts plaminogen to plasmin, which controls ECM turnover by activating some members of matrix metalloproteinase (MMP) family, which help in tissue remodelling and repair (Wathes et al., 2009). Blastocysts resulting in successful pregnancy and calf delivery were reported to have an increased *PLAU* expression by El-Sayed et al. (2006) and an increased expression has been observed in elongating d 15 bovine conceptus as well (Ribeiro et al., 2016a). Upregulation of *PLAU* indicates that there is tissue remodelling occurring in the endometrium on d 14, to prepare for implantation.

2.4.2.6 Nutrient transporter

We analyzed a number of nutrient transporters in the endometrium and observed that *FOLR1* was a transcript upregulated in VB cows. This gene codes for folate receptor 1 which helps in transporting folic acid and its derivatives, primarily 5-methyltetrahydrofolate (5-MTHF), to the inside of the cells. 5-MTHF is the biologically active form of Vitamin B_9 (folate) and is an important player in DNA synthesis (Pauwels et al., 2016) and along with choline and methionine, in epigenetic changes such as methylation of DNA and RNA (Fenech, 2012) and one-carbon metabolism (Depeint et al., 2006b). Although folate deficiency doesn't impact implantation, it indeed plays an important role in fetal growth and development and its deficiency has been reported to cause fetal defects and even preterm death in humans (Guèant et al., 2013). Upregulation of *FOLR1* indicates an active folate cycle in the endometrium in the pre-implantation period. The *FOLR1* could potentially play a part in cell modification at the implantation site and later support of the growing embryo caused by DNA synthesis and regulation.

The mechanisms by which a dietary supplementation of vitamin B in lactating dairy cows affects the above-mentioned transcripts are still unclear. There could be either direct or indirect mechanisms at play here. The current study was not designed to clearly differentiate between direct and indirect effects of vitamin B complex supplementation on endometrium gene expression. In fact, the experiment cannot isolate the effect of a specific vitamin B, but only the overall effect from both the transition and early lactation period dietary supplementation. Considering the non-significant differences in BHBA, haptoglobin and progesterone between the treatments, we are inclined to propose that the changes here observed were direct in nature rather than caused by metabolic status and health. Direct mechanism would pertain to the involvement

of B-vitamins in genomic modifications. Important members in this group would be riboflavin, folate, vitamin B_{12} and choline. These B-vitamins are important in one carbon metabolism pathways and influence nucleotide synthesis, DNA stability and methylation changes on the DNA (Depeint et al., 2006b; Fenech, 2012), which impacts tissue remodeling and cell division. Tissue and genomic modifications in the endometrial wall are important for implantation and successful pregnancy and B-vitamins can directly influence these changes and hence, embryonic survival.

Table 2.1 Composition of supplemented vitamin blends fed to cows and their functions

Vitamin	Phase	Function
Riboflavin	Transition	Co-enzyme in energy, carbohydrate, lipid and amino acid metabolism
Choline	Transition	Lipid metabolism, one-carbon metabolism and cellular signaling
Folic Acid	Transition + Lactation	One carbon metabolism and DNA synthesis (purine production)
B_{12}	Transition + Lactation	One carbon metabolism, methionine synthesis and DNA synthesis
Pyridoxine	Lactation	Co-factor in protein, lipid and carbohydrate metabolism
Pantothenic Acid	Lactation	Co-factor as coenzyme A in pathways involving oxidative respiration, lipid metabolism and steroid and prostaglandin synthesis
Biotin	Lactation	Keratin synthesis and as co-factor for carboxylase enzymes involved in fatty acid synthesis, amino acid catabolism and gluconeogenesis

Table 2.2 Ingredient and nutrient composition of feed during close up and lactation phases

Ingredient Component (% of DM)	Close Up	Lactation
Corn Silage	22.56	32.55
Grass Silage	-	14.22
Alfalfa Hay	38.07	5.30
Rye Grass Seed Straw	25.28	-
Dry Cow Mix	14.09	-
Regular Complete Mash	-	47.93
Nutrient (% of DM)		
CP	14.53	16.81
Soluble protein	5.95	6.43
ADF	33.38	18.36
aNDF	43.60	30.64
Lignin	6.08	1.70
Starch	8.72	23.60
CF	2.32	4.13
Ash	8.83	8.86
Ca	0.98	0.84
P	0.31	0.45
Mg	0.28	0.33
K	1.99	1.83
S	0.27	0.28
Met (g/kg of DM)	0.87	0.37
Se (ppm)	0.52	0.27

Table 2.3 List of genes analyzed, grouped according to their role in the tissue

Function	Genes
Adhesion molecules	*MMP19, CLD4, GLYCAM1, TIMP2, SPP1, LGALSBP3, SERPINN, EMMPRIN, CDH1, MYH9, MYH10, MYL12A, CADM3, MUC4, MUC5B, CTNNA2,PRKCG, HOXA10*
Immune system	*IGLL1, SELL, CXCL10, PTX3, TRD, MX2, IL10, IDO, LIFR, IGHG1, SLPI, LYZ2, UHRF1, IL8, IL1β, TNFα, NFκB1, MUC1, βDefensin, B3GAT1, NR1I2*
Growth and development	*CTNNB1, WNT2, DKK1, AXIN1, AXIN2, APC,FZD7,GSK3β, MSX1, RELN, FZD8,WNT3, FZD4, AGPAT, IGF1, IGF2, IGF1R, IGFBP1, IGFBP2, IGFBP3, VEGFA, WNT3A, CXXC4, CAPN6, PFKFB2, MOGAT1, DGKA, CALB2*
Steroid biosynthesis and regulation	*WISP2, OXYTOCIN, PTGES, CYP3A4, CYP4X1,CYP4F2, OXTR, PGR, ERα, ERβ, PTGS2,HPGD*
Nutrient transporters	*FOLR1, TC1, SLC27A6, SLC5A6, SLC2A5, SLC7A10, SGK1*
Nutrient metabolism	*NNMT,*
Morphogenesis	*PLAU, HOXB7,BMP15, GPX4, EEF1A1, IL6, FTH1*
Maternal recognition of pregnancy	*IFNT, ISG15*
Housekeeping	*ACTB, GAPDH, PGK1, RPL19,*

Table 2.4 Average counts of differential genes in the endometrium of pregnant Vitamin B (VB) and Control (Con) cows

Gene	VB	Con	P-Value
OXTR	498.5±105.6	246.3±97.7	0.04
SELL	179.9±29.5	118±27.3	0.1
MUC5B	812.4±1.5	244.7±1.5	0.05
MUC1	1808.1±1.1	735.1±1.1	0.02
IL1β	109.9±1.2	54.6±1.2	0.05
TRD	1324±247	720±223	0.03
SPP1	447.6±1.2	222.1±1.2	0.03
PLAU	366.4±1.1	245.5±1.1	0.1
MYH9	5962.7±219.9	5461.7±203.7	0.1
FZD8	211.2±12.1	179.5±11.2	0.05
FOLR1	1570.2±102.7	1022±95.0	0.05

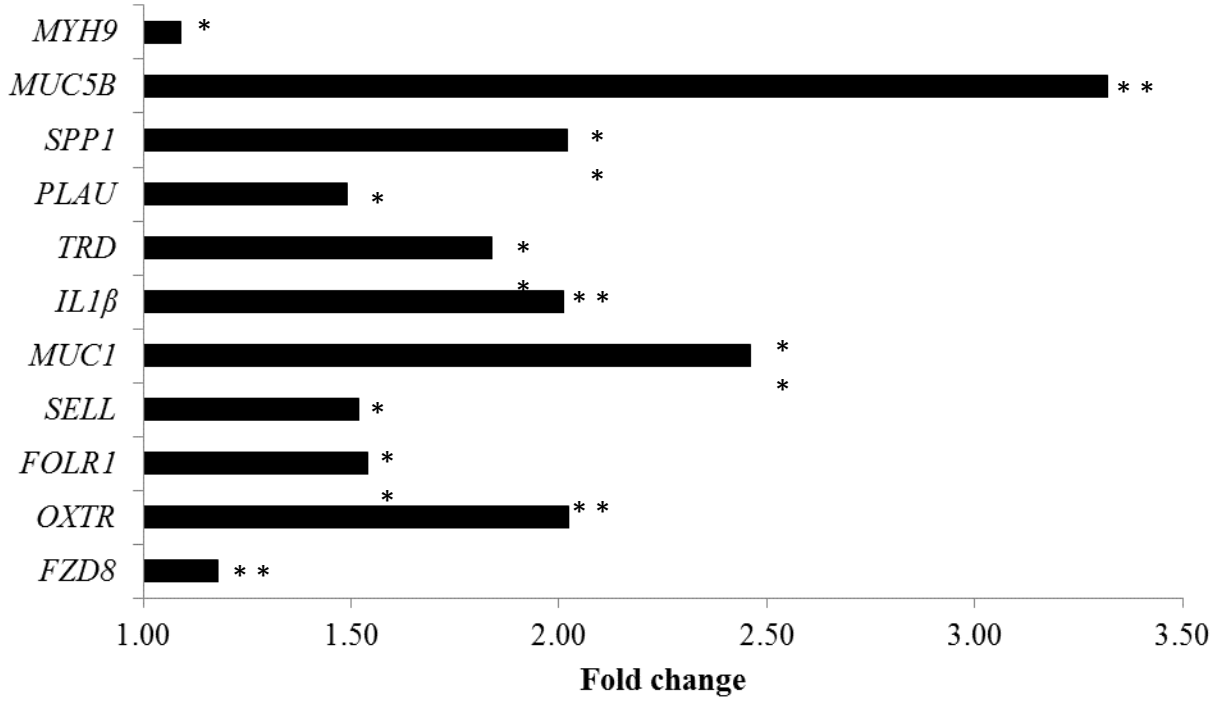

Figure 2.1 Relative fold change of differential genes between pregnant Vitamin B and Control animals with Control referrent (P ≤ 0.05, *P = 0.1)**

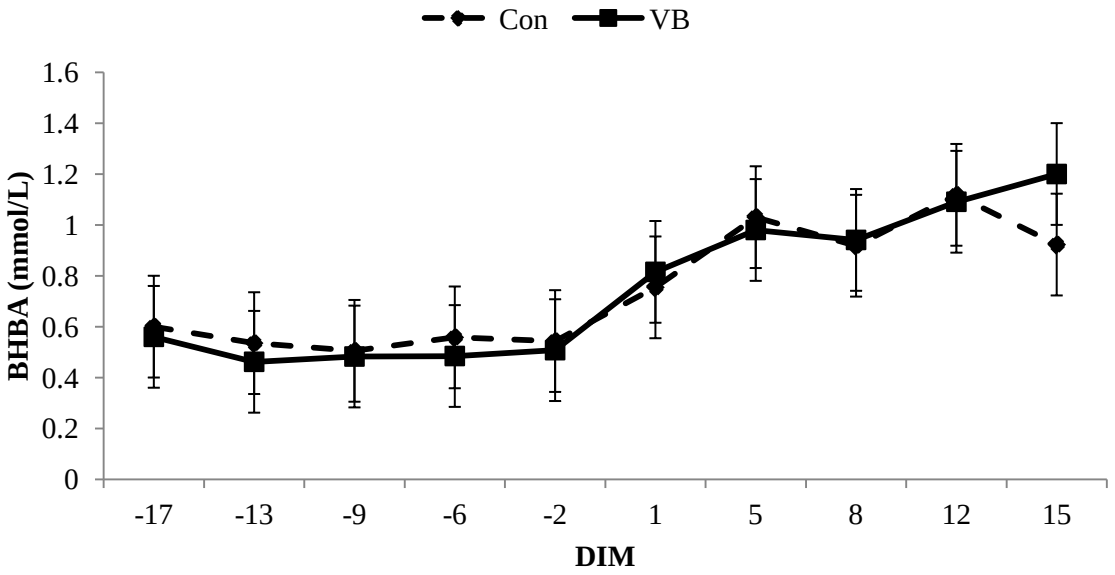

Figure 2.2 levels of Control (Con) and Vitamin B (VB) groups during transition period (P = 0.84)

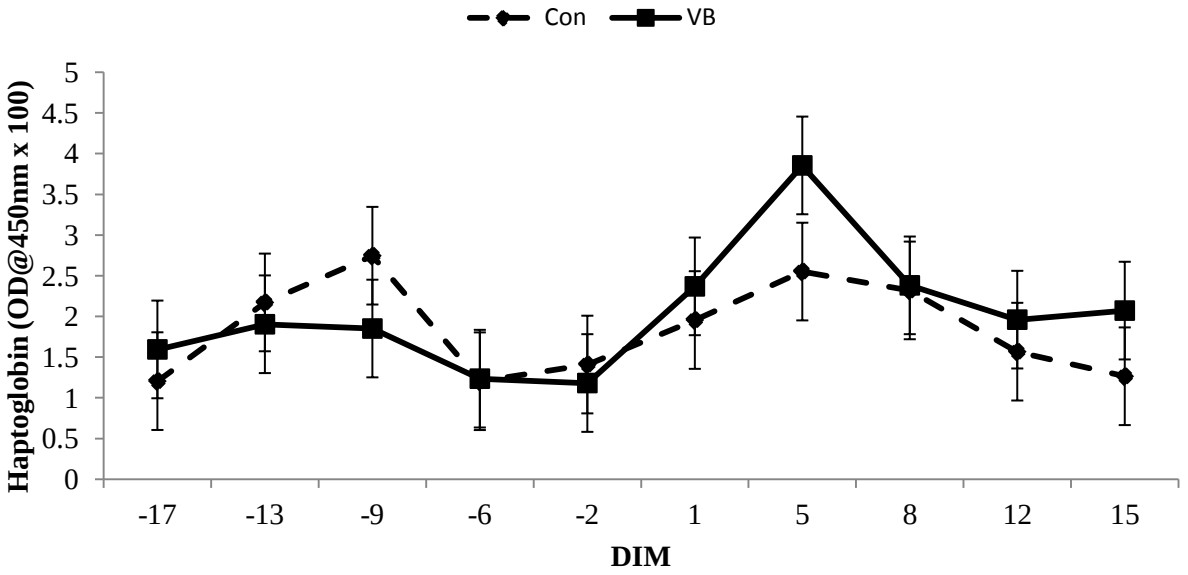

Figure 2.3 Mean haptoglobin levels of Control (Con) and Vitamin B (VB) groups during transition period (P = 0.3)

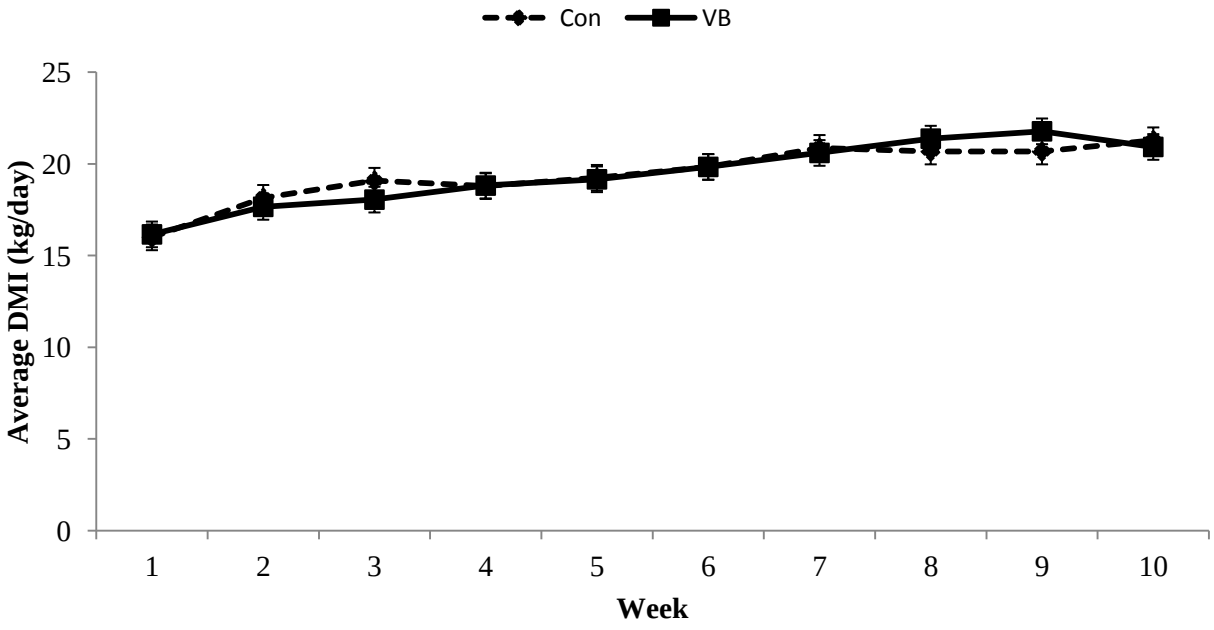

Figure 2.4 Average weekly milk production (kg/day) of Control (Con) and Vitamin B (VB) groups for first 10 weeks of lactation (P=0.9)

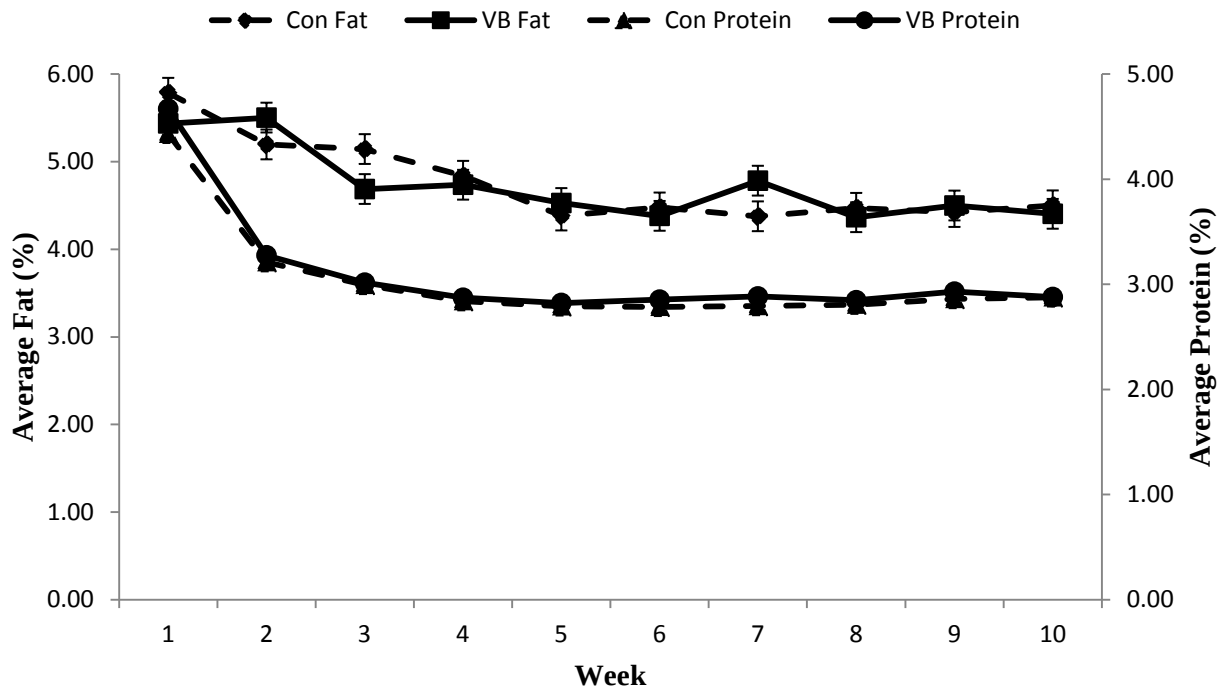

Figure 2.5 Average weekly fat (P = 0.86) and protein (P = 0.37) of Control (Con) and Vitamin B (VB) groups for first 10 weeks of lactation

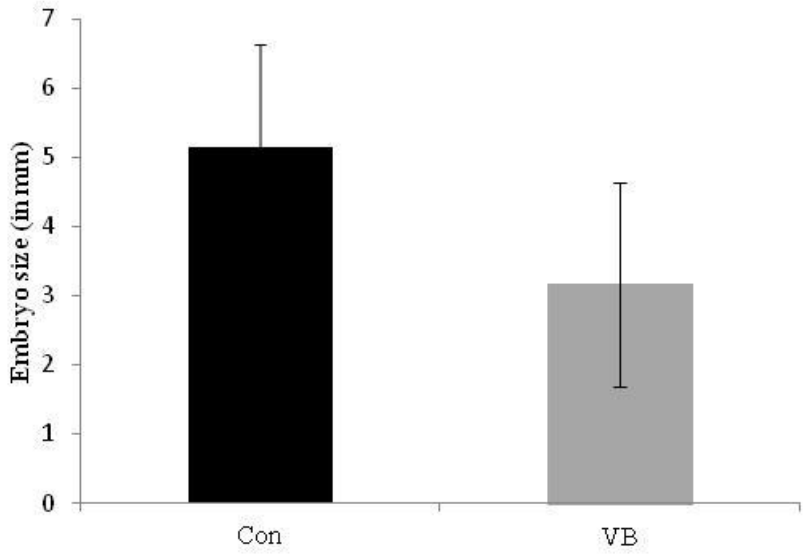

Figure 2.6 Average size of embryos collected from Control (Con; n = 7) and Vitamin B (VB; n = 6) cows on d 14 of pregnancy (P = 0.49)

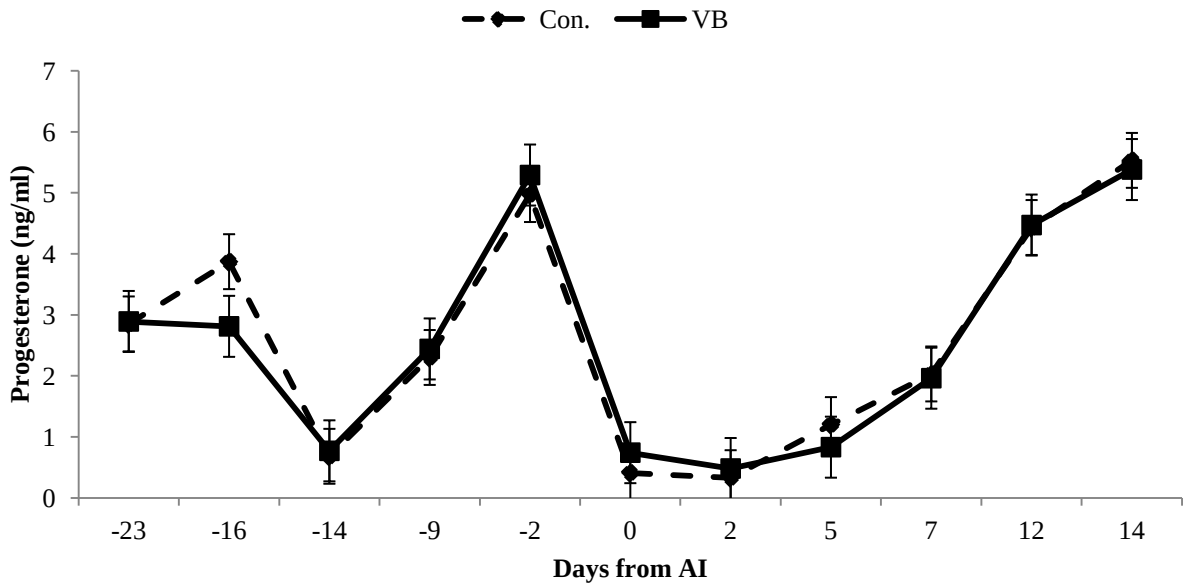

Figure 2.7 Progesterone concentrations of Control (Con) and Vitamin B (VB) groups at different time points during the synchronization protocol with Day 0 = AI (P = 0.8)

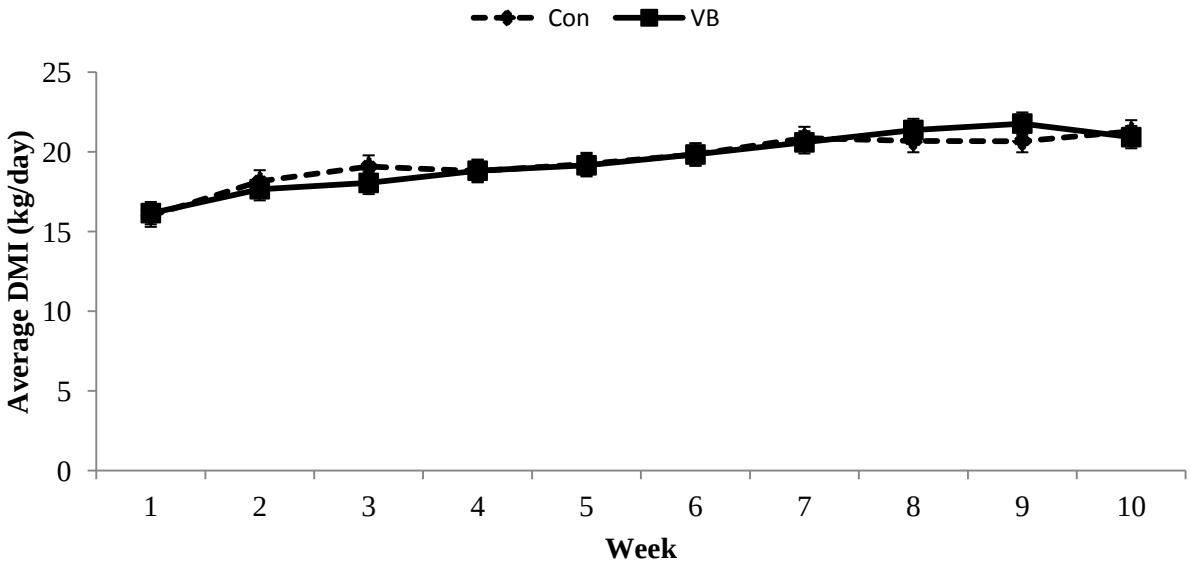

Figure 2.8 Average weekly DMI (kg/day) of Control (Con) and Vitamin B (VB) groups for first 10 weeks of lactation (P=0.94)

Chapter 3: Conclusion

3.1 Summary

Pregnancy loss, especially early embryonic loss, is one of the critical fertility issues in dairy industry. Most of these embryonic losses occur prior to maternal recognition of pregnancy, around d 16 of pregnancy. Embryo losses in dairy industry are substantial, with an estimated embryo mortality of 40% in dairy cattle (Thatcher et al., 2003). Unfavorable endometrial environment has been implicated to play a major role in these early pregnancy failures.

There are a number of studies that have looked at the effect of feed additives such as fatty acids, micro-minerals and amino acids on health and reproduction in dairy cattle, with results indicating that strategic supplementation of some nutrients having a potential beneficial effect on fertility (Santos et al., 1998; Thatcher et al., 2011; Ribeiro et al., 2016b). There has been an increasing interest in studying the effects of vitamin B complex on health and milk production of dairy cattle, due to the important role that these molecules play in metabolic cycles. In a more recent study, a positive effect of vitamin B-complex supplementation was seen as improvement of pregnancy rates at first AI in dairy cattle (Juchem et al., 2012). However, the molecular mechanisms behind the action of these molecules are relatively unknown, especially in the reproductive tract of animals. This gap in literature exists not only in dairy cattle research, but also in other species. The primary aim of this project was to study the underlying mechanisms behind the action of B-vitamins in the endometrium, to further our knowledge of micronutrient supplementation on domestic animals. The secondary aims of the project were to look at the effect of B-vitamin supplementation on milk production and metabolic parameters in high producing dairy cows. In the current study, we did not see any significant improvement in milk production or health of cows supplemented with the vitamin B complex. However, analysis of

expression of genes in endometrium around the time of maternal recognition of pregnancy revealed an upregulation of genes that are important in regulation of immune system, adhesion molecules, Wnt signaling, folate transport and morphogenesis. An improved markup of these genes is known to be play an important role in pregnancy establishment and helps explain the results observed by Juchem et al. (2012). Canadian dairy producers loose approximately $250 per cow per year due to metabolic disease incidence and poor reproductive performance (McArt et al., 2015) and strategic protected Vitamin B supplementation might be an innovative tool to improve the reproductive performance of the cows.

3.2 Limitations

One limitation of the study was the season of embryo collection. Due to unexpectedly hot weather the cows suffered from heat stress resulting in unsuccessful uterine flushing for two months, with no embryo detection. The negative impact of heat stress has been quantified in numerous studies in the past and the higher than average temperatures definitely impacted our results and could be one of the reasons why we were not able to see any differences in pregnancy rates between the two groups. A retrospective analysis of a subset of cows that were part of our trial revealed a greater pregnancy rates in VB cows compared to CON animals, further supporting this postulation. Another limitation was the time point of sample collection. Conceptus elongation starts around d 14, when we collected our samples. During the time of elongation, the conceptus doubles in length every day and sample collection on d 15 could have helped us better understand the effect of B-vitamins of growth rate of embryo as well as on IFNT production.

3.3 Future directions

This is one of the first studies to show the action of B-vitamins on the endometrium of dairy cows. Since there were no production or health effects seen, the results indicate that there is a direct effect of vitamin B complex molecules on the endometrium and not an indirect effect via improved metabolic efficiency. However, the exact mechanisms behind these changes are still not clear. Follow up studies are required to look at specific roles of these molecules in the bovine uterus and also to establish a better understanding of ruminal B-vitamin requirements.

Even though we proposed the direct action of B-vitamin molecules on the uterus, we cannot completely rule out the indirect mechanisms at play. Studies designed to look at the indirect effects of vitamin B complex on reproductive efficiency via improved metabolic activities can help us answer this conundrum. As stated in the limitations, the VB cows had greater pregnancy rates at first AI after embryo collection as well as fewer times bred, compared to CON animals, even though supplementation ended with embryo collection. This indicates a longer term carryover effect of B-complex supplementation and warrants more investigation. Also, this study adds on to the increasing evidence that the B-vitamin requirements need to be re-evaluated. Numerous studies have shown a positive impact of supplementation on health, production and fertility. Future studies should aim to re-evaluate the ruminant B-vitamin production as well as establish current requirements of dairy cattle and to update guidelines for supplementation.

3.4 Conclusions

There is increasing evidence that indicates that there is a need to re-evaluate the requirements of vitamin B complex molecules in dairy cattle. As seen in the current study, strategic supplementation with rumen-protected vitamin B complex improved the markup of

some key genes necessary for pregnancy establishment. The exact mechanisms behind these changes are still not clear; however, the results indicate a direct action of vitamin B molecules on the endometrium. A better understanding of ruminal B-vitamin requirements and more studies to look at specific roles of these molecules in the bovine uterus is required to improve the reproductive health of dairy cattle.

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Appendix A

List of genes and their target sequences

Gene	Accession	Target Sequence
ACTB	NM_173979.3	CGCCTTCGCCGCCGGTCGACACCGCAACCAGTTCGCCATGGATGATGATATTGCTGCGCTCGTGGTCGACAACGGCTCCGGCATGTGCAAGGCCGGCTTC
AGPAT1	NM_177518.1	GTGCAGGCCCAGGTTCCCATTGTGCCCATCGTCATGTCCTCCTATCAAGACTTCTACTGCAAGAAGGAGCGCCGCTTCACTTCGGGGCGATGTCAGGTCC
APC	NM_001075986.2	CACCGTACTTAAACACTACAGTATTGCCCAGCTCTTCTTCATCAAGGGGAAGTTTAGATAGCTCTCGTTCTGAGAAAGATAGAAGTCTGGAGAGAGAACG
AXIN1	NM_001191398.1	TGCTGACCAAGAAGGGCAACTACAGGTTCTACTTCAAGAAAGTGAGCGACGAGTTCGAGTGCGGCGTGGTGTTCGAAGAGGTGCGTGAGGACACGGCTGT
AXIN2	NM_001192299.1	ACCGGTATTACTTCAAAAAGGCGAGCGACGAGTTCGCGTGCGGAGCGGTGTTTGAGGAGGTCTGGGACGACGAGGTGGTCTTGCCCATGTACGAGGGCCG
B3GAT1	NM_001014393.1	CGGGGCACCATGCAGCGCAACCTGGCGCTGCGCTGGCTGCGGGAGACCTTCCCGCGCAATTCCAGCCAGCCTGGCGTGGTGTACTCTTCGAGGGACGACA
BMP15	NM_001031752.1	CCAAACTACTGTAAGGGAGTATGTCCTCGGGTACTACACTATGGTCTCAATTCTCCCAATCATGCCATCATCCAGAACCTTGTCAATGAGCTGGTGGATC
BSG	NM_001075371.2	CCTGGGCATCGTGGCCGAGGTGCTTGTGCTGGTCACCATCATCTTCATCTACGAGAAGAGGCGGAAGCCAGACGAGGTCCTGGATGATGAAGACATAGGC
CADM3	NM_001075946.1	CATCTTCACTATGCCCGTGAGAACTGCCAAGTCCCTCGTCACCGTGCTCGGAATCCCACAGAAGCCTATAATCAGTGGCTACAAGTCATCATTACGGGAA
CALB2	NM_001035288.1	AGCTCAAGGGATTCCTGTCTGATCTGCTGAAGAAGGCGAACCGACCATATGATGAACCCAAGCTCCAAGAGTACACCCAAACCATACTACGGATGTTTGA
CAPN6	NM_001192231.1	AGGATGAGCACAAGGTCATCATGTCTCTACAGCAAAAGGACCTGCGCACTTACCGCCGCATGGGAAGACCTGACAATTACATCATCGGCTTTGAGCTCTT
CDH1	NM_001002763.1	GGATTGCAAGTTCCCGCCATCCTGGGGATCCTTGGAGGCATCCTTGCTTTTCTGATCCTTATTTTGCTGCTTCTGCTACTTGTTCGGAGGAGAAGGGTGG
CLDN4	NM_001014391.2	GGCTCCTGCTGGCCAGGACAGTTCAGCCTTGACTTTGCTTGGGCTCTGCCTCTGAGTGGGTGCTCAGTGGACCATGGGGCCCACGTTGACCACCGTCCCC
CTNNA2	NM_001102110.1	CTGACTCATATGCCTCTGGCATGGGGAAAATAAGGAACAGTGTCTGTTTGCATGTAAGATGAGATGTGATCAATACCACTGATCCGTCTCTAGCCTGGGG
CTNNB1	NM_001076141.1	ATCCCACTGGCCTCTGATCAAGGCTACTGTTGGATTGATTCGAAATCTTGCCCTTTGTCCAGCAAATCATGCACCTTTGCGTGAGCAGGGTGCCATTCCA
CXCL10	NM_001046551.1	TTATCCTTCTGACTCTGAGTCAAGGTGTACCTCTCTCTAGGAATACACGCTGTTCCTGCATCGAGATCAGTAATGGATCTGTTAATCCAAGGTCCTTAGA
CXCL8	NM_173925.2	CAGAAGAAACCTGACAAAAAGCCTCTTGTTCAATATGACTTCCAAGCTGGCTGTTGCTCTCTTGGCAGCTTTCCTGCTCTCTGCAGCTCTGTGTGAAGCT
CXXC4	ENSBTAT00000002291.1	GACTGCCCGCAGAACCATTCCTCCTCCTCCTCGTCCTCCTCAGGGGGAGCTGGCGGAGCCAACCCGGCCAAGAAGAAGAGGAAAAGGTGTGGGGTCTGCG
CYP3A4	NM_001099367.1	ATGTTCCCTATCATTGGGAAGTATGGAGATGTGTTGGTGAGGAACCTGAGGAAGGAAGCAGAGAAAGGCACGTCTGTCGACATTAAAGACATCTTTGGAG
CYP4F2	NM_001035042.1	AGCAATTGCCAGGAGAAGCCCAGTGAATATATTGCTGCCATCTTGGAGCTCAGTGCCCTTGTTGCAAAACGACACCAGGAGATTTTTTTGCACATGGACT

DEFB1	NM_175703.3	ACCGACTACAGGTCACGTCCCTTCATGGCGCCTCCGGGCCCTCGTCAGTGAGATGCCAGAGCTGGCCATCGTGGAATTCCTCCCAGAACCTGG GACCTTT
DGKA	NM_001077860.1	TGCTTTGGAAATTCCAGTATCTGCTGAACCCTCGACAGGTGTTCAACCTCCTAAAGGATGGTCCTGAGCCAGGACTCAGATTCTTCAGAGATGT TCCTGA
DKK1	NM_001205544.1	ATGTATCATACCAAAGGACAAGAAGGTTCTGCCTGTCTTCGCTCATCAGACTGTGCTGCAGGATTGTGTTGTGCTAGACATTTCTGGTCCAAGA TCTGTA
EEF1A1	NM_174535.2	TGGCCAGATTAACTATGCTTCAGGGGTTTTTTCAAGAGCATGAGCAATTCATGTGGTTGGGTTCTGGTCTGCCCAAAAGCAAATTGCATATACT GAAGAG
ESR1	NM_001001443.1	CCATGGAATCTGCCAAGGAGACTCGCTACTGTGCAGTGTGCAATGACTATGCCTCAGGCTACCATTACGGAGTTTGGTCTTGCGAGGGCTGTA AGGCCTT
ESR2	NM_174051.3	GGGGCTGATGTGGCGCTCCATCGACCACCCTGGCAAGCTCATCTTTGCTCCAGACCTCATTCTGGACAGGGATGAAGGGAAATGTGTTGAAGG AATTCTA
FOLR1	NM_001206532.1	CTCCATGAGGCCTGGCCTCTGCGTTGCGGCCTGCTCTTATTGTTACTCTGGCTGCTCAGTTGAGCTCCTATTATTTTTTGACACCTGGAAATCCC TGCCC
FTH1	NM_174062.3	ACCCCGGCCGGCCACTCAGAGCCAGCCCTCGTCACCACTTGACAGCGCCCTCCGACCGGCCCAAGGTCCCCGCCACCGCTCCAGTGCCGCTCG GCCGTCG
FZD4	NM_001206269.1	GCTGTTCACCTACCTGGTGATTGGGACTTTGTTCATTGCTGCGGGGTTGGTGGCCTTGTTCAAAATTCGGTCGAATCTTCAAAAGGATGGGACG AAGACA
FZD7	NM_001144091.1	GTAGATATATGTGGGTTGGGAAAAGAGGCCTGGGTGGAAAGACGGTTTGGATGAAAAGACTTCTGGCAAAGACTTGCAGGATGATGCTGGTG ATGTTAAC
FZD8	XM_005214320.3	GCTGAGCTTGAAGCCAGACCTACAGATTTCACCTGAGGGACCTAAGTTTTCTCCCTCGACTCTGCTACGTAAACTCTTACTCCTAACACGTCCT AGATCT
GAPDH	NM_001034034.1	TGATTCCACCCACGGCAAGTTCAACGGCACAGTCAAGGCAGAGAACGGGAAGCTCGTCATCAATGGAAAGGCCATCACCATCTTCCAGGAGC GAGATCCT
GLYCAM1	NM_174828.2	GATCTCCAATGAGGACCTTTCTAAGGAGCCTTCCATCTCCAGAGAAGATTTGATTTCCAAAGAGCAAATTGTGATCAGATCTTCCAGGCAACC ACAGAGT
GPX4	NM_174770.3	GGGAGTAATGCAGAGATCAAAGAGTTCGCCGCTGGCTATAACGTCAAATTCGATTTGTTCAGCAAGATCTGTGTAAATGGGGACGACGCCCAC CCTCTGT
GSK3B	NM_001101310.1	CTGTTCTCGGTACTATAGGGCACCAGAGTTGATCTTTGGAGCCACTGATTATACCTCTAGTATAGATGTATGGTCTGCAGGCTGTGTGTTGGCT GAGCTG
HOXA10	NM_001105017.1	ATGCTGTCCTAAGTGTCAAGTGGTGACTGGGATGGTTTGCTGTCTCGGGGTTCCACTGCTTGAAATGGCCTCTGTCCTCCGGGTGCAGCTGGTT TCCTGC
HOXB7	NM_174342.2	AGAACCTCTCCGGGGTGTGTCCCGGCGACTCTGCCAAGGCGGCGGGCGCCAAGGAGCAGAGGGACTCGGACTTGGCGGCCGAGAGTAACTTC CGGATCTA
HPGD	NM_001034419.2	GCGCCAAGGTAGCGCTGGTCGATTGGAATCTCGAAGCAGGTGTCAAGTGTAAAGCCGCCCTGGATGAGCAGTTTGAACCTCAGAAGACTCTCT TTATTCA
IDO1	NM_001101866.1	CTTGGTTCTGGGATACATCACGATGGCGTATGTGTGGGGTCAAGGCGATGGAGACATCCGAAAGGTCTTGCCAAGCAATATCGCTGTTCCTTA CTGCAAA
IFNT2	NM_001015511.3	TCGTGCTCTCTCTACTGATGGCCCTGGTGCTGGTCAGCTACGGCCCGGGACGATCTCTGGGTTGTTACCTGTCTGAGGACCACATGCTAGGTGC CAGGGA
IGF1	NM_001077828.1	GGGACCCGAGACCCTCTGCGGGGCTGAGTTGGTGGATGCTCTCCAGTTCGTGTGCGGAGACAGGGGCTTTTATTTCAACAAGCCCACGGGGTA TGGCTCG
IGF1R	NM_001244612.1	ACCTGATGATCGCTCTGCCCATTGCGGTTCTGTTGATAGTGGGAGGCTTGGTTATAATGCTGTACGTCTTCCATAGAAAAAGAAATAGCAGCA GGCTGGG
IGF2	NM_174087.3	TCCAGTTTGTCTGTGGGGACCGCGGCTTCTACTTCAGCCGACCATCCAGCCGCATAAACCGACGCAGCCGTGGCATCGTGGAAGAGTGTTGCT TCCGAAG
IGFBP1	NM_174554.2	GAATGTGTCCCCAGAGAGCTCAGAGATAACTCAGGAGCAGCTTCTGGACAATTTCCACTTGATGGCCGAGTCCAGTGAGGACCTGCCCATCCT CTGGAAT

IGFBP2	NM_174555.1	CAGGAATTGGACCAGGTCCTGGAGCGGATCTCCACCATGCGCCTTCCGGATGAGCGGGGTCCCCTGGAGCACCTCTACTCCCTACACATCCCCAACTGTG
IGFBP3	NM_174556.1	TTCATGCCTTAGCAACGCGTGTGGCTCATGTTAGACGCGCTTCGTCTGCACTTGTAAGACGAGACAAGCCTCATCAAGAAGAAGAACGCCCTGTCCTTTA
IGHG1	DQ452014.1	TGCAAACGACCCTGTGATTGTTGCCCACCGCCTGAGCTCCCCGGAGGACCCTCTGTCTTCATCTTCCCACCGAAACCCAAGGACACCCTCACAATCTCGG
IGLL1	NM_001083800.1	GGAAGCAGCACGAATATCGGCATTTATGGTGTAAACTGGTACCAACAGGTCCCAGGATCGGGCCTCAAAACCATCATCTATGAAGATAAGTATCGACCCT
IL10	NM_174088.1	GGCGAGGCGAAGACTTTCTTTCAAATGAAGGACCAACTGCACAGCTTACTGTTGACCCAGTCTCTGCTGGATGACTTTAAGGGTTACCTGGGTTGCCAAG
IL1B	NM_174093.1	TGACCTGAGGAGCATCCTTTCATTCATCTTTGAAGAAGAGCCTGTCATCTTCGAAACGTCCTCCGACGAGTTTCTGTGTGACGCACCCGTGCAGTCAATA
IL6	NM_173923.2	CAAAAATGGAGGAAAAGGACGGATGCTTCCAATCTGGGTTCAATCAGGCGATTTGCTTGATCAGAACCACTGCTGGTCTTCTGGAGTATCAGATATACCT
ISG15	NM_174366.1	GCAGCAGGTGTGCCAAAAGGAGCGTGTACAAGCAGACCAGTTCTGGCTGTCTTTTGAAGGGAGGCCCATGGATGATGAGCACCCGCTGGAGGAATACGGC
LGALS3BP	NM_001046316.2	GGTGGGGCAGGTGCGGTTCCCCATGATGCCACCCCAGGACCTCTTCTCGCTGCAGTTTAACCTGTCCCTGTACTGGAGTCACGAGGCGCTCTTCCAGAAG
LIFR	NM_001192263.1	AATTCTGCCGGCTCATCACCACCTTCTAAAATAGCTAGTATGGAAATTCCCAATGATGATCTCAAAATAGAGCAGGCTCTTGGAATGGGAAATAGGATCC
CYP4X1	XM_003585903.4	GGTGATGGCTCAATCTGTGAACATCATGCTGAATAAGTGGGAGAAGATTTGTGGCTCTCAGAATACACTTCTGGATATCTATGAGCACATCAACTTGATG
NNMT	XM_015474625.1	GTATTACAGGATGAACCAGGATCCCGTCGGGGATGAAGTCCTCCATTTCCTGCTAAAACACTACAATGCCACCTTTAAACCAGGTGGGCTGGAAGGGAAA
LYZ2	NM_180999.1	TAATGATGGCAAAACCCCTAATGCAGTTGACGGCTGTCATGTATCCTGCAGCGAATTAATGGAAAATGACATCGCTAAAGCTGTAGCGTGTGCAAAGCAT
MMP19	NM_001075983.1	TTAATTTCAAGATGTCTCCTGGCTTTCCCAAGAAGCTGAATAGGGTAGGACCCAACCTGGATGCTGCTCTCTATTGGCCTATCAACAAAAAGGTGTTCCT
MOGAT1	NM_001001153.2	CCCATGGAGTGCTTGTGGTTGGAGCCTTTGGAAACTTCTGTACAAATTATTCGGCCTTCAAGGAGCTGTTTCCCGGCTTTACCTCCTATCTTCACGTGCT
MSX1	NM_174798.2	TTCCCACGAGGTGCTCCCTTGTTCAGCACCGCCTGGCACCTTCTCTTTAACGCCCACACTGCTCTGGTTTGTCCTCCGTTGCTACCGGAGTAAACTCTCT
MUC1	NM_174115.1	CAGGATCTGTGGTGGTAGAATTAACTCTGGCCTTCCGAGAGGGTACCACGGCCGAGTGGGTGAAGGCACAGTTCAGTCAGCTTGAAGCACACGCAGCCAG
MUC4	XM_015471614.1	GCCTGCACCCTATCACAGTTCAATGGCTCCTTAAGCCCAATAACACAATCCATGTTCAGCTCAATAACCAGACCATAGCATTTGAGACTAACGGTGAAGA
MUC5B	XM_015461377.1	GGTGTCCATCTCTAGTCCCTCTGCCTTCCCGAGGTCCTCACCCATGACCACCAAGCCAGACTTGTCCAGTTACTCACCCACAGGTCACCTGTTGACCTCT
MX2	NM_173941.2	AAGCATTACCTGGGGCAGCTGATAGACCCAGCACTCAAGATGCTCCAGAAGGCCATGGAAATTGTCTGGCAAACTTTCAAGGACACAGCCAAAAAGCATT
MYH10	NM_174834.1	AGATACGAGATCCTCACTCCAAATGCTATCCCTAAGGGTTTCATGGATGGCAAACAGGCTTGTGAACGAATGATCCGGGCTTTAGAATTGGACCCAAACT
MYH9	NM_001192762.1	CTGCAGGTCGAGCTGGACAATGTGACAGGTCTTCTCACTCAGTCGGACAGCAAGTCTAGCAAGCTCACCAAGGACTTCTCTGCTCTGGAGTCACAGCTGC
MYL12A	NM_001015640.2	GGGCACATGCATCTCTATAATCAGACTGGATATGGGACTTCTTGTCATTTTAAGAGTAGAAAATAGGGTAATTTAACTTACCAGCTGCCGTCTACCCTCC
NFKB1	NM_001076409.1	CCACTATGGATTCCCCACGTATGGCGGAATTACCTTCCATACTGGAACCACTAAATCTAATGCTGGGATGAAGCATGGAACCATAGACACCCCATCTAAA

NR1I2	NM_001103226.1	CCACATCGCTGACGTGTCCACCTACATGTTCAAAGGCATCATCAACTTTGCCAAGGTCATCTCCTATTTCAGGGACCTGCCCATAGAGGACCAGATCTCC
OXT	NM_176855.1	GGGCAAAGGCCGCTGCTTCGGGCCCAGCATCTGCTGCGGGGACGAGCTGGGCTGCTTCGTGGGCACGGCCGAGGCGCTGCGCTGCCAAGAGGAGAACTAC
OXTR	NM_174134.2	TGGATCACGCTCGCCGTCTACATTGTGCCCGTCATCGTCCTTGCCACCTGCTATGGCCTTATCAGCTTCAAGATCTGGCAGAATTTACGGCTCAAGACGG
PFKFB2	NM_174812.4	CGAGGAAAACAGTTTGCCCAGGCTCTAAGGAAGTTTCTGGAGGAACAGGAGATAGCAGACCTCAAAGTGTGGACGAGCCAGTTGAAAAGGACTATCCAGA
PGK1	NM_001034299.1	CTGATGGTGTCCCCATGCCTGATAAGTACTCCTTGCAGCCAGTTGCTGTAGAACTCAAATCTCTGCTGGGCAAGGATGTTTTGTTCTTGAAGGACTGTGT
PGR	NM_001205356.1	AGAAAGTGCTGTCAGGCTGGCATGGTTCTTGGAGGCCGAAAGTTTAAAAAGTTCAATAAAGTTAGAGTTATGAGAACACTAGATGCTGTTGCCCTCCCAC
PLAU	NM_174147.2	ACACTGCTTCATTGATCACCCAAAGAAGGAGAACTACATTGTCTACTTGGGTCAGTCACGGCTTAACTCCGACACACGTGGGGAGATGCAGTTTGAGGTG
PRKCG	NM_001166502.2	AACGGTCTCTCCGATCCCTATGTGAAGCTGAAGCTCATCCCAGACCCTCGGAATTTGACCAAGCAGAAGACCCGCACGGTGAAAGCTACGCTAAACCCTG
PTGES	NM_174443.2	TCTTTCCTCGGACCGAACCCCTTCGTCGCCCGGATGCACTTCCTGGTCTTCTTCCTGGGCCGTATGGTACACACCGTGGCATACCTGGGGAAACTGCGGG
PTGS2	NM_174445.2	GAAATGATCTACCCGCCTCATGTTCCTGAACACTTGAAGTTTGCTGTGGGCCAGGAAGTCTTTGGTCTGGTGCCTGGTCTGATGATGTATGCCACCATTT
PTX3	NM_001076259.1	CCCGACAGCGGTGGGAAGAACGTCGTCTCTCCAGCAATGCATATCTCTGTGATTCTGTTTTGTGCGCTCTGGTCTGCAGTGTCGGCGGAGAACTCAGATG
RELN	NM_001206458.1	CTCTATATTTCAGTAAGGCTGGGAAAAGACAGTTGGTGAGCTGGGATCTGGATACTTCTTGGGTGGACTTTGTCCAGTTCTACATCCAAATCGGTGGAGA
RPL19	NM_001040516.1	GCCTGTGACTGTCCATTCCCGGGCTCGATGCCGGAAAAACACCTTGGCTCGCCGGAAAGGCAGGCATATGGGTATAGGTAAGCGAAAGGGTACTGCCAAT
SELL	NM_174182.1	GAAGTGTGTCTCTCCCAGCATATCTGAAGAGTTTTCAACATGACCAGCTGCTCCTTCTGTTCCCGCTTTGCTCCATCTCCCATCCCTCAACTTTCAGCCT
SERPING1	NM_174821.2	TGGAGCAGGCTCTCAGCACCGCCGTCTTCAAAGCTGTCATAAAGAAGCTGGAGATGACCAAGTTCCATCCCACTCACCTGACGATGCCTCGCATCAAAGT
SGK1	NM_001102033.1	CCTGAGGTGCTTCATAAGCAGCCCTATGATAGAACCGTGGACTGGTGGTGCCTGGGGGCCGTCTTATATGAGATGCTGTATGGCCTGCCTCCCTTTTATA
SLC27A6	NM_001101169.1	TTCCAAGACTCCCAGAAGGTATCAGCATTTGGGCAATGAAAGACTCTGTTCCACAAGGCATAATTTCACTCAAAGAAAAACTGAGCACAGCATCTGACTG
SLC2A5	NM_001101042.1	CACCACCGTCTACATCTTCCTGATCATCCCTGAAACCAAGTCCAAGACCTTCATAGAAATCAATCGGATCTTCATCAAGATGAACAAAGTGCCAGGGGTG
SLC5A6	NM_001046219.2	TAATTCACTGGCAACTGTTACAATGGAAGACTTGATTCGACCCTGGTTCCCTCATGTCTCTGAAGTCCGGGCCACCATGCTTTCCAGAATCATCGCCTTT
SLC7A10	NM_001104989.1	GTGGGGACTACGCCTATGTCACTGAGATCTTTGGGGGCCTGGCTGGCTTCCTGCTGCTCTGGAGCGCCGTGCTCATCATGTACCCCACGAGCCTGGCCGT
SLPI	NM_001098865.1	GAAAGTCTGCCTTTCCCCTATGAAAGCCTGATTCCTGCCGTTTGGGAGAGTCTCTGGATTCCTACTCTGTGTGGTGTGGGAGTTCCATTTCTACGCCCAG
SPP1	NM_174187.2	CTTTCCAAAGAACTCACGCCAAAGGCCAAGGATAAGAACAAGCATTCCAATCTGATTGAGAGTCAGGAAAATTCCAAACTCAGCCAAGAATTCCATAGCC
TCN1	NM_001193105.1	CACCAACCCTTCTTGTGGCCTCAATCCAGTCAAATTCAACACCTCCACTGAAAAGCCTGGTACTGTGACACCTACAACCGCACCCTTGAATATCTTAGTC
TIMP2	NM_174472.4	CAGGACTCCTGGGGCCAATTGACAGTGTCCAAGAGTTCAGACTGGTCCAGCTCCGACATCCCTTCCTGGAAACAGCATGAATAAAAACAGTCATCCAAGT

TNF	NM_173966.2	TTCGCAACATTCCTTGAGAAGATCTCACCTAGAACTTGACATGCGTGGACTTCAACTCTCCCTTCCTGCCAATGTTTCCAGACTCCCCTGAGGTGGGAAG
TRD	ENSBTAT00000052114.1	CCCCGATTCAGTGACATGTTCGGTTGAACACAACAAGCAAACCTGGCACTCTACTGACTTTGAACCAAAGAAAACTATTCCAGAAACAACTCCAAAACCG
UHRF1	NM_001103098.1	GGCCGCAAGCACAGCAAGTATGCGCCCATCGAGGGCAACCGGTACGACGGCATCTATAAGGTTGTCAGGTACTGGCCCGAGAAGGGCAAGTCCGGCTTCC
VEGFA	NM_174216.2	GCCAGGCCTCCGAAACCATGAACTTTCTGCTCTCTTGGGTACATTGGAGCCTTGCCTTGCTGCTCTACCTTCACCATGCCAAGTGGTCCCAGGCTGCACC
WISP2	NM_001102176.1	ACTCGTATTAGTAAGAACTAACCGTTTTCACGACTCCGCAATGCACCAGGCCAAGCTACACATTATACAAGTCAGTGCTGGGAAACATTCTGGACTAGAG
WNT2	NM_001013001.1	CGTGCCATCGGCCTGGGCGTTACTGAGTGGACGATGGAGTGCCAGCACCAGTTCCGCCAGCACCGCTGGAACTGCAACACCCTGGACAGGGATCACAGCC
WNT3	NM_001206024.1	CAGGAGTGCCAGCACCAGTTTCGGGGCCGCCGCTGGAACTGCACCACCATCGACGACAGCCTGGCCATCTTCGGGCCCGTCCTCGACAAAGCCACCCGGG
WNT3A	XM_015471858.1	GAGTCCGCCTTCGTGCACGCCATCGCCTCTGCCGGCGTAGCCTTCGCGGTGACGCGCTCTTGCGCCGAGGGCTCCGCTGCCATCTGCGGCTGCAGCAGCC