Absorption and Utilization of Choline and Vitamin B12 in Lactating Dairy Cows using Different Delivery Methods.

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DEDICATION

Dedicated to

the memory of my beloved mother Graciela M. Etcheverry Rossello

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ABSTRACT

Choline and vitamin B12 are essential nutrients for growth and performances of production animals. However, both nutrients are extensively degraded during digestion in the rumen. This thesis comprised three experiments. First, four cows equipped with a rumen cannula and catheters in the portal vein and a mesenteric artery received a post-ruminal bolus of: 1) cyanocobalamin (CN-CBL) alone (0.1 g) [gram], 2) CN-CBL (0.1 g) + casein (10 g) or 3) CN-CBL (0.1 g) + whey proteins (10 g). After the bolus, blood samples were taken until 24 h [hour] post-bolus. The intestinal absorption of CN-CBL was greater when the vitamin was given in solution with casein (4 μg [micro-gram]/h) compared with CN-CBL given alone or with whey protein (-25 μg/h and -19 μg/h, respectively). Second, a LC-MS/MS methodology was established for differentiation of choline metabolites in blood and milk, from different physiological states of the lactating cow. Total choline concentration in plasma, which was almost entirely phosphatidylcholine, increased 10-fold from early to late lactation (1,305 to 13,535 μmol/L [micro-mol per liter]). In milk, phosphocholine was the main metabolite in early lactation (492 μmol/L), but decreased exponentially through lactation to 43 μmol/L in late lactation. In contrast, phosphatidylcholine in milk was the main metabolite in mid and late lactation (188 μmol/L and 659 μmol/L, respectively). Third, the choline metabolites were measured in milk and blood after post-ruminal infusion (ABO) of choline chloride or dietary supplemented with rumen protected choline (RPC) in a low dose 12.5 g (L) and high dose 25 g (H) choline/d, respectively. Although lipid soluble metabolites or total choline in plasma were not affected by treatments, total choline was transfer into milk at a low level, 2% RPC-H and 5% ABO-H. For practical application of our findings, first, dietary formulation of CN-CBL with

addition of casein may improve CN-CBL absorption in dairy cows; second, milk from dairy cows at early lactation is higher in phosphocholine and thus might be used for infant formula to better match breast milk; third, prediction of choline supply may be possible based on betaine and phosphocholine yields in milk

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LIST OF ABBREVIATIONS

ACho, Acetylcholine

Bet, Betaine

Cho, Free choline

GC-MS Gas Chromatography-Mass Spectrometry

GPCho, Glycerophosphocholine

HILIC, Hydrophilic Interaction Chromatography

IS, Internal Standard

LC-MS/MS, Liquid Chromatography-Tandem Mass Spectrometry

LPC, Lysophosphatidylcholine

PC Phosphatidylcholine

PCho, phosphocholine

PEMT Phosphatidylethanolamine N-Methyltransferase

RPC Rumen Protected Choline

SAM S-adenosylmethionine

SM, Sphingomyelin

TAG Triacylglycerol

THF, Tetrahydrofolate

TMR Total mixed ration

VLDL Very Low Density Lipoproteins

WOL, Week of Lactation

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

Introduction

Choline and vitamin B_{12} have several essential metabolic functions in mammalian animals, including the methylneogenesis or one-carbon metabolism, by which methyl groups are transferred to homocysteine to form methionine. Methionine is the precursor for s-adenosylmethionine, the main intermediate in the methylation of nucleic acids, lipids and proteins.

In dairy cows, the majority of choline and vitamin B_{12} in feed ingredients is degraded in the rumen (NRC, 2001). To prevent ruminal degradation, rumen protected (RP) products were developed that were successful in reducing liver triglycerides (TAG), consistent with the biological action of choline. However, the amount of choline absorbed in the small intestine (i.e., choline bioavailability) and its metabolism remains unknown. The combination with folic acid and vitamin B_{12} delivered by intramuscular injection increased milk components yield (e.g., fat and total solid yield), owing to improved metabolic efficiency. However, such performance improvements are too costly and not feasible under commercial conditions. The following dissertation will be divided in four chapters. The first chapter will focus on reviewing the literature on structure and functions of choline and vitamin B12 as well as describing the net-flux of a nutrient through the gastrointestinal tract. The second chapter will evaluate the casein and whey proteins as a delivery system to increase the intestinal absorption in dairy cows. The third chapter will focus on characterization of choline metabolites in blood and milk during lactation in dairy cows. The fourth chapter will evaluate the effect of post-ruminal infusion and rumen protected delivery of choline on blood and milk choline metabolites in the lactating dairy cow.

Vitamin B_{12}

Structure and synthesis of isomeric forms

Vitamin B_{12}, also called cobalamin (Cbl), is a water-soluble vitamin with a key role in the normal functioning of the brain and nervous system. It is the largest (MW=1355.4 Da) and most chemically complex of all the vitamins (Kozyraki and Cases, 2013). It consists of a central cobalt atom surrounded by six coordination sites. Four coordination sites contain corrin rings, one contains a dimethylbenzimidazole group, and one coordination site, the center of reactivity, is variable giving the different isomeric forms of vitamin B_{12}: a cyano group (-CN), a hydroxyl group (-OH), a methyl group (-CH3), or a 5'-deoxyadenosyl group (Watanabe, 2007). All the vitamin B_{12} isomers could form deeply red-colored crystals, due to the exposure of the cobalt-corrin complex to water (Herbert, 1988).

The synthetic isomers of cobalamin are cyanocobalamin and hydroxycobalamin, produced by bacterial synthesis, and converted to the biologically active isomers (i.e. methylcobalamin and adenosylcobalamin) after absorption. The industrial synthesis of cobalamin is a multi-step bio-synthetic fermentation process that requires selected microbial strains able produce high yields of vitamin B_{12} (Martens et al., 2002). Cyanocobalamin (CN-CBL) is commercially prepared adding a cyano group to stabilize cobalamin otherwise destroyed under light exposure. Since the cyanocobalamin form of vitamin B_{12} is easy to crystallize and is not sensitive to air-oxidation, it is typically used as a form of vitamin B_{12} for food additives and multivitamin supplements (Herbert, 1988). Hydroxycobalamin is another form of vitamin B_{12} commonly found in pharmaceutical products, which is produced by bacteria and typically supplied as a liquid solution for injection (Solomon, 2007).

Distribution in food

The synthesis of vitamin B_{12} is limited exclusively to bacteria. Thus, this vitamin is present only in foods that were bacterially fermented and those derived from the tissues of animals that obtained vitamin B_{12} from their rumen or dietary supplement (Combs Jr, 1998). Animal tissues that accumulate vitamin B_{12} (e.g., liver and kidney) are excellent food sources of vitamin B_{12} (Table 1.1). The richest food sources of vitamin B_{12} are dairy products, meats, eggs, fish, and shellfish. The main vitamin B_{12} isomeric forms in foods are methylcobalamin, deoxyadenosylcobalamin, and hydroxycobalamin. The main natural sources of vitamin B_{12} for animal feedstuffs are animal by-products such as meat and bone meal, fish meal, and cheese whey (Combs Jr, 1998).

Digestion, Absorption and Metabolism

Inhumans, vitamin B_{12} absorption is mediated by protein binding occurring in specific sections of the gastrointestinal tract. Vitamin B_{12} initially binds to the salivary R-binder (haptocorrins) in the mouth, which has high affinity for vitamin B_{12} under acid conditions (optimum pH <3) (Petrus et al., 2009). In the stomach, dietary vitamin B_{12} is released from proteins by pepsin, a proteolytic enzyme produced by gastric chief and parietal cells. However, the salivary R-binder protects vitamin B_{12} from acid hydrolysis (Petrus et al., 2009). Then, vitamin B_{12} binds to R-binder and passes into the small intestine where the R-binder is hydrolyzed by pancreatic proteases, and the free vitamin B_{12} binds to the intrinsic factor (IF) secreted by the gastric parietal cells (Combs Jr, 1998, Stipanuk and Caudill, 2012). In the small intestine, little vitamin B_{12} is absorbed by passive diffusion (ca. 1%). The main mechanism of vitamin B12 absorption is via active receptors located at the microvilli of the distal ileum. These receptors contain the

multi-ligand apical membrane protein cubulin, which recognizes and binds the IF–vitamin B_{12} complex, but not the unbound or free vitamin B_{12}. The enterocyte internalizes the IF–vitamin B12 complex by endocytosis and the IF is degraded in the cytosol (Figure 1.1). Vitamin B_{12} then traverses the endothelial cell and enters the portal circulation.

The intestinal absorption of vitamin B_{12} is limited by saturated rate of the proteins transports (Carmel, 2008). At doses of 3 μg, approximately 50% of the vitamin B_{12} will be absorbed, and at higher doses the net absorption decreases progressively (Carmel, 2011).

Vitamin B_{12} is transported in the blood bound to the proteins transcobalamin (or transcobalamin II) and haptocorrin (or transcobalamin I). Transcobalamin carries only about 20-30% of the total circulating vitamin B_{12} because it has a very short life, and haptocorrin carries about 70-80% of circulating vitamin B_{12} (Combs Jr, 1998, Stipanuk and Caudill, 2012). In humans, vitamin B_{12} is mainly stored as 5'deoxyadenosylcobalamin (ca. 2-3 mg) in the liver, and as methylcobalamin in plasma. Excess of vitamin B_{12} is excreted via urine and bile. Generally, the enterohepatic circulation results in effective re-uptake of biliary vitamin B_{12} via the IF-mediated process (Combs Jr, 1998, Stipanuk and Caudill, 2012).

The mechanism of absorption of vitamin B_{12} in ruminants has not been described in detail. The concentration of transcobalamin in bovine blood is similar to humans; however the binding capacity is lower in bovine transcobalamins (Polak et al. 1979).

Mammals use vitamin B_{12} as a cofactor of two enzymes (Figure 1.2) (Kozyraki and Cases, 2013).

1- Methionine synthase (also called methyl-FH4 methyltransferase). By converting homocysteine to methionine in the cytosol, methionine synthase generates the essential

aminoacid methionine involved in several methylation reactions necessary for nucleotide, DNA, RNA and protein synthesis and to regenerate tetrahydrofolate.

2- Methylmalonyl-CoA mutase. This mitochondrial enzyme participates in the catabolism of odd-chain fatty acids, branched amino acids, and cholesterol, to generate succinyl-CoA.

Vitamin B_{12} in the ruminant

Ruminants do not have dietary requirements for vitamin B_{12} (NRC, 2001) and it is generally agreed that ruminal microorganisms produce enough vitamin B_{12} when cobalt is available in the diet (NRC, 2001). Synthesis of vitamin B_{12} in the rumen is dependent on a continuous supply of dietary cobalt, as microorganisms can synthesize the organic portion of the corrin nucleus in the presence of cobalt (Combs Jr, 1998). Cobalt dietary requirement for dairy cattle is 0.11 mg/kg of dry matter (NRC, 2001). As dietary cobalt increases, the ruminal microbes also produce an increasing amount of non-physiologically active analogs to vitamin $B_{12,}$ (NRC, 2001).

In lactating cows, vitamin B_{12} is needed for propionate metabolism (gluconeogenesis), methionine synthesis, and folate metabolism. At the beginning of the lactation in dairy cows, the concentration of serum vitamin B_{12} is reduced due to the increased demands for lactation (Girard and Matte, 1999). Serum vitamin B_{12} concentration dropped from 5.7 ng/mL at 55 d prepartum, to 2.0 and 1.9 ng/mL at 7 and 120 d of lactation, respectively (Kincaid and Socha, 2007). Studies using both dietary supplements (Graulet et al., 2007, Girard and Desrochers, 2010) or intramuscular injections (Girard and Matte, 2005a, Preynat et al., 2009) of CN-CBL combined with folic acid, biotin or rumen protected methionine supplementation, resulted in increased of milk fat and total solids, suggesting that the amount of vitamin B_{12} produced by ruminal

microorganisms is not enough to meet vitamin B_{12} requirements (Table 1.2). In addition, vitamin B_{12} concentration in milk was increased from 1.2 to 4.0 ng/ml when parental and dietary supplementation was used (Girard and Matte, 2005a; Graulet et al., 2007).

A glass of milk from supplemented cows will cover 42% of adult human daily requirements for vitamin B_{12} (Girard and Matte, 2005a; Graulet et al., 2007). Therefore milk from supplemented cows with vitamin B_{12} has the potential to be an excellent source of vitamin B_{12} for human consumption. However, such production responses require high amounts of dietary vitamin B_{12} (500 mg) in order to increase plasma concentration (Graulet et al., 2007) at levels similar to those observed after 10 mg vitamin B_{12} given by intramuscular injections. A study conducted in dairy cows equipped with cannulas in rumen and duodenum, determined that 80% of a dietary supplement of cyanocobalamin (CN-CBL) is degraded in the rumen and less than 25% of the amount of vitamin B_{12} bypassing the rumen is absorbed in the small intestine of dairy cows (Girard et al., 2009). Although there is evidence that a dairy cow needs vitamin B_{12} supplementation for optimal performance, current dietary supplementations for vitamin B_{12} are costly and the use of intramuscular injections is not feasible under commercial conditions.

Choline

Structure and Functions

Free choline [2-hydroxy-N,N,N-trimethylethanaminium or (b-hydroxyethyl) trimethylammonium], $(CH_3)_3N+CH_2CH_2CH_2OH$, (2-hydroxymethyl-trimethyl-ammonium) is a trimethylated, positively charged, quaternary, saturated amine that serves as precursor of several metabolites and is commonly grouped with the water-soluble B vitamins. Choline is a family of compounds present in cells as a free choline and within subunits of a macromolecule (e.g., phosphocholine in biological membranes) (Wurtman et al., 2010). Pure choline is a colorless, viscous, strongly alkaline hygroscopic liquid. Choline is soluble in water, formaldehyde, and alcohol and has no definite melting or boiling point (McDowell, 1989). The chloride salt of this compound, choline chloride, is produced by chemical synthesis for the feed industry. Choline chloride exists as white crystals very soluble in water and organic solvents. Aqueous solutions have neutral pH. The prominent biological relevance is derived from three methyl groups that enable choline chloride to serve as a methyl donor (Combs Jr, 1998).

Distribution in food

Zeisel et al. (2003) evaluated the choline content of 145 commonly consumed foods. Choline and choline metabolites determinations included free choline (Cho), phosphocholine (PCho), glycerophosphocholine (GpCho), phosphatidylcholine (PC), betaine, and sphingomyelin (SM). All raw and unprocessed foods contained some choline. Foods with the highest total choline concentration (mg/100 g) were: beef liver (418), chicken liver (290), eggs (251), wheat germ (152), bacon (125), dried soybeans (116) and pork (103). The predominant form was PC, which

accounted for 95% of total choline in eggs and about 55-70% in meats and soybeans. In wheat germ, the predominant form was Cho (45%), while PC was the second most abundant form (30%). In general, whole fruits and vegetables contained about 5-40 mg total choline/100 g (Table 1.2). The amount of total choline in vegetables was stable during cooking process, but free choline decreased and PC increased when vegetables were boiled (Zeisel et al., 2003). In addition, finely minced processing in vegetables decreased the concentration of PC and increased Cho concentration, suggesting that phospholipase D was activated (Zeisel et al., 2003).

Digestion, Absorption and Metabolism

Digestion and absorption of choline vary depending on the specific choline ester (Stipanuk and Caudill, 2012). The absorption of free choline is mainly in the jejunum and ileum via a sodium dependent carrier-mediated transport that is saturable and substrate-specific (Stipanuk and Caudill, 2012; Garrow 2007). Free choline in the enterocyte is rapidly phosphorylated to PCho by the cytosolic enzyme choline kinase. In the nucleus, PCho is activated to CDP-choline in a CTP-dependent reaction by CTP-phosphocholine cytidyltransferase. The synthesis of PtdCho by the CDP-choline pathway is regulated by the rate-limiting enzyme CTP:phosphocholine cytidyltransferase (CCT). The decrease of PC levels or fatty acid /diacylglycerol concentrations increases the activity responds of CTP:phosphocholine cytidylyltransferase (CCT) enzyme in the nucleus high (Gehrig et al., 2009). Finally, CDP-choline is catalyzed by choline phosphotransferase to PC in the endoplasmatic reticulum. The new PC is then transported out of the cell via membrane lateral diffusion by vesicular transport or a protein carrier.

An increase in choline supply in the small intestine increases the uptake of choline by the liver, the major site of choline metabolism, through the cystidine diphosphate (CDP)-choline pathways

for phospholipids synthesis (i.e phosphatidylcholine, sphingomyelin), whereas excess choline is converted to the methyl donor betaine (Fig. 1.3). In non-ruminants, the CDP-choline pathway is the main source of choline (Zeisel et al., 1991) but in ruminants because almost all dietary free choline is degraded in the rumen (Sharma and Erdman, 1989) this pathways is limited (Grummer, 2012).

Choline can be synthesized endogenously by the enzyme phosphatidylethanolamine N-methyltransferase (PEMT) that catalyzes the methylation of phosphatidylethanolamine to PC by the transfer of 3 methyl groups from S-adenosylmethionine (Cole et al., 2012). In non-ruminants, this enzyme accounts for *ca.* 30% of the PC made in hepatocytes (Vance et al., 1997). PEMT knockout mice have an absolute requirement for dietary choline and the deficiency of dietary choline in these mice results in a dramatic reduction of PC, accumulation of triglycerides in the liver, and death after 3-5 days (Vance et al., 1997).

Although PEMT was detected in several tissues including brain and mammary epithelial cells, PEMT activity is quantitatively significant in the liver (Reo et al., 2002). The PEMT gene is estrogen-responsive in human and mouse hepatocytes (Resseguie et al., 2007). When deprived of dietary choline, almost 80% of men and postmenopausal women developed liver or muscle damage, whereas only 43% of premenopausal women developed such signs of organ dysfunction (Fischer et al., 2007). Therefore, from an evolutionary perspective, women during pregnancy and lactation can supply more choline from endogenous biosynthesis to support nervous system development in neonates (Fischer et al., 2010).

A single nucleotide polymorphism in the sequence of the genes of PEMT and choline dehydrogenase (CHDH) are associated with altered susceptibility to develop organ dysfunction

on a low choline diet, and they likely affect dietary requirements for choline (da Costa et al., 2006).

Although the product of both CDP-choline and the PEMT pathways is PC, the fatty acid profile of the PC molecules differs between pathways synthesis. The combination of esterified fatty acyl moieties in the PC molecules as defined by at the sn -1 and sn -2 positions of the glycerophosphate backbone, is specific of the synthesis by both pathway. An study in human and mouse livers using radioisotopes of PC metabolic labeling found that PC produced via the PEMT-mediated phosphatidylethanolamine (PE) pathway was enriched in long-chain PUFAs such as DHA (22:6n23) and arachidonic acid (ARA; 20:4n26) (DeLong et al., 1999). In contrast, PC generated by the cytidine diphosphate (CDP)–choline pathway is enriched in di- and mono-unsaturated fatty acids such as linoleic acid (18:2n2) and oleic acid (18:1n2). The portion of PtdCho species containing DHA (pmol PtdCho-DHA/nmol PtdCho) is useful as a surrogate marker for *in vivo* hepatic PEMT activity in humans (da Costa et al., 2011).

Physiological Functions of Choline

The physiological functions of choline include lipid biosynthesis and metabolism, membrane formation, neurotransmission, and 1-carbon metabolism. Choline is present in bile at high concentrations (95% as PC). Particularly, PC acts directly on the intestinal mucosa to restore its protective hydrophobic lining, otherwise under bile salts attack (Barrios and Lichtenberger, 2000). Approximately 95% of biliary PC are reabsorbed by the intestine and incorporated into chylomicrons. Although newly synthetized PC contributes only a small percentage of biliary PC, both betaine homocysteine methyltransferase and PEMT are localized into the canalicular

membranes and thereby enable the synthesis of PC close to the biliary excretion site (Sehayek et al., 2003).

Phospholipids are the structural elements of biological membranes, and are critical for cellular division and transmembrane signal transduction (Combs Jr, 1998). The main phospholipid constituent of the bilayer membrane is PC. In the cell membrane, most of the phosphatidylcholine is located in the outer leaflet of the lipid membrane. Sphingomyelin and lysophosphocholine are also found in all membranes within the cell, but at much lower levels than PC. Like PC, the sphingomyelin and lysophosphocholine content in the outer leaflet of the plasma membrane is greater than that in the inner leaflet.

The main phospholipid on the surface monolayer of VLDL particles is PC which is essential for packaging and transporting the lipids delivered from the diet as chylomicron and non-esterified fatty acids into lipoprotein lipids for export to extra hepatic tissues.

Choline is required for several processes in the brain. It is needed for neurotransmitter synthesis (acetylcholine), essential for synapsis formation and myelation (Micheau and Marighetto, 2011). Studies in rodents have shown that the availability of choline in utero and during the post-natal period produces important changes in neural systems that underlie learning and memory and exerts life-long effects on cognitive functioning (Zeisel, 2012).

The importance of dietary methyl donors, and in particular choline, requires a broad understanding of the interrelationship and balances of one carbon metabolic pathways. Figure 1.4 integrates metabolic pathways involved in one carbon metabolism.

The endogenous biosynthesis requires the methylation of PE to PC catalysed by PE-N-methyltransferase (PEMT), which requires the methyl donor S-adenosylmethionine (SAM) (Reo et al., 2002). This reaction is influenced not only by the availability of SAM, but is also inhibited

by S-adenosylhomocysteine (SAH), and the ratio of SAM:SAH (an indicator of methylation capacity) therefore affects the activity of PEMT (Vance et al., 1997). Since there is no storage for free choline, at cellular level choline is oxidized to betaine (Garrow, 2007). The increase of free choline at the small intestine can saturate and inhibit choline kinase, the first enzyme in the CDP-choline pathway and then, the majority of choline then enters the mitochondria where it is oxidized to betaine in a two-step reaction (Stipanuk and Caudill, 2012).

In ruminants, only small quantities of methyl group nutrients (choline, folate and methionine) are available from the diet and methionine synthase assumes a much more important role (Neill et al., 1979). Methionine synthase is responsible for the *de novo* synthesis of methionine from one-carbon units furnished by 5-methyl-tetrahydrofolate (Pinotti, 2012). In studies on interchangeability of choline and methionine in lactating goats by infusing radiolabelled choline and methionine, Emmanuel and Kennelly (1984) concluded that 28% of methionine was used for choline synthesis. In dairy ruminants, methionine is also the first limiting amino acid required for transmethylation reactions and milk protein synthesis (Lobley et al., 1996). Vitamin B_{12} is a cofactor for methionine synthase to generate methionine when a methyl group from 5-methyl-THF is transferred to homocysteine. If one carbon-unit from the folate metabolism is available, vitamin B_{12} supports PEMT activity by reducing homocysteine level and by increasing methionine levels, resulting in an increased SAM: SAH ratio, and thus increased methylation capacity (van Wijk et al., 2012).

Analytical methods for choline

Several analytical methods have been proposed to identify and quantify choline metabolites with different performance and specificity.

Total choline can be determined by the quantification/addition of all free and esterified choline isomers, or by the hydrolysis of the metabolites before quantification of free choline. The digestion or hydrolysis is not a preferred method as it loses information on the individual choline-containing compounds.

Currently, the only widely accepted quality control assay for total choline determination is the AOAC 999.14 method, modified for various matrices (Rader et al., 2004). The method consists in digesting the sample in acid or alkali to release most of the choline, followed by an enzymatic hydrolysis using phospholipase D. Then, the free choline moiety is exposed to choline oxidase with subsequent liberation of hydrogen peroxide. In the presence of peroxidase, phenol is oxidized and a quinoneimine chromphore is formed with 4-aminoantipyrine. Both enzymatic and color-forming reactions are synchronized to occur concurrently. Absorbance is measured at 505 nm, corrected with a choline blank, and then quantified using a calibration curve. This method is relatively simple and does not require specialized or dedicated instrumentation. Moreover, the method was assessed in a collaborative study including 29 laboratories prior to reaching official status by AOAC (Phillips, 2012). The limitation of this methodology is that it does not quantify the acid-resistant of PCho (Phillips, 2012).

The measurement of choline-related compounds and phospholipids is important to understand the biological relevance of specific isomers. Various analytical techniques were developed for the quantification of some of these choline moieties, including ^{31}P nuclear magnetic resonance (^{31}P NMR) (Brinkmann-Trettenes et al., 2012), gas chromatography coupled with mass

spectrometry (GC/MS) (Pomfret et al., 1989), liquid chromatography coupled with evaporative light scattering, fluorescence, or mass spectrometry (Sala Vila et al., 2003, Zhao et al., 2011).

Nuclear magnetic resonance (^{31}P NMR) has been used to quantify phospholipids including PC, phosphatidylethanolamine, and phosphatidylinositol in different biological matrices (Klein et al., 2010, Brinkmann-Trettenes et al., 2012). Its main drawback is that this method uses chemical shifts observed for phosphorus-containing molecules, so it cannot quantify species without P such as choline (Zhao et al., 2011). Moreover, although it is a very convenient method requiring little sample preparation, it cannot differentiate fatty acid chains in choline esters (e.g., PC; Zhao et al., 2011).

Previous methods using GC-MS (Pomfret et al., 1989) and thin-layer chromatography (Dickens and Thompson Jr, 1982) are time-consuming and have relatively low sensitivity. For instance, despite of the fact GC-MS successfully quantified choline, PCho, GPCho, PC, and SM, as well as AcCho and LPC, Pomfret et al. (1989) suggested that only 40 samples could be analyzed in a single week, which it is not practical for routine use in the determination/quantification of total choline in foods and dietary supplements (Phillips, 2012). Koc et al. (2002) developed a LC-MS technique using isotope dilution MS (IDMS) to analyze choline compounds. This method was utilized in the analysis of 630 food items to generate a USDA database (Howe et al., 2004). Although improved, this method still requires pre-fractionation of the extracted samples into an aqueous phase containing betaine, choline, GPC, PCho and a chloroformic phase containing phosphatidylcholine and sphingomyelin. The two phases are analyzed in two LC runs using the same mobile phase but different gradients.

Recently, a new analytical method was developed and validated to simultaneously quantify all choline-related compounds and phospholipids (Zhao et al., 2011, Xiong et al., 2012). Due to both

the diversity of polar head groups found in phospholipid molecules and the polarity of choline-related compounds, a separation scheme based on hydrophilic interaction chromatography (HILIC) was used to determine either a limited number of water soluble choline compounds or phospholipids classes such as PC and SM. In addition, mass spectrometry was added as an additional dimension of separation based on mass-to-charge ratio. The molecular weight and fragment ion information provided by the mass spectrometer made it possible to differentiate choline metabolites with various combinations of fatty acyl substitutes within the PC or LPC fractions (Zhao et al., 2011).

Choline in the ruminant

Best and Huntsman (1930) demonstrated that choline is required in the diet by showing that a choline-deficient diet caused fatty liver disease in rodents. The essentiality of choline in the diet was determined for several mammalian species such as rat, dog, human and production animals namely poultry and swine (Stipanuk and Caudill, 2012). However, the extensive degradation of dietary choline in the rumen and the endogenous choline synthesis made it difficult to estimate choline requirements in ruminants (NRC, 2001).

Plant and animal tissues as well as feedstuffs derived from them contain free choline and choline-containing phospholipids (Pinotti et al., 2002). Choline is present mainly as a PC (lecithin) in feed ingredients and crude unprocessed fat sources, while in compound feeds, choline chloride is usually added as a supplement. Choline in feed (predominantly lecithin) and supplemental choline chloride were shown to be extensively degraded (from 79 to 99%) in the rumen (Sharma and Erdman, 1989). In sheep equipped with rumen fistula and abomasal cannula, 76% of [^{14}C]-choline injected into the rumen was expelled as methane over 6h (Neill et al., 1978)

and the ^{14}C phosphatidylcholine in the rumen was initially rapidly broken down (<3 h). Approximately 28% of the radioactivity remained constant in the rumen, suggesting that part of the dietary choline was incorporated into protozoa PC (Neill et al., 1979). Only 0.07% of the administrated [^{14}C] –choline dose was found in the liver and only traces in other tissues (Neill et al., 1979). Therefore, choline present in the diet of ruminants largely as phosphatidylcholine from plant membranes is rapidly released and degraded in the rumen by the microbes to trimethylamine and ultimately methane (Neill et al., 1978, Neill et al., 1979).

In dairy cows, the output of choline compounds in milk is high but the dietary availability of choline is still low and precursors from the *de novo* pathway may be limiting, especially at the onset of lactation (Baldi and Pinotti 2006). Several studies showed that 50 to 60% of the transition cows (3 weeks before and after lactation) experience moderate to severe fatty liver disease at the onset of lactation (Bobe et al., 2004), which is a clear symptom for choline deficiency. Based on those considerations, microencapsulated technology has been developed to protect choline from degradation in the rumen. The formulated microcapsule allows controlled release of the supplement into the small intestine, thereby improving its effectiveness and ensuring optimal dosage. Commercially available rumen protected choline, containing 28–50% choline chloride, was found to protect *ca.* 85% of the choline from rumen degradation *in vitro* (Pinotti et al., 2002). These products can be added directly to the compound feed or administered as a top-dress.

Several studies were conducted to elucidate the effect of rumen-protected choline (RPC) supplementation on transition dairy ruminants. RPC supplementation reduced triacylglycerol accumulation in the liver and increased milk yield and milk fat concentration, consistent with the native choline biological effect (Table 1.4). Indeed, gene expression for proteins involved with

lipoprotein synthesis, i.e. VLDL, and assembly in liver was greater in cows supplemented with RPC during the transition period (Goselink et al., 2012). A study conducted in dairy cows from 25 days prepartum to 80 days postpartum, reported that cows fed with RPC showed reduced incidence of clinical ketosis, mastitis, and morbidity (Lima et al., 2012). The supplementation during transition period on dairy cows with rumen-protected choline (20 g of choline chloride) increased plasma levels of choline-containing phospholipids and folates, from 127 to 171 mg dL^{-1} and 6.74 vs. 9.68 ng mL^{-1}, respectively (Baldi et al., 2004). Therefore, RPC supplementation not only increased choline availability but also optimized methyl group metabolism. The increase in plasma folates was attributed to a sparing effect of choline on one-carbon metabolism.

The supplementation of choline plays a significant role in the transition cow but requirement for choline may continue throughout lactation. At the mammary gland level in dairy ruminants, choline plays a major role in lipid metabolism, particularly in lipid transport and milk fat secretion. When availability of choline from natural or sources or synthetic is restricted, the rate of choline-containing phospholipids synthesis decreases, affecting lipoprotein lipid transport (Pinotti et al. 2002). Therefore, it is necessary to define choline requirements and feeding guidelines for choline on dairy cows.

The choline content of milk is a useful measure of choline status. The milk choline secretion increased from 1.95 to 3.95 g/d when dairy cows were infused during a 14-d period with 60 g/d of choline chloride (Deuchler et al., 1998). In addition, when dairy cows were abomasally infused with 0, 25, 50, and 75 g/d of choline chloride, the total choline in milk increased from 2.56 to 3.82 g/d (Deuchler et al., 1998). Therefore, the total choline concentration in milk is a marker of choline supply and bioavailability in dairy cows.

Choline in milk

Despite the lack of dietary-derived choline in the ruminant, milk and dairy products are an important source of choline in the human diet. One cup of retail milk (2% fat) is expected to provide approximately 40 mg of choline (Howe et al., 2004), which represents nearly 10% of an adult woman's daily choline requirement (Zeisel and Da Costa, 2009). The major choline-containing compounds in bovine milk are free choline, glycerophosphocholine and phosphatidylcholine (Patterson et al., 2008). The total choline content is similar in both, whole and skim milk (16.40 mg/100g vs 15.63 mg/100g, respectively). However, PC concentration is the only metabolite greater in whole milk than skim milk (1.15 mg/100g vs 0.75 mg/100g, respectively) whereas the other choline metabolites are in similar concentrations for both milks.

Phospholipids account for about 1% of the total bovine milk lipids (Bitman and Wood, 1990), and about 60% of these are found as part of the milk fat globule membrane (MFGM), with the rest located in the residual MFGM material found in the milk serum phase (Keenan, 2001). Distribution of the fatty acids and the head groups of the individual phospholipids determine the physical characteristics of the dairy products and the MFGM structure. The degree of saturation and the length of the fatty acids of phospholipids play a key role in the fluidity/rigidity of the MFGM surrounding the liquid lipid core of the fat globule (Singh, 2006). Phospholipids in milk are easily hydrolyzed or oxidized (Singh, 2006). Upon processing (e.g. homogenization, heat treatment), the MFGM is altered and loses some phospholipids, which will consequently influence its functional properties (Keenan, 2001). It is uncertain whether this occurs due to heat treatment and/or during agitation when membrane material desorbs because of coalescence of fat globules (Singh, 2006).

Net flux of the nutrients across the gastrointestinal tract and the liver

The stomach allows extensive digestion, absorption and metabolism of dietary nutrients before they reach the small intestine, where further digestion and absorption occurs. *In vivo* measurements of the relative contribution of stomach or post-stomach tissues to the absorption or metabolism by the entire gastrointestinal tract, have been limited to measurements of nutrient disappearance from digestion in cannulated animals (Reynolds and Huntington, 1988). These measurements did not account for the extensive metabolism of nutrients by the gut tissue which determined their net appearance in the hepatic portal vein. At the small intestine, the digestion products are transported into the enterocyte where some nutrients are taken up for maintenance of the structural and functional integrity of the small intestinal mucosa or enter to portal blood or the lymphatic vessels for passage and delivery to the rest of the body (Stipanuk and Caudill, 2012).

The portal vein is a vessel that conducts blood from the gastrointestinal tract and spleen to the liver. This blood is rich in nutrients that were extracted from the ingested food and will be metabolized in the liver. Therefore, blood sampled at the portal vein compared with arterial blood represents the cumulative effects of metabolism across a range of diverse tissues (Cronjé and Boomker, 2000). For that reason, the use of chronic indwelling catheters in appropriate blood vessels has been successful in obtaining *in vivo* measurements of net flux of metabolites across gastrointestinal tissues (Figure 1.4). Moreover, the combination of arterial–venous (A-V) differences, blood flow and measurements including isotopic extraction, provides invaluable insight into the quantitative metabolism of absorbed nutrients by the splanchnic tissues (Dijkstra et al., 2005). The technique involves the implantation (under general anesthesia) of catheters in an artery and in the mesenteric, portal and hepatic veins. Any artery may be used as the

concentration of metabolites is virtually the same in all arteries (Dijkstra et al., 2005). As the arterial sample is critical, the carotid artery is often elevated to a subcutaneous position to allow insertion of a temporary catheter (Huntington et al., 1989). This methodology allows getting direct, quantitative information on nutrient absorption from the gut, including identifying limitations on rate and extent of digestion and absorption. Indeed, experiments altering diet composition and feeding protocols, obtain information needed to minimize digestive and absorptive losses, thereby improving efficiency of nutrient uptake from the gut (Huntington, 1982).

It has been demonstrated that the plasma flow is affected by feeding time, body weight and physiological state (e.g., pregnancy) (Katz and Bergman, 1969, Huntington, 1982). For instance, portal blood flow in cows ranged from 1.14 to 2.65 liters/h per kg of body weight (Huntington, 1982). Also, fasting significantly decreased the mean rates of blood flow in sheep (Katz and Bergman, 1969). Estimation values for blood flow and metabolite concentration for 24-h is ideal but rarely done. Hourly feeding reduces but does not eliminate variation over 24 h, depending in part on the level of intake, and may well distort 'normal' metabolism (Dijkstra et al., 2005).

The net flux of a nutrient is calculated as follows:

$$\text{Net flux of the compound} = (Pm - Am)*PBF$$

Where

PBF is the portal blood flow (l/h);

Pm is the Portal concentration of the metabolite (μmol/l)

Am is the Arterial concentration of the metabolite (μmol/l)

If Pm > Am, net flux of the metabolite is positive (there is net release or absorption into the venous blood).

If Pm < Am, net flux of the metabolite is negative (there is net uptake or removal from the arterial blood).

Para-aminohippuric acid (PAH) can be used as an indicator of blood flow because it is not metabolized and it is rapidly excreted by the kidney (Dijkstra et al., 2005). Other techniques have been described for measuring blood flow, including electronic methods using a probe around a vessel such as the portal vein or hepatic artery or an electromagnetic or ultrasound/Doppler shift detector. These methods provide instant and time-averaged flows but are not feasible in cattle, because of difficulties in probe placement on the portal vein (Lindsay and Reynolds, 2005). The method of PAH involves infusion into the blood at one point and measurement of concentration downstream (Dijkstra et al., 2005). Blood flow is calculated by determining the difference in indicator dye concentration, PAH, between the target vessel and the peripheral circulation relative to the dye infusion rate (Isserty et al., 1998):

$$Fpv = I / Cpv - Ca$$

Where, Fpv is the rate of blood flow (ml/min) in the portal vein; I is the infusion rate of PAH (mg/min); and Cpv and Ca are the concentrations of PAH (mg/ml) in the portal venous and arterial blood, respectively.

The concentration of PAH in the blood should remain constant after a period of continuous infusion in which the PAH is allowed to equilibrate with the extracellular fluid, because PAH is rapidly excreted by the kidney (Katz and Bergman, 1969). The PAH is infused continuously at least 20 min before the first sampling, then through the experimental session (Huntington et al.,

1989). Simultaneous blood samples are taken from catheters in the portal vein, hepatic vein and artery to form a sample set.

Huntington et al. (1989) chose to insert catheters into two mesenteric veins and to infuse PAH. Other anatomical sites for PAH infusion were tried (e.g., aorta caudal) but the higher blood pressure in the aorta compared with the mesenteric veins, created a strong resistance to infusion and resulted in expansion of the inner volume of the infusion lines (Ortigues et al., 1994). Another alternative is infusing PAH through the mesenteric and ruminal veins, both at the same time. This technique reduces the variability compared to mesenteric-only infusions (Ortigues et al., 1994). However, high incidence of phlebitis was observed in sheep when the ruminal vein was catheterized and infused with PAH (Webster and White, 1973).

The concentration of PAH varies with the size of animals infused. Animals weighing 250 kg or less can be infused with 7 to 10 % PAH (wt/vol) at 1 ml/min, but larger animals require 10 % PAH at 2 ml/min, to provide an adequate blood concentration. The goal is to achieve 20 mg/L in arterial blood, to obtain a reliable concentration difference for calculation of blood flow. Incomplete mixing in blood vessels may result in over- or under-estimation of the A–V difference and error in blood flow estimation (Huntington et al., 1989). Therefore, mesenteric infusion catheters should be established with their tips as far as possible from portal sites, and portal vein sampling catheters should be inserted downstream, where turbulence helps to mix portal blood as it is delivered to the liver. In addition, the use of PAH for measuring blood flow is dependent on an appropriately placed mesenteric vein catheter for PAH infusion (Dijkstra et al., 2005).

The PAH has to be dissolved in sterile saline solution and NaOH, titrated to pH 7.4 with HCL and filtered before infusion. An advantage of using PAH is that is not metabolized in the liver

compared to other markers (e.g., bromophthalein, BSP) (Katz and Bergman, 1969). However, chemical alteration occurred in the liver (acetylation) and Katz and Bergman (1969) suggested that the samples should be deactylated before analysis.

Portal drained viscera applications in choline and vitamin B_{12} studies

Choline and water soluble vitamins (biotin, folic acid, niacin, pantothenic acid, pyridoxine, riboflavin, thiamin, and vitamin B_{12}) requirements are generally considered to be met through synthesis by ruminal microorganisms (NRC, 2001). However, over the last 50 years, milk and milk component yields increased drastically. Therefore, requirements increased accordingly and synthesis by ruminal microflora alone may not be sufficient to meet these increased needs (Girard and Matte, 2005b). True deficiencies of these vitamins are rare in ruminants but their supply might be limiting optimal performance. Therefore, information on bioavailability and ruminal synthesis, for most water-soluble vitamins it is needed to determine requirements (NRC, 2001).

Techniques used to obtain *in vivo* quantitative measurements of nutrients absorbed in lactating dairy cows include gut cannulation, multi-catheterization of the hepatic portal vasculature, and isotope dilution (Reynolds et al., 1994). Placement of cannula in rumen cannulation and duodenum enable measurements of ruminal digestion and dynamics, microbial protein synthesis, and the flow of microbial and dietary constituents to the small intestine. With the addition of multi-catheterization techniques for measurements of nutrient absorption, it is possible to estimate the net movement of metabolites across sections, including the mesenteric-drained viscera or the rumen or the net flux of nutrients across the portal-drained viscera (PDV) tissues. The PDV represents a heterogeneous group of tissues and cell types which metabolize nutrients

to varying degrees or in divergent directions (Reynolds et al., 1994). The interpretation of net PDV flux measurements is further complicated by the fact that nutrients enter and leave the system via the intestinal lumen, blood, and lymph. Nevertheless, net nutrient appearance across PDV represent the amounts of nutrients absorbed into blood available to the rest of the body after PDV metabolism is subtracted (Reynolds et al., 1994).

The net flux of vitamins from the rumen is the difference between the amount absorbed from the lumen and the amount used (or stored) in cells of the rumen wall. In a study on cows equipped with rumen cannula and catheter in the ruminal vein, no detectable net flux of folates and vitamin B_{12} across the rumen wall was detected before the infusion of vitamins. When high doses of folates and vitamin B_{12} were infused, both reached the blood circulation across the rumen wall in low concentrations, especially for vitamin B_{12} (Girard et al., 2001). Indeed, when cows were equipped with rumen and duodenal cannula, only 20% of supplemented cyanocobalamin was recovered at duodenal level (Girard et al., 2009). Therefore, 80% of dietary supplement of CN-CBL was degraded in the rumen. Moreover, low dietary choline efficiency was found when U-14^C labeled grass was introduced into the rumen of cannulated sheep, as the [14^C] PC was rapidly broken down (~75%) (Neill et al., 1979).

The net flux of vitamins and choline at PDV level is the difference between the intake by the intestinal cells and the release to the portal vein. When cows were supplemented with CN-CBL (500 mg), the net flux across PDV was positive after 24hr with a slow release (49.3% was released during 4–10 h post-ingestion and 34.9% 20–24 h post-ingestion) and a biphasic absorption pattern (Girard et al., 2001). The PDV net flux of choline when choline is supplemented has not been studied. The use of animals surgically prepared with gastrointestinal

and hepatic venous catheters, will provide an understanding of vitamin limitations and absorption in the lactating dairy cow.

Summary and objectives

There has been renewed interest in one-carbon metabolism, prompted by recent reports indicating that modest dietary inadequacies of choline and vitamin B_{12} can contribute to important diseases such as fatty liver diseases in the lactating dairy cow. The overall objective of this thesis was to determined choline and vitamin B_{12} absorption and utilization patterns in lactating dairy cows. This thesis comprised three objectives.

1) Evaluate the efficacy of casein and whey protein as delivery methods to improve intestinal absorption of vitamin B_{12} in cows.

2) Establish a methodology to measure choline and its metabolites in blood and milk and determine changes in their relative proportions occurring during early, mid and late lactation.

3) Determine the bioavailability of abomasally infused choline and rumen protected choline and evaluate potential biomarkers for choline absorption.

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Appendix

Table 1.1: Sources of vitamin B_{12} in foods

Food	Vitamin B12 μg/100 g
Beef	1.9-3.6
Beef kidney	38
Beef liver	69-122
Milk	0.4
Yogurt	0.1-0.6
Chicken	0.3
Eggs	1.26
Pork	0.6
Salmon	3.2

[1] Adapted from Combs Jr (2012)

Table 1.2: Choline metabolite concentrations (mg/100 g) in common foods[1]

	Choline	GPC	PCho	PC	SM	Betaine	Total choline[2]
Beef liver	56.67	77.93	11.77	247.75	24.10	6.34	418.22
Chicken liver	47.87	8.80	4.86	213.70	14.8	12.86	290.04
Eggs	0.62	0.60	0.61	238.43	10.74	0.59	251.00
Pork	2.19	1.18	22.51	70.45	6.42	3.54	102.76
Bacon	12.06	14.52	2.68	85.58	10.05	1.57	124.89
Milk 2%	2.82	9.98	1.58	1.15	0.87	0.94	16.40
Soybean	47.27	2.92	1.12	64.56	ND	2.08	115.87

[1] Adapted from Zeisel et al., 2003

Betaine (Bet), free choline (Cho), phosphocholine (PC), glycerophosphocholine (GPC), sphingomyelin (SM), phosphatidylcholine (PtC)

[2] Total choline (TC) is the sum of Cho, LPC, PtC, PC, SM and GPC.

Table 1.3: Summary of studies on effects of vitamin B12 on milk production and milk composition on the lactating dairy cow

Reference	Lactation stage	Dose (mg/d)	Via	Dosage	DMI (kg/d)	Milk yield (kg/d)	Fat yield (kg/d)	Solids yield (kg/d)
Croom et al. (1981)	9 wk 11 wk 13wk 15wk	0 150	i.m.	1d/wk	No differences within treatments			
Elliot et al. (1979)	2 wk before 8wk after calving	0 10	i.m.	2d/wk	NA	26.2 30.0	0.73 0.84	NA
Girard-Matte (2005a)	4wk to 18 wk	0 10	i.m.	1d/wk	20.7 21.7	28.5 31.1	0.87^{y} 1.00^{x}	3.52^{b} 3.90^{a}
Graulet et al. (2007)	3wk before 8 wk after calving	0 500	oral	1/d	21.3 22.0	39.5 36.5	1.42^{y} 1.49^{x}	4.69 4.58
Preynant et al. (2009)	3 wk before 16 wk after calving	0 10	i.m	1d/wk	23.7 23.1	34.1^{b} 39.2^{a}	1.18^{y} 1.34^{x}	3.79^{b} 4.29^{a}
Girard and Desrochers (2010)	17 wk after calving	0 500	oral	1/d	23.1 23.1	30.4^{b} 31.6^{a}	1.30 1.28	4.01 4.09

^{abc} Means within a column within the same study among treatments with different superscripts differ ($P < 0.05$).

^{xy} Means within a column within the same study among treatments with different superscripts differ ($P < 0.1$).

Table 1.4: Summary of studies on effects of rumen-protected choline (RPC) on milk production and milk composition on the lactating dairy cow [1]

Reference	Lactation stage	RPC (g/d)	DMI (kg/d)	Milk yield (kg/d)	Fat yield (kg/d)	Protein yield (kg/d)
Erdman and Sharma (1991)	0 to 147 d	0	21.8	36.7	1354	1211
		16.9	21.7	37.7	1198	1187
		36.1	23.2	38.9	1384	1205
		51.1	21.9	37.4	1387	1208
Erdman and Sharma (1991) 13% CP	121 to 205 d postpartum	0	21.8	31.7[c]	1271	1088
		18.5	22.1	31.7[c]	1243	1079
		37.2	22.2	33.4[b]	1498	1107
		56.9	22.7	34.8[a]	1207	1178
Erdman and Sharma (1991) 16.5% CP	121 to 205 d postpartum	0	23.7	31.6[b]	1209	1147
		19.6	23.4	31.6[b]	1017	1124
		40.4	23.9	33.9[a]	1108	1226
		57.9	23.2	33.8[a]	1160	1205
Erdman (1994)	Early lactation	0	No reported	30.0[a]	1047[a]	954[a]
		33.3		31.1[b]	1128[b]	982[b]
DiConstanzo and Spain (1995)	28 d prepartum to 120 d postpartum	0, 15, 30, or 45g		No differences within treatments		
Deuchler et al (1998)	Mid-lactation	0	22.2	30.6	1232	1091
		50	21.7	30.6	1153	1137
Piepenbrink and Overton (2000)	21 d prepartum to 63 d postpartum	0	12.8-18.4	39.4	1592	1174
		45	12.0-18.9	43.5	1836	1314
		60	12.9-18.3	40.2	1596	1206
		75	12.5-18.7	41.1	1763	1262
Hartwell et al. (2000) Low RUP (4.0%)	28 d prepartum to 120 d postpartum	0	12.9-23.1	38.6	1530	1200
		6	12.3-22.7	38.0	1600	1160
		12	12.6-22.3	39.8	1490	1170
Hartwell et al. (2000) High RUP (6.2%)		0	11.7-20.9	37.2	1430	1070
		6	12.4-21.4	38.7	1500	1120
		12	12.6-20.1	34.4	1320	1020
Piepenbrink and Overton (2003)	21 d prepartum to 63 postpartum	0	12.6-17.8	12.6-17.8	1580	1170
		45	11.9-18.7	11.9-18.7	1780	1280
		60	12.8-18.3	12.8-18.3	1560	1180
		75	12.5-18.8	12.5-18.8	1710	1240
Pinotti et al. (2003)	14 d prepartum to 30 d postpartum	0	11.3-19.4	28.5[b]	880[b]	868
		20	11.4-19.9	31.4[a]	1056[a]	966

Continued Table 1.4

Reference	Lactation stage	RPC (g/d)	DMI (kg/d)	Milk yield (kg/d)	Fat yield (kg/d)	Protein yield (kg/d)
Guretzky *et*	21 d prepartum to	0	12.0-14.8	29.6[b]	1380[b]	1050
al. (2006)	21 d postpartum	15	12.1-15.7	31.6[a]	1460[a]	1090
Davidson et al.	21 to 91 d	0	27.9	27.9	840	730
(2008)	postpartum	40	20.2	27.5	790	740
Primiparous						
Davidson et al.	21 to 91 d	0	21.9[b]	37.7[b]	1030[b]	920[b]
(2008)	postpartum	40	24.3[a]	44.1[a]	1116[a]	1100[a]
Multiparous						
Chung et al	41 d postpartum	0	26.0[b]	44.5[b]	No reported	
(2009)		25	28.2[a]	48.9[a]		
		50	28.5[a]	47.8[a]		
Ardalan et al.	4 wk before to 20	0	No reported	30.71[b]	1071	1009
(2009)	wk postpartum	60		34.23[a]	958	902
Zom et al.	3 wk before to	0	13.1-20.5	No differences within		1075[b]
(2011)	6wk postpartum	60	13.1-21.3	treatments		1235[a]

[1] Adapted from Benoit S. 2009.

[abc] Means within a column within the same study among treatments with different superscripts differ ($P < 0.05$).

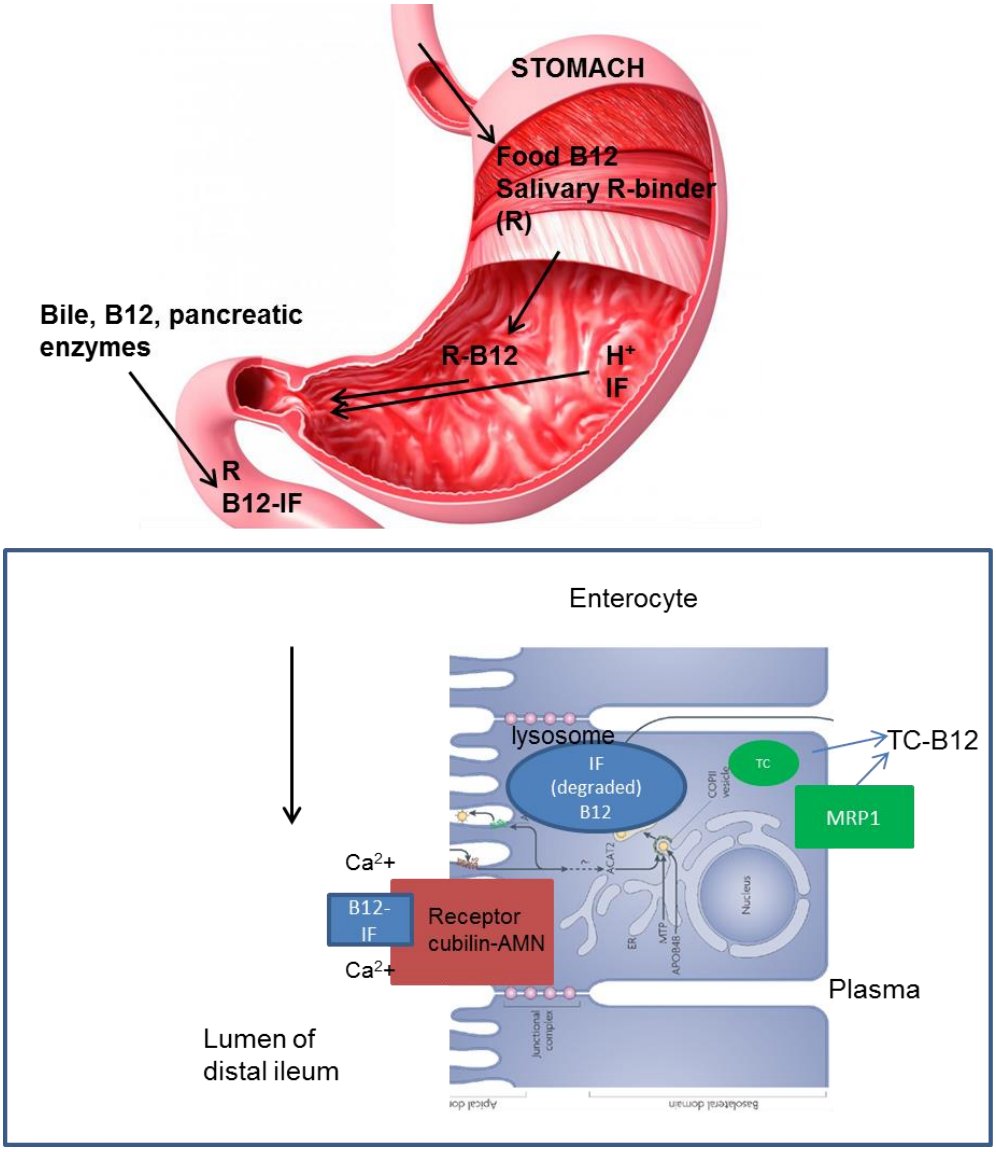

Figure 1:1: Absorption and processing of dietary vitamin B12. IF, intrinsic factor; MRP1, multi drug resistant protein 1; TC, transcobalamin. Adapted from Stipanuk and Caudill, 2012.

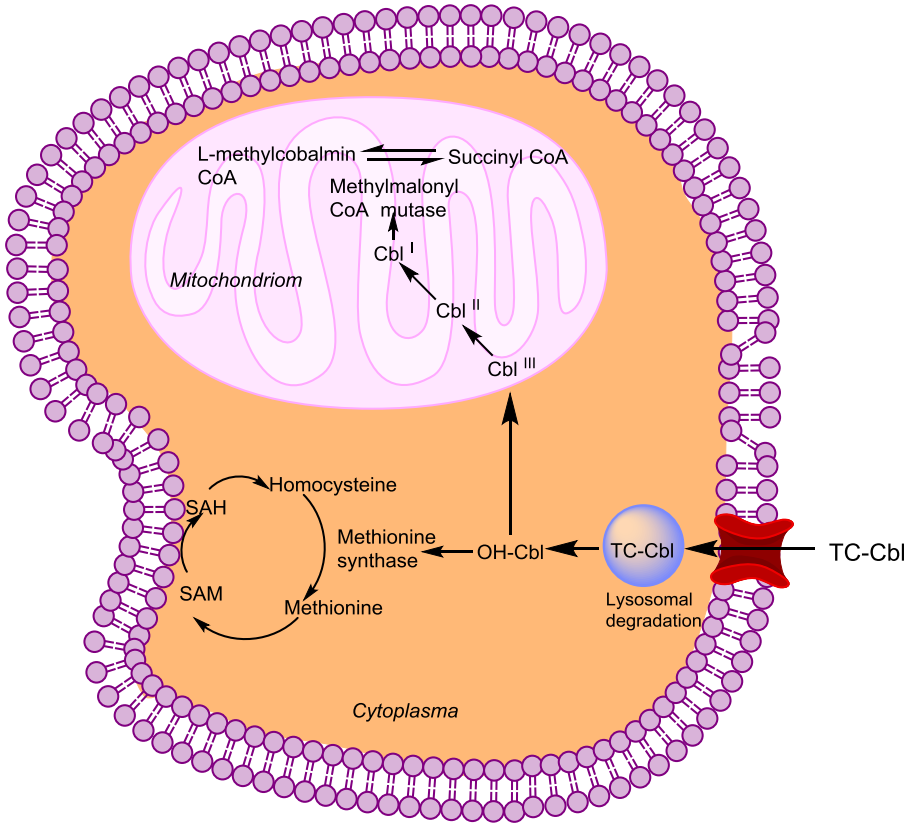

Figure 1.2: Cellular uptake and metabolism of cobalamin (Cbl). OH-Cbl, hydroxycobalamin; SAH; S-adenosylhomocysteine, SAM; S-adenosylmethionine. TC; transcobalamin.

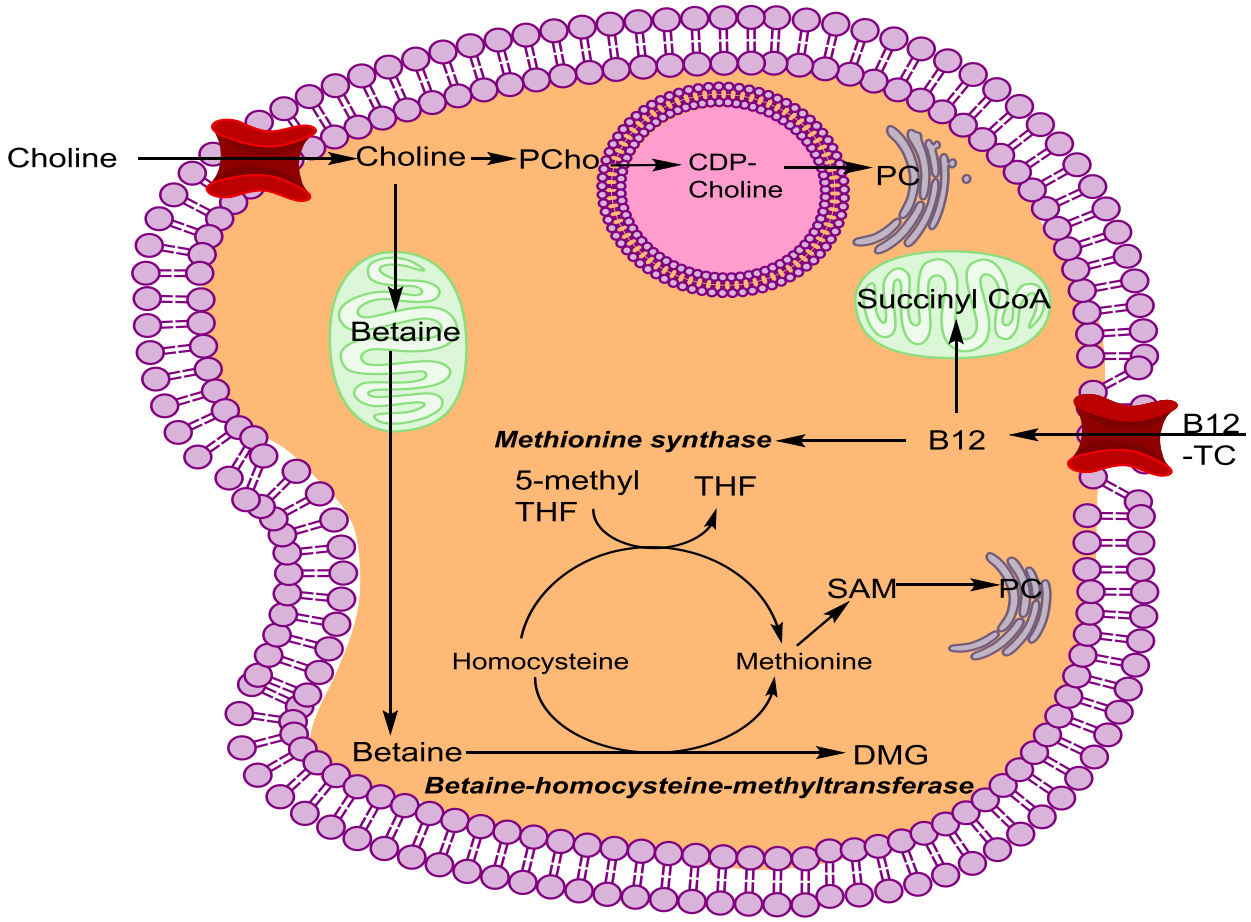

Figure 1.3: Choline and vitamin B12 on one carbon metabolism pathways. CDP-Choline, cytidine diphosphate-Choline; DMG, dimethylglycine; PC, Phosphatidylcholine; PCho, Phosphocholine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.; TC, transcobalamin; 5-Methyl-THF, 5-Methyl-tetrahydrofolate; THF, Tetrahydrofolate system.

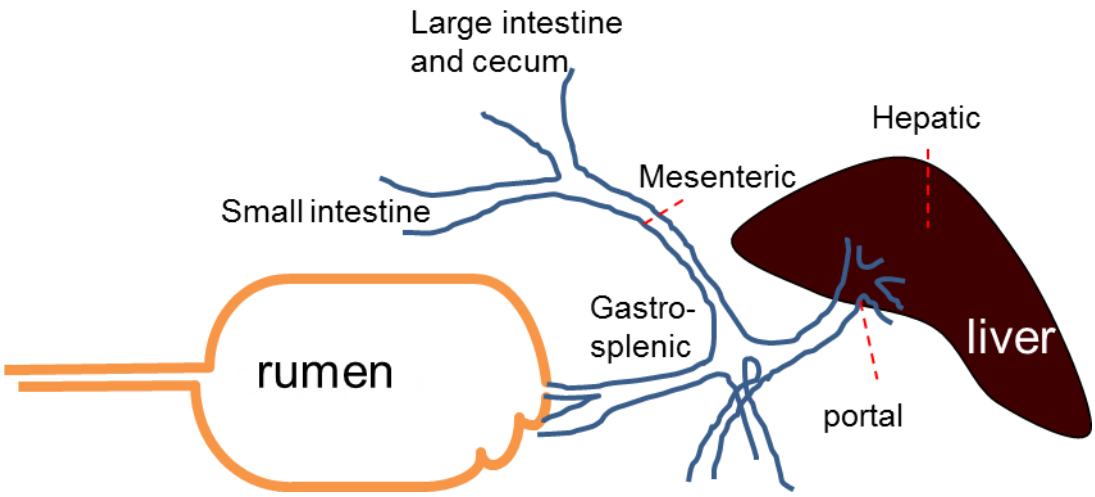

Figure 1.4: Scheme of the siting of catheters (dash red lines) in splanchnic studies in dairy cows. Adapted from (Dijkstra et al., 2005). The catheter at portal vein is located 3 to 7 cm craniad to the connection of the liver. The catheter at hepatic vein was located 2 to 6 cm from the lateral edge of the liver. The mesenteric artery is selected in the area of the proximal duodenum.

CHAPTER 2: CASEIN AND WHEY PROTEINS AS DELIVERY METHODS FOR CYANOCOBALAMIN TO INCREASE INTESTINAL ABSORPTION IN LACTATING DAIRY COWS

This chapter is a lightly revised version of a manuscript by V. M. Artegoitia, M. J. de Veth, F. Harte, D. R. Ouellet, C. L. Girard, The use of "our" in this chapter refers to my co-authors and I. My primary contributions to this paper include (1) the sample preparation, (2) the collection and analysis of data, (3) the gathering and interpretation of literature, and (4) the manuscript writing.

Abstract

Improving vitamin B_{12} absorption is important for optimal performance in dairy cows and for increasing vitamin B_{12} concentrations in milk for human consumption. However, 80% of a supplement of synthetic vitamin B_{12}, cyanocobalamin (CN-CBL), is degraded in the rumen of dairy cows and only 25% of the amount escaping destruction in the rumen disappears in the small intestine. The objective of this study was to evaluate the efficacy of casein and whey proteins as delivery methods for CN-CBL in solution to increase intestinal absorption of vitamin B_{12} in cows measuring portal drained viscera (PDV) flux. Four multiparous lactating Holstein cows (236 ± 17 DIM) equipped with a rumen cannula and catheters in the portal vein and a mesenteric artery were used in a randomized cross-over design. They were fed 12 equal meals per day to maintain steady-state. On experimental days, they received a post-ruminal bolus of: 1) CN-CBL alone (0.1g; CA), 2) CN-CBL (0.1g) + casein (10 g; CC) or 3) CN-CBL (0.1g) + whey proteins (10 g; CW). After the bolus, blood samples were taken simultaneously from the 2 catheters every 15 min during the first 2 h and then every 2 h until 24 h post-bolus. Milk yield, DMI, arterial and PDV flux of vitamin B_{12} were analyzed by the MIXED procedure. Milk yield and DMI were not affected by treatments. Arterial plasma concentrations of vitamin B_{12} increased when CN-CBL was infused with dairy proteins (average 408 pg/ml, CC and CW) compared with CA (323 pg/ml). On average the PDV net flux of vitamin B_{12} was positive during the first 45 min after the post-ruminal bolus; the maximal value was already reached 30

min after the bolus and decreased rapidly thereafter. The PDV net flux of vitamin B_{12} during the 24 h period following the bolus of vitamin was greater when the vitamin was given in solution with casein (CC, 4 μg/h) whereas the PDV net flux of vitamin B_{12} was negative for CA and CW (-25 μg/h and -19 μg/h, respectively). Only 0.74% of the total amount of vitamin B_{12} infused post-ruminally reached the portal blood within the first 45 min and then there was no further release until 24 h after the bolus. The present results suggest that CN-CBL given with casein increased vitamin B_{12} absorption as compared to CN-CBL given alone. For practical applications of our findings, development of a casein-based formulation may improve CN-CBL absorption in dairy cows.

Key words: Cyanocobalamin, bioavailability, vitamin B_{12}, dairy cow

Introduction

Bacteria present in the rumen synthesize vitamin B_{12} and unless dietary Co supply is inadequate, the amount of vitamin B_{12} produced in rumen is sufficient to prevent apparition of deficiency symptoms (NRC, 2001). However, supplements of vitamin B_{12}, intramuscular injection (10 mg/d) or oral dose (500 mg/d), given to dairy cows in early lactation combined with folic acid supplements, improve lactational performance and metabolic efficiency, suggesting that rumen microbial synthesis might not be always sufficient to optimize animal performance (Girard and Matte, 2005, Preynat et al., 2009). However, under commercial conditions, systematic use of intramuscular injections of the vitamin is not practical whereas 80% of a dietary supplement of the synthetic form of vitamin B_{12}, cyanocobalamin (CN-CBL) is degraded in the rumen and less than 25% of the amount of vitamin B_{12} bypassing the rumen is absorbed in the small intestine of dairy cows (Girard et al., 2009).

Previous studies in pigs estimated the bioavailability of dietary vitamin B_{12} using portal drained viscera (PDV) flux (Matte et al., 2010, Matte et al., 2012).The cumulative net PDV fluxes of vitamin B_{12} after ingestion of meals supplemented with 44 and 71 μg of CN-CBL without dairy protein in the diet were not different from 0 (Matte et al. 2012) However, when vitamin-free casein was present in the diet the mean net PDV fluxes of vitamin B_{12} after boluses of 25 and 250 μg of dietary CN-CBL were positive at 2.4 and 5.1 μg, respectively, and differed between doses (Matte et al., 2010) Therefore, milk proteins might be an approach to increase the level of absorption of vitamin B_{12}.

The objective of this study was to determine the efficacy of casein and whey proteins as delivery methods for CN-CBL in solution to increase intestinal absorption in dairy cows estimate by measuring porto-arterial difference and calculating PDV flux of the vitamin.

Materials and Methods

Animals were cared for according to the recommended Code of Practice for the Care and Handling of Dairy Cattle (2009) and the guidelines of the Canadian Council on Animal Care (CCAC, 2009). Four multiparous lactating Holstein cows (236±17 DIM and averaging 708 kg ± 89 kg of BW), equipped with a rumen cannula and chronic indwelling catheters in the portal and 2 mesenteric veins as well as in a mesenteric artery (Huntington et al., 1989) at least five months before the start of the experiment, were used. The catheters were exteriorized individually over the paralumbar shelf and spine. The right carotid artery was surgically raised to a subcutaneous position to allow access to arterial blood if the catheter placed in a mesenteric artery failed. In cases both arterial catheters failed, an auricular artery was used. Cows were housed in a tie-stall barn with free access to water under 16 h of light per day (0500 to 2200 h) and were fed in the morning 1 kg of long hay per day and a TMR (Table 2.1) served in 12 equal meals per day (2-h

intervals) by automatic feeders (Ankom, Fairport, NY) at 95% of their voluntary feed intake measured the week before the start of the study. Feed intake and refusals, when present, were weighed and recorded daily. Cows were milked twice a day (0700 and 1900 h).

The cows were randomly assigned to 1 of 3 treatments in a cross-over design with 1 d and 3 d of wash-out between periods 1 and 2 and 2 and 3, respectively. The treatments were a post-ruminal bolus infusion (Vicini et al., 1988) of 60 mL of the following solutions: 1) CN-CBL alone (0.1g; CA), 2) CN-CBL (0.1g) + casein (Casein acid hydrolysate vitamin free, Sigma, St-Louis, MO); 10 g; CC) and 3) CN-CBL (0.1g) + milk whey protein isolate (Hillmar^{TM} 9400 whey protein isolate, Canada Colors and Chemicals Ltd, St-Laurent, QC, Canada); 10 g; CW). Assuming that 1 μmole of CN-CBL links to 1 μmole of casein (molecular weight between 19 and 23 mg per μmole), linking 73.8 μmoles of cyanocobalamin should require 73.8 μmoles of casein (approximately 1.4-1.7 g caseins). Using 10 g of casein should assure that milk protein is well in excess of cyanocobalamin. The same weight of whey proteins was used. The post-ruminal bolus infusion of 60 mL was given in less than 60 s and was followed by a similar bolus of water.

All feed ingredients were sampled at the beginning of the study and frozen at -20°C until analysis. Samples were dried, ground and analyzed for DM and CP (AOAC, 2000), ADF and NDF (Ankom200 fiber analyzer, Ankom Technology Corp., Fairport, NY) and cobalt (inductively coupled plasma emission spectrometry) (Agri-Food Laboratories, Guelph, ON, Canada).

Starting 30 min after the post-ruminal infusion, blood samples (6 mL) were simultaneously taken from the portal vein and an artery every 15 min for the first 2 h and then, every 2 h until 24 h after the post-ruminal bolus. Immediately after collection, blood samples

were put in Vacutainer tubes with EDTA (Becton Dickinson Inc, Franklin Lakes, NJ), the tubes were then placed on ice until centrifuged (2000 × *g* for 10 min at -4°C) and plasma was stored at –20 °C. Vitamin B_{12} in plasma was analyzed in duplicate by radioassay with a commercial kit designed for human plasma (SimulTRAC-S Radioasssay kit, Vitamin B12 (^{57}Co)/Folate (^{125}I), MP Biomedicals, Diagnostics Division, Orangeburg, NY).

Porto-arterial difference of CN-CBL across portal drained viscera was calculated as described by Girard et al. (2001) for each sampling time. Blood flow was estimated based on values obtained from the same cows fed the same diet in a previous study (Maxin, personal communication) according to the following equation,:

$$\gamma = 400.38 + 47.351 \times DMI$$

Where γ is the average blood flow (l/h) and DMI is dry matter intake (kg/d).

A positive net flux indicates a release by the tissues into the portal vein whereas a negative flux indicates an uptake by the tissues.

All variables were analyzed as a cross-over design using the MIXED procedure (SAS version 9.3 Institute Inc., Cary, NC, USA) according to the following model:

$$Y_{ijkl} = \mu + P_i + B_j + P_i(cow_k) + T_l + BT_{jl} + \varepsilon_{ijkl}$$

where Y_{ijkl} indicated the dependent variable; μ was the overall mean; P_i, the effect of period; B_j, the effect of treatment, and T_l, the effect of time. The errors terms were $P_i(cow_k)$ used to test the main effects (error a) and ε_{ijkl}, the residual error. As the time intervals were different, the following covariance structures were compared: SP(POW), SP(GAU), SP(EXP), SP(LIN), SP(LINL), SP(SPH), ANTE(1) and UN. For each variable, the statistical analysis retained was the one with the smallest fit statistic values. Results are reported as least squares means and standard errors of the means. Means were assumed to be different at $P \leq 0.05$ and tended to

differ at $0.05 < P \leq 0.10$, then the 2 following *a priori* comparisons were used: CC vs. CA + CW and CA vs. CW.

Results and Discussion

There was no effect of treatments ($P \geq 0.6$) on milk production and DMI, averaging 33 ± 2.5 kg/d and 19.6± 1.0kg/d, respectively. Arterial plasma concentrations of vitamin B_{12} increased when CN-CBL was infused with dairy proteins, from 323 pg/ml with CA to 408 pg/ml on average CC and CW (treatment effect, P=0.02; Table 2.2). Although portal plasma concentration of vitamin B_{12} was not changed by treatments (P=0.42, Table 2.2), concentration of vitamin B_{12} varied over the time within treatments (interaction time × treatment, P<0.01, Table 2.1, in where CC and CW presented higher concentrations during the first hour after post-ruminal infusion compared with CA, and CC was higher than CA and CW 2 h after the post-ruminal infusion. On average porto-arterial differences of vitamin B_{12} were affected by the time after the post-ruminal infusion (time effect, $P < 0.01$; Table 2.2), being positive during the first 90 min after the abomasal bolus. The difference was maximal 30 min post-bolus and rapidly decreased, then from 120 min after the abomasal bolus until the end of the sampling period, porto-arterial differences of vitamin B_{12} were negative or not different from 0 (data not shown). On average for the 24 h period following the post-ruminal bolus, there was a treatment effect ($P < 0.05$) for porto-arterial difference and net PDV flux of vitamin B_{12}; CA and CW were negative whereas they were positive for CC (Table 2.2). On average the PDV net flux of vitamin B_{12} was positive during the first 45 min after the post-ruminal bolus, the maximal value was already reached 30 min after the bolus and decreased rapidly thereafter; from 1 h until 24 h after the post-ruminal bolus, PDV net flux of vitamin B_{12} was negative (time effect, $P < 0.01$; Figure 2.2). Only 0.64% of the total amount of vitamin B_{12} given post-ruminally reached the portal blood within the first 30 min and

0.74% at 45 min and then, there was no release until the end of the sampling period. In contrast with our findings the net flux of this vitamin across PDV in mid-lactation dairy cows was still positive 24 h after ingestion of a supplement of 500 mg CN-CBL (Girard et al., 2001). The post-ruminal infusion dose in the present study was 5-fold less than the amount of CN-CBL used in the previous study (Girard et al., 2001) because Girard et al. (2009) observed that, in dairy cows, only 20 % of a dietary supplement of CN-CBL was recovered at the duodenal level. However, the results in the present study suggest that the dose infused post-ruminally might not be sufficient to reach similar net release of vitamin B_{12} across PDV-flux.

In pigs, PDV flux of vitamin B_{12} was positive when dietary CN-CBL was given with a semi-purified diet containing vitamin-free casein (Matte et al., 2010). However, the PDV flux of vitamin B_{12} was negative or not different from 0 when CN-CBL was given in a meal based on corn, wheat and soybean meal (Matte et al., 2012). Indeed, in pigs intestinal absorption of vitamin B_{12} during the 24 h period after ingestion of milk was greater than after ingestion of the synthetic form of the vitamin (Matte et al., 2012). Little is known about the efficacy of milk protein as well as differences on the effect of casein vs. whey proteins on the intestinal absorption of CN-CBL. Milk proteins are natural vehicles of bioactives compounds (e.g. calcium, amino acids and inmunoglobulins) to the neonate (Livney, 2010). During digestion, milk proteins offer a variety of possibilities for reversible binding of active molecules and for protecting them until their release at the desired site within the body (Chen et al., 2006, Elzoghby et al., 2011). For instance, in non-ruminant animals, the rates at which peptides and AA are released during digestion differ among milk proteins (Boirie et al., 1997). Caseins form clots that slowly empty from the stomach, whereas whey proteins are rapidly digested resulting in high concentrations of AAs in the bloodstream (Lacroix et al., 2006). A patented oral insulin

formulation for humans, called Cap-PEG-Insulin-Casein (CAPIC, BioSante Pharmaceuticals Inc., Baudette, MN), aggregates caseins around a proprietary formulation of insulin (Morçöl and Bell, 2001, Morçöl et al., 2004). Due to its mucoadhesive properties and its capacity to resist degradation under acidic conditions, casein protects the target drug as it passes through the stomach, allowing the drug to be released at the site of absorption (Park et al., 2011). Indeed, vitamin B_{12} has been used as a model drug incorporated into a casein hydrogel matrix which has an *in vitro* prolonged release behavior (Song et al. 2010). Thus, milk casein seems suitable as vehicle or as component for the construction of vehicles for delivering various bioactive molecules, especially those that require slow intestinal delivery, such as vitamin B_{12}.

In summary, our data show that post-ruminal infusion of CN-CBL given with casein increased vitamin B_{12} intestinal absorption as compared to CN-CBL given alone. For practical applications of our findings, development a rumen protected vitamin B_{12} with casein may improve CN-CBL absorption in dairy cows.

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Appendix

Table 2.1 Ingredient and chemical composition of the total mixed ration[1]

Ingredient, % DM basis	Value
Soybean hulls	5.8
Corn silage[2]	29.9
Grass silage[3]	18.2
Corn	18.1
Canola meal	21.9
Beet pulp	2.0
Mineral and vitamin supplement[4]	1.8
Calcium Carbonate	0.3
Urea	0.2
Megalac®	1.7
Nutrient composition	
MS, %	50.0
CP, % of DM	15.1
RDP, % of CP	49.4
Soluble protein, % of CP	31.1
ADF, % of DM	18.9
NDF, % of DM	32.6
NE_L, Mcal/d	1.59
Co, mg/kg of DM	0.40

[1]All cows were served daily 1 kg of long hay (85% DM) on a DM basis, 10.5% CP; 2.5% soluble protein; 30.6% ADF; 54.4% NDF
[2]8.6% CP; 3.4% soluble protein; 21.7% ADF; 40.0% NDF
[3]16.7% CP; 7.7% soluble protein; 31.2% ADF; 47.6% NDF
[4]Contained per kilogram: 91.8 g of Ca; 47.9 g of P; 47.9 g of Mg; 11.9 g of K; 80.8 g of Cl; 136.8 g of Na; 15 g of S; 1,946 mg of Fe; 2,656 mg of Zn; 440 mg of Cu; 1,798 mg of Mn; 23 mg of I; 19.50 mg of Se; 441.6 kUI of vitamin A; 56.6 kUI of vitamin D and 2,630 UI of vitamin E.

Table 2.2 Arterial and portal plasma concentrations, porto- arterial difference and net flux across portal-drained viscera (PDV) of vitamin B_{12} during the 24 h following a post-ruminal bolus of cyanocobalamin alone (CA) or in solution with casein (CC) or whey protein isolate (CW)

	Treatments			*P* values			*P*-value contrast	
	CA	CC	CW	Treat[1]	Time	Time x treat[1]	CA vs. CC + CW	CC vs. CW
Artery, pg/mL	322.9 ± 22.7	403.2 ± 19.2	412.9 ± 18.6	0.05	0.21	0.41	0.02	0.73
Portal, pg/mL	332.5 ± 39.8	405.6 ± 34.5	388.7 ± 33.9	0.42	<0.01	<0.01	0.22	0.74
Portal-Arterial , pg/mL[2]	-17.5 ± 5.2	2.9 ± 4.6	-13.4 ± 4.2	0.05	<0.01	0.34	0.04	0.05
PDV-net flux, µg/h	-25.4 ± 7.4	3.93 ± 6.6	-18.94 ± 6.0	0.05	<0.01	0.39	0.09	0.04

[1] Treat= treatment

[2] Differences between portal and arterial concentrations of vitamin B_{12} (pg/ml)

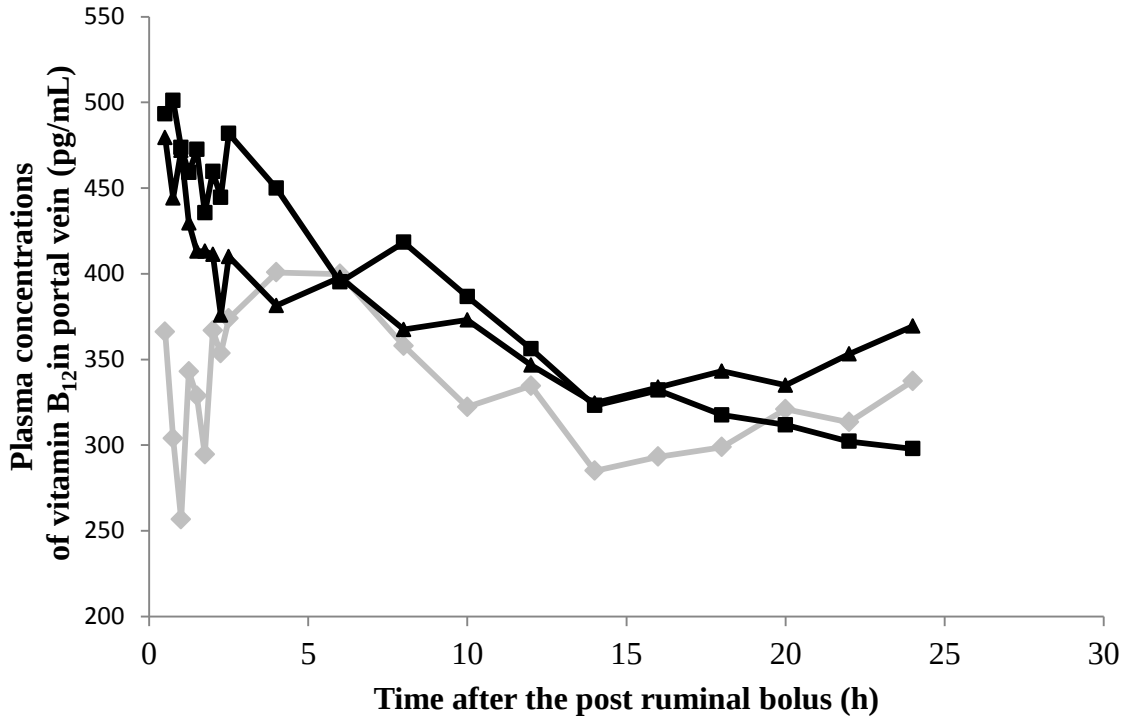

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Figure 2.1 Plasma concentrations of vitamin B12 in the portal vein of lactating dairy cows during the 24 h following a post-ruminal bolus of 100 mg of cyanocobalamin alone (CA) or in solution with casein (CC) or whey protein isolate (CW). Each symbol represents either CA (♦), CC (■) or CW (▲).Values are means from 4 cows per treatment, standard errors of the mean = 54.33 (pg/ml) (time × treatment effect, P > 0.01).

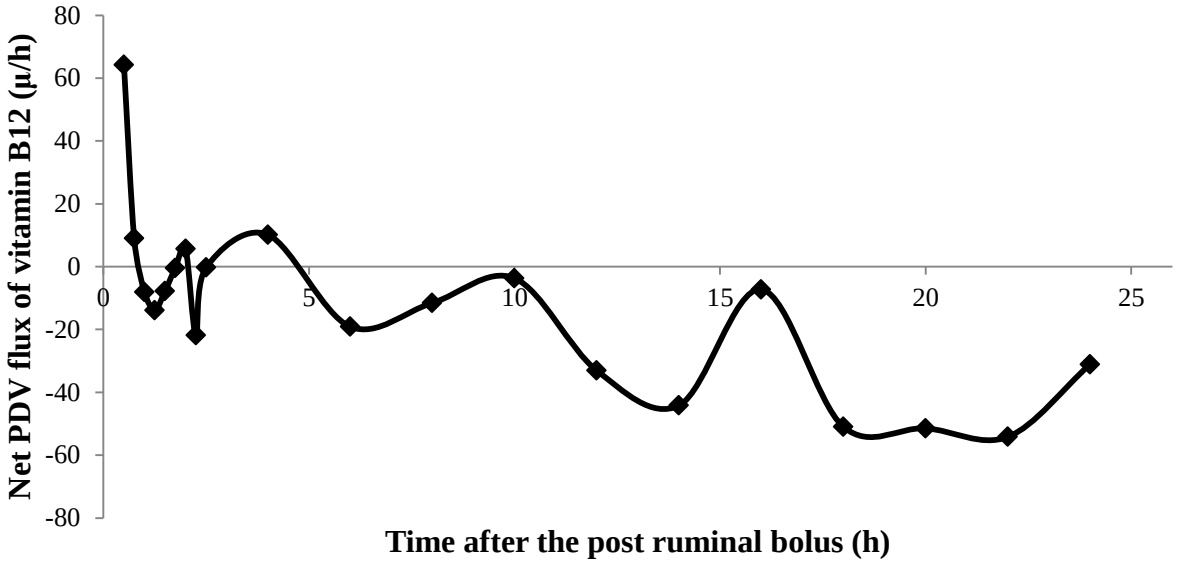

Figure 2.2 Net flux of vitamin B12 from the portal-drained viscera (PDV) of lactating dairy cows during the 24 h following a post-ruminal bolus of 100 mg of CN-CBL. Values are means from 4 cows and 3 treatments, with standard errors of the mean = 16.27 (µ/h) (time effect, P = 0.05).

CHAPTER 3: CHOLINE AND CHOLINE METABOLITE PATTERNS AND ASSOCIATIONS IN BLOOD AND MILK DURING LACTATION IN DAIRY COWS

This chapter is a revised version of a paper by the same title in press on PlosOne by Virginia M. Artegoitia, Jesse L. Middleton, Federico M. Harte, Shawn R. Campagna, Michael J. de Veth. My primary contributions to this paper include (1) taking samples and preparation, (2) the collection and analysis of data, (3) the gathering and interpretation of literature, and (4) the manuscript writing

Abstract

Milk and dairy products are an important source of choline, a nutrient essential for human health. Infant formula derived from bovine milk contains a number of metabolic forms of choline, all contribute to the growth and development of the newborn. At present, little is known about the factors that influence the concentrations of choline metabolites in milk. The objectives of this study were to characterize and then evaluate associations for choline and its metabolites in blood and milk through the first 37 weeks of lactation in the dairy cow. Milk and blood samples from twelve Holstein cows were collected in early, mid and late lactation and analyzed for acetylcholine, free choline, betaine, glycerophosphocholine, lysophosphatidylcholine, phosphatidylcholine, phosphocholine and sphingomyelin using hydrophilic interaction liquid chromatography-tandem mass spectrometry, and quantified using stable isotope-labeled internal standards. Total choline concentration in plasma, which was almost entirely phosphatidylcholine, increased 10-times from early to late lactation (1305 to 13,535 μmol/L). In milk, phosphocholine was the main metabolite in early lactation (492 μmol/L), which is a similar concentration to that found in human milk, however, phosphocholine concentration decreased exponentially through lactation to 43 μmol/L in late lactation. In contrast, phosphatidylcholine was the main metabolite in mid and late lactation (188 μmol/L and 659 μmol/L, respectively), with the increase through lactation positively correlated with phosphatidylcholine in plasma ($R^2 = 0.78$). Unlike previously reported with human milk we found no correlation between plasma free choline concentration and milk choline metabolites. The changes in pattern of phosphocholine and phosphatidylcholine

in milk through lactation observed in the bovine suggests that it is possible to manufacture infant formula that more closely matches these metabolites profile in human milk.

Introduction

Choline is found in various forms in humans and animals (Zeisel et al., 1991; Pinotti et al., 2002) (Figure 3.1). The water-soluble choline metabolites include acetylcholine (ACho), an important neurotransmitter for brain and neuromuscular function (Zeisel et al., 1991), betaine (Bet), an oxidative intermediate of choline which supplies a methyl group for the conversion of homocysteine to methionine (Zeisel and Blusztajn, 1994), and glycerophosphocholine (GPCho), which like Bet acts as an organic osmolyte in cells (Gallazini and Burg, 2009). The lipid-soluble choline-containing metabolites phosphatidylcholine (PC), sphingomyelin (SM), and lysophosphatidylcholine (LPC) are all structural components of mammalian membranes (Zeisel 1990). PC is essential for synthesis of VLDL, a key mechanism to export triacylglycerol from the liver (Yao and Vance, 1988).

Choline can be obtained from the diet (Zeisel et al., 2003) and from endogenous biosynthesis that predominantly occurs in the liver via the phosphatidylethanolamine N-methyltransferase (PEMT) pathway (Reo et al., 2002). Although the PEMT pathway represents an important source of choline (Zeisel and Blusztajn, 1994), dietary intake of choline is necessary because the pathway supplies insufficient choline moiety to maintain the normal function of cells, tissues and organs (Zeisel et al., 2003; Reo et al., 2002). In the case of the ruminant, however, dietary choline is extensively degraded (>80%) by rumen bacteria (Neill et al., 1979; Sharma and Erdman 1989) and therefore endogenous synthesis via the PEMT pathway represents a critical source of choline (Baldi and Pinotti, 2006). For the lactating dairy cow, the periparturient period is characterized by a high incidence of fatty liver disease and supplementation of rumen-

protected choline has been found to reduce the extent of hepatic fatty infiltration and increase expression of genes involved in VLDL transport (Zom et al., 2011; Goselink et al., 2012.

Despite the lack of dietary-derived choline in the ruminant, milk and dairy products are an important source of choline in the human diet. One cup of retail milk (2% fat) is expected to provide approximately 40 mg choline (Howe et al., 2004), which represents nearly 10% of an adult woman's daily choline requirement (Zeisel and da Costa, 2009). In addition, bovine milk is the main ingredient used in the manufacture of infant formula (IOM, 2004), which is used during a period of human development where choline requirements are high for organ growth and membrane biosynthesis (Caudill 2010). Studies that have compared the choline content of breast milk and infant formula have found that whilst the total choline in breast milk was relatively constant [1250-1481 µmol/L), choline in bovine-derived infant formula varied by over 7-fold (311-2270 µmol/L) (Ilcol et al., 2005 Holmes-McNary 1996). Further, these studies have found the proportion of the various choline metabolites in milk differs for breast milk and infant formula, which may be important for the neonate as the bioavailability of the choline metabolites can vary considerably (Cheng et al., 1996). Despite the variation in choline content and choline metabolite proportions of bovine-derived infant formula, it is not known to what extent this variation may be attributed to the milk initially harvested from cows or due to post-harvest processing.

Lactation is characterized by extensive physiological adaptations that are coordinated to provide the appropriate quantity and pattern of nutrients for milk synthesis (Bauman and Currie, 1980). These adaptations have been associated with changes in choline metabolism, with differences in choline metabolite concentrations during lactation reported in breast milk and porcine milk (Ilcol et al., 2005, Donovan et al., 1997). However, there has not been a systematic evaluation of

changes in choline metabolites in plasma and milk during lactation in dairy cows. Recently, a method based on hydrophilic interaction liquid chromatography-tandem MS (HILIC LC-MS/MS) was developed and validated to identify and quantify all of the major choline-containing compounds as well as their fatty acid composition within a phospholipid class (Zhao et al., 2011, Xiong et al., 2012).

The objectives of the current study were threefold. Firstly, we sought to characterize the changes in choline and choline metabolites in blood and milk over the first 37 weeks of lactation (WOL) by sampling during early, mid and late lactation, which is indicative of periods in lactation where the physiological state differs greatly in the dairy cow. The quantification of choline output in milk will contribute to an understanding of the mammary glands' requirement for choline during lactation. In addition, changes in the different PC species were of interest as recent research has shown that PC species with long chain polyunsaturated fatty acids, primarily docosahexaenoic acid (DHA), originated from PEMT activity in the liver of rodents and humans (da Costa et al., 2011, Pynn et al., 2011). Secondly, we sought to determine whether the yield of choline and choline metabolites in milk were associated with changes in blood choline metabolite concentrations. Previously, weak correlations between blood and milk choline concentrations were found in nursing women (Ilcol et al., 2005). In the present study, we had the advantage of monitoring the same animals throughout lactation, which was expected to reduce the variation and improve the likelihood of observing interrelationships. Thirdly, we sought to determine the relationship between milk choline yield and common animal performance variables (i.e. milk yield, fat and protein content, and WOL) to evaluate whether easily measurable end points could be used to predict total milk choline output.

Materials and Methods

Animal experimentation was approved by The University of Tennessee Institutional Animal Care and Use Committee.

Twelve multiparous Holstein cows averaging 653 ± 55 kg (mean ± SEM) body weight were recruited at calving and managed under the same diet, without choline supplementation, throughout the study.

The diet was a total mixed ration (TMR) formulated using the National Research Council requirements (NRC, 2001) and were fed ad libitum at 0700 and 1900 daily (Table 3.1). Water was available at all times. TMR samples were collected once each month during the experiment and stored at −40°C before being analyzed for chemical composition by wet chemistry (Dairy One Cooperative, Inc., Ithaca, NY). The individual ingredients that comprised the TMR diet were analyzed for choline metabolites (Table 3.2).

Milk and blood samples were collected on one day each week during three periods of the lactation, early lactation (weeks 1, 2 and 3; n=12), mid lactation (weeks 7, 10 and 13; n=12) and late lactation (weeks 31, 34 and 37; n=10). Cows were milked at 0600 and 1800 h daily. Milk was sampled and yield determined at both daily milkings (pm and am) of each week. One aliquot was stored at 4°C with a preservative (bronopol tablet; D&F Control System, San Ramon, CA) until analyzed for fat and true protein content (Tennessee DHIA, Inc., TN) by mid-infrared spectrophotometry (Milko-Scan, Foss Electric, Hillerød, Denmark). A second aliquot of milk was stored at −40°C for choline metabolite analysis. Blood samples were taken after the am milking from the coccygeal artery/vein with a Vacutainer-EDTA (Becton Dickinson Inc, Franklin Lakes, NJ), immediately placed on ice, centrifuged (2000×g at 4 C° for 10 min) and plasma samples were stored at -40 °C.

The absolute quantification of choline and choline metabolites at each WOL was determined using isotope dilution MS with the exception of GPCho where an external calibration curve was used because no commercial internal standard (IS) was available. Stock solutions of the IS in methanol were prepared so that the final concentration in the autosampler vial was ca. 5 times greater than the lower limit of linearity of the target compound to ensure an analyte to IS signal ratio greater than 10:1 to avoid suppressing the target compound signal (Zhao et al., 2011). The following standards were used: Cho chloride-trimethyl-d9 (Cho-d9, Cambridge Isotope Laboratories DLM 549-1, Tewksbury, MA), Bet (Bet-d11 Cambridge Isotope Laboratories DLM 407, Tewksbury, MA), PCho chloride (PCho-d9, Cambridge Isotope Laboratories, DLM-298, Tewksbury, MA) PC-d9 (Avanti polar lipids, 860362, Alabaster, AL), SM-d13-c13 (Ricerca-custom made, Painesville, OH) ACho-d13(C/D/N Isotopes ICN D-1780, Quebec, Ca), LPC-D3 (Larodan 71-2826, Malmo, Sweden). The ratio between the area of the target compound and area of the IS was multiplied by the known IS concentration to quantify the various phospholipids. A calibration curve was used to calculate the absolute quantitation of GPCho concentration (1-O-Octadecyl-2-O-methyl-sn-glycero-3-phosphorylcholine, Sigma, 09262). Serial dilutions were made from 0.0012 µl to 11857.55 µl concentration of GPCho in 10 mL of methanol resulting in a linear equation using for quantification.

Milk samples from consecutive milkings (pm, am) on each sampling day were composited proportional to milk yield at each milking prior to extraction for choline analysis. Plasma, milk and feed samples were extracted based on the Bligh and Dyer (1959) method with modifications described by Zhao et al. (2011). The samples were kept on ice throughout the extraction process. Briefly, 1 ml of extraction solvent (chloroform, methanol, water, 1:2:0.8) was added to 200 µL of plasma or milk, or 100 mg of feed, and 40 µl of the internal standard

solution. The samples were centrifuged at 28620×g and 4°C for 5 min. The resultant supernatant was transferred into a separate glass vial. The extraction procedure was repeated twice and each time the supernatants were collected into the same vial. The combined extracts were dried under constant nitrogen steam and then re-dissolved in 5 ml of methanol.

Metabolites of choline (Figure 1) were analyzed using HILIC LC-MS/MS based on methods outlined by Zhao et al. (2011). A Finnigan Surveyor MS Pump Plus coupled to a Finnigan Surveyor Autosampler was used to introduce the samples onto an Ascentis Express HILIC column (150 × 2.1 mm, 2.7 µm particles) HPLC separation. The autosampler tray temperature was 4°C and full loop injections of 10 µL were used. The column temperature was set at 25°C and 200 µL/min flow rate was used. The mobile phases were acetonitrile (ACN, solvent A) and 10mM ammonium formate in water buffered to pH 3.0 with formic acid (solvent B). A 30 min gradient was performed: t) 0.1 min, 8% solvent B; t) 10 min, 30% solvent B; t) 15 min, 70% solvent B; t) 18 min, 70% solvent B; t) 18.01 min, 8% solvent B; t) 30 min, 8% solvent B. At the end of analysis the column was flushed with 100% ACN for 30 min prior to storage to preserve the stationary phase and to prevent retention time shifts in subsequent analyses.

Fused silica tubing (0.10 ID 0.19 mm OD) was connected to the ion source housing of a Finnigan TSQ Quantum Discovery MAX operating in electrospray ionization (ESI) mode for sample introduction. Ion source parameters were 4500 ESI spray voltage and 290°C ion transfer capillary temperature. Nitrogen was used as a sheath gas set at 40 arbitrary units at the instrument and 100 psi from the source. Argon collision gas was set to 1.5 mTorr in the collision cell and 20 psi from the source. The selected reaction monitoring (SRM) transitions were used with the following parameters: 0.05 s scan time, 1 m/z scan width, and Q1 & Q3 peak widths full

width at half maximum of 0.7 Da. Parent and product masses were rounded to 0.1 m/z unit. A complete list of masses and collision energies used were reported by Zhao et al. (2011). SRM transitions of internal standards were: PC 799.7 to 193, SM 735.6 to 188, AC 159.4 to 91.2, LPC 499.3 to 184, Cho 113.2 to 69.1, Bet 129.1 to 66.2, and PCho 193 to 125.1. Two segments were used to lower duty cycle. Segment 1 was from 0-10.8 min, and contained the SRM transitions for all PC and SM. Segment 2 was from 10.8-30 min, and contained the SRM transitions for SM, AC, LPC, Cho, Bet, GPCho, and PCho. All Finnigan instruments were operated using Xcalibur (Thermo Fisher Scientific Inc., Waltham, MA, version 2.0.7) installed on a Dell Precision 390 computer. The data was processed with the Xcalibur 2.0.7 Quan Browser software (Thermo Fisher Scientific Inc., Waltham, MA).

Effects of WOL on milk composition and milk and plasma choline metabolites were analyzed using the GLIMMIX procedure of SAS (SAS version 9.3 Institute Inc., Cary, NC, USA). The model included WOL as fixed effect, cow as a random effect, with WOL as the repeated measure, and spatial power as the covariance structure because of unequal sampling intervals. Means are reported as least squares means with their respective pooled standard errors and considered to differ when $P \leq 0.05$. For choline metabolites that had unequal variance the data was Log10 transformed.

Hepatic and mammary PEMT activity was evaluated from the PEMT ratio which was calculated based on the ratio of PC species containing DHA to total PC in blood and milk, respectively (da Costa et al., 2011; DeLong et al., 1999). While in two instances there was coelution of PC containing DHA with other long-chain polyunsaturated fatty acids, DeLong et al. (1999) reported the same coelution of PC species associated with PEMT activity. Relationships between WOL, milk and plasma choline metabolites and PEMT activity were

explored. When effects were found significant, polynomial (PROC GLIMMIX) and exponential (PROC NLIN) relationships were evaluated. Model adequacy was assessed based on mean square error, residuals against both fitted and predicted values, and normal probability plot. In addition, relationships between choline metabolites in milk and blood were examined. Variable selection was performed and relationships between selected variables were evaluated using PROC REG. Finally, multiple regression analysis was conducted using PROC REG to assess the relationship between milk total choline yield and animal performance variables (i.e. milk yield and milk composition) during lactation.

Results

Animals were sampled on each of three days during early, mid and late stages of lactation; their yield and composition of milk is shown in Table 3.3. As expected, milk yield followed the typical lactation curve, increasing from early to mid-lactation and then decreasing during late lactation. Milk protein yield did not change during lactation; however, milk fat yield was higher in early lactation and then decreased as lactation progressed. Milk fat and protein concentration were greater at the start of lactation, lowest at mid and intermediate at the end of lactation.

The main choline metabolites detected in plasma were PC, SM and LPC, with the concentration of all metabolites changing over lactation (Table 3.4). PC was the predominant choline metabolite found in plasma, with its proportion of total choline increasing from 77% at the onset of lactation to 95% by late lactation. Also PCho and Bet concentrations in plasma were the highest at the end of the lactation. In contrast, SM and LPC concentrations in plasma increased from early to mid-lactation and then decreased in late lactation. ACho was not detected in plasma and as its internal standard was also not detected, it is likely the metabolite was

degraded. The concentration of total choline in plasma showed a positive quadratic relationship with WOL (Figure 3.2), increasing up to 10-times from early to late of lactation, which was mainly explained by the increase in PC. The profile of fatty acids bound to LPC, containing a single fatty acid, and PC, containing a combination of two fatty acids, were quantified in plasma and milk during lactation. The plasma concentration of all forms of LPC and PC changed during lactation (Table 3.5). The main fatty acid associated with LPC in plasma was linoleic acid (C18:2) and all forms, except LPC containing stearic acid (C18:0), increased from early to mid-lactation and then decreased in late lactation. For PC, the concentrations of all species increased in plasma from early to late lactation regardless of fatty acyl composition.

The concentration and yield of all choline and choline metabolites in milk, except for LPC yield, changed during lactation (Table 3.6). The two main choline metabolites in milk were PC and PCho, which together represented 60 to 80% of the total choline in the milk. The yield of PtdCho increased linearly through lactation, from <5 g/d in early lactation to >12 g/d by late lactation (Figure 3.3A). In contrast PCho was the main choline metabolite in early lactation (averaged 2.7 g/d), and its yield displayed an exponential decay as lactation progressed (Figure 3.3B).

The total output of choline in milk (based on the moiety alone) was lowest in mid lactation, averaging 1.8 g/d, whereas it averaged 2.7 g/d and 3.1 g/d in early and late lactation, respectively (Table 3.6). The yield of free choline in milk increased during the initial 3 weeks of lactation and then remained constant until the end of lactation (Table 3.6). In milk, except for 18:0/22:5 PC, the concentrations of all double fatty acid pairings within PC increased from early to late lactation (Table 3.7). The main fatty acids found in LPC were palmitic acid whereas palmitic and oleic acid together were the main combination found in PC.

When evaluating relationships between the choline metabolites in plasma and milk we found that milk PC yield increased linearly as PC concentration in plasma increased (Figure 4A). In contrast, milk PCho yield showed a negative exponential decay with increased PC concentration in plasma (Figure 4B). We also evaluated the relationship between WOL and the PEMT ratio in plasma and milk. There was no consistent pattern in the plasma PEMT ratio over lactation, whereas in milk the ratio was highest during early lactation

Finally, the multiple regression analysis found that WOL, milk yield and the content of fat and protein in milk, were able to explain a moderate proportion of the variation in total choline in milk during lactation (R^2=0.67; p<0.001). The model obtained for total choline yield in milk (γ; g/d) was

$$\gamma = -11.6 - 0.221WOL + 0.015WOL^2 + 0.492\,fat + 2.04\,protein + 0.351yield$$

Where WOL is week of lactation, fat is percentage of fat in milk, protein is the percentage of protein in milk, and yield is daily milk yield (kg); SEE were 4.88, 0.166, 0.004, 1.24, 0.377, 0.048, respectively.

Discussion

Milk and dairy products derived from the bovine are a rich source of choline for the human diet; however; little is known about how choline varies in milk during lactation in the dairy cow. In addition, choline is found in various water- and lipid-soluble forms in milk, which can differ in their absorption, transport and metabolism in the nursing infant (Davenport and Caudill, 2013). When comparing the concentration of serum choline metabolites in infants fed either breast milk or bovine-based infant formula, Ilcol et al. (2005) reported around 2-fold greater serum Cho levels in breast milk fed infants, with these levels positively correlated with the concentrations of

water-soluble choline metabolites (i.e. Cho, PCho and GPCho) in breast milk. Therefore, characterizing the changes in choline metabolites in bovine milk during the course of lactation may form the basis to enhance the choline metabolite profile in bovine-derived infant formula. We adopted methodology that has been recently developed and validated to detect the major choline metabolites in egg yolk (Zhao et al., 2011; Xiong et al., 2012). This technique utilizes HILIC LC-MS/MS that, as well as providing improved sensitivity, has an advantage over other analytical methods as it identifies all the major choline metabolites (Figure 3.1) and differentiates the fatty acid chains in PC and LPC in a single chromatographic run. We also used matching IS for each analyte class, except GPCho, for absolute quantification for a member of each choline metabolite class as well as for enhanced quantification of all other compounds.

Choline and choline metabolites in blood were of interest as it is unknown how their concentrations may change during lactation in the dairy cow. When comparing free choline concentration in plasma, dairy cows in our study had 3- to 4-fold lower levels than previously reported for breastfeeding woman (Ilcol et al., 2005). In ruminants dietary choline is extensively degraded in the rumen (Sharma and Erdman, 1989; Baldi and Pinotti, 2006), which may explain the low and constant levels of free choline in plasma during lactation in the present study. We did find an increase of over 10-times in the total choline concentration in plasma from early to late lactation, with the majority of the increase coming from elevated PC levels. The plasma PC concentrations in early lactation were similar to that previously reported in breast feeding women (Ilcol et al., 2005); however, levels in blood were much higher at mid and late lactation in our study. PC is the main phospholipid component in all the lipoprotein classes, with its proportion ranging from 60 to 80% of total phospholipids (Cole et al., 2012). As lactation progresses there is an increase in lipoprotein concentration in blood, primarily in HDL concentration in dairy

cows (Raphael et al., 1973). Therefore, the increased concentrations of PC are expected to have been associated with the changes in types and levels of lipoproteins in plasma that occur during lactation.

The levels of the choline moiety in milk during lactation were important to consider as milk from the bovine is an important source of choline for the human diet. The USDA database for choline content in foods indicates that retail milk with 2% fat has 16 mg/100 g (Howe et al., 2004). In the present study the total choline moiety concentration in milk was lower than that reported by USDA throughout lactation; with mid lactation levels less than half of late lactation levels (averaged 8.3, 5.0 and 11.1 mg/100 g in early, mid and late lactation, respectively). Similar choline moiety levels in milk of dairy cows, when not supplemented with choline, have previously been reported by Deuchler et al. (1998) across 3 experiments (ranged from 6.6 to 8.9 mg/100 g) and by Pinotti et al. (2003) (10.0 mg/100 g). The basis for the difference between these animal studies which have analyzed choline in milk collected at harvest and the USDA database is not clear, although it may relate to milk processing and/or the low number of retail samples (n=2) used to develop the USDA database estimate. Considering this large difference in choline levels in milk and the importance of choline originating from dairy to the human population, further evaluation of choline levels in milk may be warranted to ensure choline intake meets requirements.

Although choline is considered an essential nutrient and dietary requirements have been developed for almost all domestic animal species, presently there is no requirement established for lactating dairy cows by the NRC (2001). Nutrient requirements for lactating dairy cows are partitioned by the NRC based on their use for maintenance, pregnancy, growth and lactation. Therefore one important step in developing requirements for dairy cattle is increasing the

understanding of the incorporation of choline in milk and how this varies through lactation. This approach has been used in the past for humans, where the dietary reference intake for choline is 30% higher (125 mg/d) for breastfeeding women than it is for non-breastfeeding woman, with the higher level of intake calculated based on predicted levels of choline secreted daily in milk (Pitkin et al., 2000). In the current study, the yield of the choline moiety increased over 2-fold from mid to late lactation (1.6 vs 3.5 g/d, respectively). Previously Deuchler et al. (1998) reported choline levels in milk of cows, also when not supplemented choline, in early lactation (2.6 g/d) and mid-lactation (2.1 g/d) that is within the range observed in our study. Although these dairy cattle studies may give an indication of the choline requirement for lactation, research is needed to better understand what levels of choline are needed for non-lactating functions. This is particularly important in the case of the peripaturient dairy cow where there is a high prevalence of fatty liver disease (Zom et al., 2011), which is the classic symptom of choline deficiency (Garrow, 2007).

Changes in choline and its metabolites during lactation have previously been reported in human and porcine milk (Ilcol et al., 2005; Donovan et al., 1997); however, there has not been a systematic evaluation of changes during lactation in choline metabolites in milk of dairy cows. Recently Klein et al. (2011) used NMR to provide relative quantification to estimate the range in concentration of four water-soluble choline metabolites in milk during lactation of dairy cows. For Cho, Bet and GPCho, the lowest level of the range (10% quantile) reported by Klein et al. (2011) were all greater throughout lactation than the average concentrations measured in our study, however, the changes in these 3 choline forms and PCho through lactation were all similar across the two studies. When comparing the concentration of all eight choline metabolites we found that PCho was the main choline metabolite during early lactation (averaged 58% of all

choline forms), whereas PC was the main choline metabolite after early lactation and increased, as a percentage of total choline forms, from 36% at week 7 to 71% at week 37 of lactation. The PC concentration in cow's milk in late lactation (ranging from 582 to 701 μmol/L) was much greater than that previously reported by Ilcol et al. (2005) in human milk at any point in lactation (ranging from 97 to 155 μmol/L). In contrast, the concentration of SM in cow's milk (38 μmol/L) was lower than found in human milk (92 μmol/L) when compared across lactation (Ilcol et al., 2005). In the present study, PCho was the main water soluble choline metabolite in cow's milk, which is similar that reported for porcine milk (Donovan et al., 1997) and human milk (Ilcol et al., 2005). In contrast to our study, Ilcol et al. (2005) found no relationship between the concentration of PCho in breast milk and day after birth, although their study was cross-sectional and did not sample individuals over time. Early research comparing the choline metabolites in breast milk and infant formula suggested that infant formula had lower levels of PCho than breast milk (Holmes-McMary et al., 1996; Holmes et al., 2000); however, recent data from Ilcol (2005) and the present study results indicate that there is a large range in PCho in both breast milk (100 to 1200 μmol/L) and unprocessed bovine milk (12 to 820 μmol/L). This suggests that, if an ideal PCho level can be determined for infant nutrition, milk from dairy cows in specific stages of lactation might be used to manufacture infant formula to better match breast milk concentrations of PCho.

The levels of the choline metabolites in milk are of interest as it has been shown that the absorption and bioavailability can differ across the various metabolites. Cheng et al. (1996) reported that when radiolabeled choline forms were added to infant formula fed to suckling rat pups, Cho and PCho rapidly appeared in blood and the liver, whereas PC took longer to appear in blood and was metabolized differently by the liver. The different appearance rates of label for

choline and PCho versus PC in blood were likely due to independent mechanisms of absorption at the small intestine (Cheng et al., 1996). PC is hydrolyzed by phospholipase A2 in the intestine and the LPC that is generated is absorbed by passive diffusion. However, PCho is hydrolyzed by phosphatase to free choline, which is absorbed via carrier-mediated transport at the intestine (Garrow, 2007). In addition, in the calf after birth intestinal phosphatase activity is high (Le Huerou et al., 1992) whereas phospholipase A2 enzyme activity is low at birth and increases after weaning (Huerou-Luron et al., 1992). Therefore, the functional importance of higher milk PCho in early lactation, observed in similar concentrations (~500 μmol/L) in the human, porcine and bovine, may be to provide a rapidly bioavailable supply of choline for sustaining growth and maintenance of new born animals.

There is little known about the uptake of choline and its metabolites from circulation by the mammary gland. In the human free choline can be taken up from circulation (via active transport) by the mammary epithelium across a steep concentration gradient (Davenport and Caudill, 2013; Chao et al., 1988), and recently Ilcol (2005) found a positive correlation between serum free choline and milk free choline concentrations ($r=0.74$, $p<0.01$). In the current study, we found a positive linear relationship between PC concentration in plasma and PC yield in milk of dairy cows ($r=0.55$, $p<0.01$; Figure 4A). However, unlike the trends observed in humans, we did not find an association between plasma free choline and choline in milk, which may relate to the low concentration of free choline in blood of the dairy cows throughout lactation (<4.5 μmol/L) compared to that reported in breast-feeding woman (>14.6 μmol/L).

PC can be synthesized in the body through phosphorylation of choline from the diet or via endogenous synthesis mediated by PEMT in the liver (Reo et al., 2002) and mammary gland (Chao et al., 1988). These two pathways of PC synthesis result in different pools of bound fatty

acids, with PEMT the main origin of long-chain polyunsaturated fatty acids, primarily DHA, in rodent and human hepatocytes (DeLong et al., 1999; Pynn et al., 2011). In addition, a recent study in humans (da Costa et al., 2011) found that the ratio of DHA to total fatty acids in plasma PC was a useful marker for in vivo hepatic PEMT activity. Although we were not able to quantify total DHA in PC, due to coelution of some of the PC molecular species, the detection of DHA occurred in combination with other long-chain polyunsaturated fatty acids (arachidonic acid and docosapentaenoic acid) that originate primarily from the PEMT pathway (DeLong et al., 1999; Pynn et al., 2011). We did not find a consistent pattern in the PEMT ratio in plasma across lactation, but a reduction in the ratio in milk from 0.009 to 0.004 was observed from early lactation to late lactation. Although the PEMT ratio has not been used to predict PEMT activity in the mammary gland, our results may suggest that mammary endogenous biosynthesis of choline dropped from early to late lactation and this was consistent with increasing plasma PC concentrations as lactation progressed. Interestingly, the plasma PEMT ratio in the current study was several-times lower than that reported by da Costa et al. (2011) and Pynn et al. (2011) in plasma of humans and mice, suggesting PC derived from hepatic PEMT activity may contribute a smaller proportion of total PC in the cow. This aligns with earlier results reported for ruminants by Robinson et al. (1984) where they found hepatic PEMT in vitro activity from sheep tissues was around one-quarter of that measured in the rat. In addition, unlike rodents and humans, where almost all PEMT activity occurs at the liver (Reo et al.,2002) Robinson et al (1987) reported that the majority of choline derived from PEMT activity came from extrahepatic tissue in sheep, with skeletal muscle contributing around 60% of this activity.

Multiple regression analysis indicated that milk production variables (i.e., fat and protein content and yield of milk), along with WOL, were associated with the total amount of choline

secreted in milk. This result suggests that choline metabolites in milk, in addition to being located in the bilayer membrane of the fat globule (Lopez et al., 2008), may also be related to the protein component in milk. As a greater understanding of the requirement for choline is developed for the lactating dairy cow there may be value in predicting milk choline output based on easily measurable variables on farm. The association we found in this study, although developed based on a small sample set of cows, suggest that the prediction of milk choline output may be possible with typically measured animal and production variables.

In conclusion, the present study quantifies the eight major choline metabolites in blood and milk during lactation in the dairy cow. Total choline in plasma increased over 10-times from early to late lactation, with the majority of the increase coming from elevated PC levels. The concentration of the choline moiety in milk decreased from early to mid-lactation before rising again in late lactation; however, throughout lactation milk choline remained below the levels reported previously in commercial milk. In milk PCho was the main metabolite in early lactation and decreased rapidly over the first weeks of lactation. The high PCho levels in early lactation have also been seen in the human and porcine and may be of importance in ensuring a rapidly bioavailable form of choline for the nursing young. In contrast, PC in milk was lowest in early lactation and increased steadily through lactation, with this change positively correlated with PC in plasma. Finally, we found that production variables (milk yield and milk fat and protein content) along with WOL were correlated with total milk choline yield, suggesting that it may be possible to predict milk choline output from easily measureable variables on the farm.

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Appendix

Table 3.1 Ingredient and chemical composition of total mixed ration.

Ingredient (g/kg dry matter)[1]	
Corn silage	644
Lactating supplement[2]	270
Corn	18
Clover hay	22
Rye grass hay	28
Alfalfa hay	18
Chemical analysis (g/kg dry matter)[3]	
Crude protein	181
Neutral detergent fiber	452
Acid detergent fiber	312
NEL[4] (Mcal/kg dry matter)	0.72

[1]Dietary dry matter averaged 840 g/kg.
[2] Contained per kilogram: ground corn 197 g, cottonseed 200 g, wheat mids 95 g, corn gluten feed 85 g, cottonseed hulls 75 g, citrus pulp 70 g, soybean meal 65 g, cottonseed meal 60 g, distillers grain with soluble 46 g, calcium salts of palm fatty acids 20 g, fish meal 20 g, cane molasses 20 g, ground limestone 20 g, sodium bicarbonate 10 g, urea 7 g, salt 5 g, Zn methionine 5 g. vitamin A 12665 IU, vitamin D 2760 IU, vitamin E 90 IU, selenium 0.3 mg, copper 12.9 mg, Zinc 50 mg, cobalt 0.23 mg, manganese 49 mg, iron 80 mg, iodine 0.50 mg.
[3]Analysis by Dairy One Inc., Ithaca, New York.
[4] Net energy for lactation

Table 3.2 Choline and choline metabolite concentration in individual feed ingredients used to make the total mixed ration.

Ingredient	Choline and choline metabolite[1], mg/100 g								
	ACho	Bet	Cho	LPC	PC	PCho	SM	GPCho	TC[2]
Corn silage	0.07	5.3	12.9	0.2	8.6	0.01	nd	0.26	22.1
Supplement[3]	0.12	65.2	23.1	27.6	974.7	0.75	0.39	40.02	1132.0
Corn	nd	5.0	9.8	4.0	30.9	0.08	nd	3.18	47.9
Clover hay	0.02	10.7	39.0	3.7	74.2	0.68	0.06	5.19	122.9
Rye grass hay	0.02	8.4	33.3	0.6	11.8	0.14	0.02	0.91	46.8
Alfalfa hay	0.06	16.6	43.3	4.0	3.8	0.14	nd	2.69	54.0

[1]Acetylcholine (ACho), betaine (Bet), free choline (Cho), glycerophosphocholine (GPCho), lysophophatidylcholine (LPC), not detected (nd), phosphatidylcholine (PC), phosphocholine (PCho), sphingomyelin (SM), total choline (TC).
[2]TC is the sum of ACho, Cho, LPC, PC, PCho, SM and GPCho.
[3]See Table 1 for ingredient composition of the supplement mix.

Table 3.3 Milk yield and composition of dairy cows during lactation.

	Week of lactation									
	1	2	3	7	10	13	31	34	37	SEM
Milk, kg/d	26.6^{ef}	32.8^{bcd}	36.0^{ab}	36.9^{ab}	38.2^{a}	35.7^{ab}	30.5^{cde}	29.1^{def}	24.8^{f}	2.01
Protein										
%	3.88^{a}	3.19^{b}	2.91^{c}	2.67^{d}	2.77^{cd}	2.75^{cd}	3.22^{b}	3.21^{b}	3.22^{b}	0.09
g/d	1020	1038	1049	983	1059	986	992	938	795	65.5
Fat										
%	5.55^{a}	5.12^{a}	5.00^{abc}	4.20^{c}	3.95^{c}	3.81^{c}	4.27^{bc}	3.94^{c}	4.37^{bc}	0.29
g/d	1478^{bc}	1628^{ab}	1808^{a}	1538^{abc}	1493^{bc}	1372^{bcd}	1307^{cd}	1146^{d}	1097^{d}	132.0

^{abcd} Means within a row with different superscripts differ ($P < 0.05$).

Table 3.4 Choline and choline metabolites concentration in plasma (µmol/L) of dairy cows during lactation.

	Stage and week of lactation											
	Early				Mid				Late			
Metabolite[1]	1	2	3	SEM	7	10	13	SEM	31	34	37	SEM
Bet[2]	25.5^{cd}	18.4^{e}	13.7^{e}	3.48	18.3^{de}	18.6^{de}	20.4^{de}	3.48	32.0^{c}	38.5^{b}	48.9^{a}	3.84
Cho	3.75	3.92	4.47	0.35	3.75	4.16	4.01	0.35	3.45	3.02	3.26	0.38
PCho[2]	0.43^{b}	0.45^{b}	0.41^{b}	0.09	1.22^{a}	1.28^{a}	1.57^{a}	0.37	1.78^{a}	1.43^{a}	1.66^{a}	0.39
GPCho[2]	1.12^{cd}	0.56^{d}	1.03^{b}	0.32	4.12^{ab}	4.37^{ab}	5.03^{a}	0.98	2.36^{abc}	2.46^{abc}	1.78^{b}	0.81
LPC	113^{d}	133^{d}	196^{c}	24.7	321^{ab}	251^{c}	363^{a}	25.8	250^{bc}	243^{c}	218^{c}	30.2
SM	189^{d}	231^{cd}	273^{c}	28.0	648^{a}	593^{a}	626^{a}	28.0	444^{b}	401^{b}	404^{b}	30.6
PC[2]	1014^{e}	1437^{d}	1757^{c}	132	5515^{b}	5491^{b}	6803^{b}	560	13535^{a}	12593^{a}	10894^{a}	1253
TC[3]	1305^{f}	1805^{e}	2220^{d}	159	6456^{bc}	6325^{c}	7866^{b}	614	14241^{a}	13255^{a}	11536^{a}	1250

[1]Betaine (Bet), free choline (Cho), glycerophosphocholine (GPCho), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphocholine (PCho), sphingomyelin (SM), total choline (TC).
[2]Variables were Log10 transformed to obtain equal variance. Means and SEM are back-transformed.
[3]TC is the sum of Cho, LPC, PC, PCho, SM and GPCho.
[abcd] Means within a row with different superscripts differ ($P < 0.001$).

Table 3.5 Concentration of lysophophatidylcholine (LPC) and phosphatidylcholine (PC) with a particular fatty acyl chain in plasma of dairy cows during lactation.

	Stage and week of lactation											
	Early				Mid				Late			
	1	2	3	SEM	7	10	13	SEM	31	34	37	SEM
Metabolite concentration[1], μmol/L												
LPC												
16:0	18.9^{e}	22.7^{de}	30.7^{c}	2.97	52.4^{a}	41.1^{ab}	50.7^{a}	5.07	33.2^{bc}	28.9^{cd}	28.0^{cd}	3.51
18:0	18.1^{d}	23.7^{d}	34.4^{c}	4.01	64.5^{ab}	53.5^{b}	73.2^{a}	8.53	65.2^{ab}	59.1^{ab}	54.1^{ab}	8.29
18:1	24.7^{d}	27.9^{d}	39.6^{cd}	5.87	62.5^{a}	50.3^{abc}	59.0^{ab}	5.87	48.4^{abc}	41.7^{bcd}	38.6^{cd}	6.46
18:2	40.2^{f}	56.0^{e}	77.2^{d}	9.6	161.9^{a}	125.7^{ab}	165.3^{a}	20.5	113.7^{bc}	99.4^{bcd}	86.6^{cd}	15.4
PC												
16:0/16:0	8^{d}	12^{c}	13^{c}	1.5	53^{b}	52^{b}	65^{b}	7.5	166^{a}	140^{a}	127^{a}	20.7
16:0/16:1	8^{d}	10^{c}	12^{c}	1.4	44^{b}	42^{b}	52^{b}	6.1	132^{a}	114^{a}	105^{b}	16.7
16:0/18:1	142^{d}	183^{c}	210^{c}	19.7	497^{b}	476^{c}	534^{b}	50.2	970^{a}	901^{a}	851^{a}	97.8
16:0/18:2	265^{f}	371^{e}	457^{d}	42	1423^{c}	1431^{d}	1694^{c}	156	3160^{a}	2896^{ab}	2413^{b}	315
16:0/20:3	86^{f}	119^{e}	148^{d}	14	435^{c}	459^{c}	577^{b}	53	1046^{a}	1009^{a}	844^{a}	104
16:0/20:4	34^{e}	50^{d}	64^{c}	6.4	204^{b}	203^{b}	253^{b}	25.3	497^{a}	445^{a}	394^{a}	53.7
16:0/20:5,16:1/20:4[2]	7^{e}	9^{d}	11^{e}	1.3	40^{b}	38^{b}	50^{b}	5.6	120^{a}	111^{a}	102^{a}	14.7
16:0/22:6,18:1/20:5, 18:2/20:4[2]	6.8^{e}	9.3^{d}	10.7^{d}	1.1	36.1^{c}	33.4^{c}	41.8^{c}	4.4	94.6^{a}	74.0^{ab}	65.0^{b}	10.9
18:0/18:1	107^{d}	153^{c}	184^{c}	18.2	501^{b}	493^{b}	594^{b}	58.8	1145^{a}	1073^{a}	934^{a}	98.9
18:0/18:2,18:1/18:1[2]	243^{g}	356^{f}	446^{e}	42	1394^{d}	1479^{d}	1856^{c}	176	3385^{a}	3269^{ab}	2633^{b}	346
18:0/20:3	24^{f}	41^{e}	58^{d}	8	286^{bc}	271^{c}	340^{b}	52	1085^{a}	1050^{a}	974^{a}	156
18:0/20:4	35^{e}	54^{d}	67^{d}	8	305^{bc}	262^{c}	369^{b}	46	948^{a}	866^{a}	825^{a}	128
18:0/22:5	12^{e}	19^{d}	22^{d}	2.7	90^{bc}	72^{c}	99^{b}	12.5	246^{a}	209^{a}	203^{a}	33.8
18:1/20:4,18:0/20:5, 16:0/22:5[2]	22^{e}	31^{d}	36^{d}	4.1	125^{bc}	109^{c}	147^{c}	16.7	348^{a}	293^{a}	269^{b}	42.9
18:1/22:6	0.9^{f}	1.5^{e}	1.7^{e}	0.20	6.6^{cd}	6.0^{d}	8.5^{c}	1.02	19.6^{a}	14.2^{ab}	12.0^{b}	2.56
18:0/22:6,18:1/22:5[2]	6^{f}	9^{e}	10^{e}	1.2	42^{cd}	35^{d}	49^{c}	5.9	121^{a}	87^{ab}	71^{b}	16.0
PEMT Ratio[3]	0.013^{c}	0.014^{bc}	0.013^{c}	0.0007	0.016^{ab}	0.014^{bc}	0.015^{bc}	0.0008	0.018^{a}	0.014^{bc}	0.014^{bc}	0.001

[1]All metabolites, except for LPC 18:1 were Log10 transformed to obtain equal variance. Means and SEM represents non-transformed data.

[2]Two or three possible combinations of fatty acids were identified by the same product ions from HILIC LC-MS/MS.

[3]Phosphatidylethanolamine N-methyltransferase (PEMT) ratio = (16:0/22:6,18:1/20:5,18:2/20:4 + 18:1/22:6 + 18:0/22:6,18:1/22:5)/ (16:0/16:1 + 16:0/20:5, 16:1/20:4 + 16:0/16:0 + 16:0/18:2 + 16:0/18:1 + 16:0/20:4 + 18:0/20:4 + 18:0/20:3 + 18:0/22:5 + 18:0/18:2, 18:1/18:1 + 18:0/18:1+ 18:1/20:4,18:0/20:5,16:0/22:5)

[abcdef] Means within a row with different superscripts differ (P < 0.001).

Table 3.6 Concentration and yield of choline and choline metabolites in milk of dairy cows during lactation.

	Stage and week of lactation											
	Early				Mid				Late			
	1	2	3	SEM	7	10	13	SEM	31	34	37	SEM
Metabolite concentration[1], μmol/L												
ACho	nd	nd	nd	-	0.05^{a}	0.04^{a}	0.04^{a}	0.005	0.01^{b}	0.01^{b}	0.01^{b}	0.006
Bet[2]	100^{a}	45^{cd}	27^{e}	6.5	17^{f}	18^{f}	19^{f}	2.2	37^{d}	49^{bc}	59^{b}	5.6
Cho	28^{d}	43^{cd}	57^{c}	11.1	101^{b}	112^{b}	117^{ab}	11.1	118^{ab}	127^{ab}	144^{a}	12.2
PCho[2]	527^{a}	537^{a}	413^{b}	76.1	155^{c}	113^{d}	64^{ef}	17.1	34^{g}	42^{fg}	52^{e}	7.6
GPCho	102^{b}	82^{bc}	76^{cd}	9.9	55^{de}	55^{de}	48^{e}	10.6	156^{a}	146^{a}	156^{a}	10.7
LPC	2.27^{cd}	2.40^{bcd}	2.45^{bc}	0.25	1.85^{d}	1.86^{d}	1.75^{d}	0.25	3.03^{ab}	3.24^{a}	3.41^{a}	0.27
SM	52.8^{a}	44.9^{ab}	45.9^{b}	2.12	33.8^{c}	30.7^{cd}	27.8^{d}	2.12	35.8^{c}	36.7^{c}	36.7^{c}	2.31
PC[2]	170^{bc}	149^{c}	167^{bc}	12.7	202^{b}	178^{bc}	183^{bc}	14.7	582^{a}	701^{a}	694^{a}	60.8
TC[3]	908^{a}	872^{a}	787^{b}	56.5	559^{c}	501^{cd}	450^{d}	56.5	886^{a}	1002^{a}	972^{a}	61.9
Metabolite yield[1], g/day												
ACho	nd	nd	nd	-	0.0003^{a}	0.0003^{a}	0.0003^{a}	0.00004	0.00004^{b}	0.00003^{b}	0.00002^{b}	0.00004
Bet[2]	0.26^{a}	0.16^{b}	0.10^{cde}	0.03	0.07^{d}	0.08^{d}	0.08^{d}	0.01	0.12^{bc}	0.16^{b}	0.15^{b}	0.02
Cho	0.07^{d}	0.13^{c}	0.20^{b}	0.04	0.37^{a}	0.43^{a}	0.41^{a}	0.04	0.36^{a}	0.36^{a}	0.34^{a}	0.04
PCho[2]	2.43^{b}	3.03^{a}	2.61^{b}	0.53	1.00^{c}	0.75^{d}	0.40^{d}	0.17	0.18^{f}	0.21^{f}	0.24^{f}	0.04
GPCho[2]	0.59^{b}	0.62^{b}	0.63^{b}	0.08	0.47^{bc}	0.49^{bc}	0.40^{c}	0.07	1.08^{a}	0.98^{a}	0.89^{bc}	0.15
LPC	0.03	0.05	0.04	0.007	0.03	0.04	0.03	0.007	0.05	0.05	0.05	0.008
SM	0.95^{abc}	0.99^{ab}	1.13^{a}	0.07	0.85^{bcd}	0.78^{cde}	0.68^{de}	0.07	0.75^{de}	0.71^{de}	0.61^{e}	0.07
PC[2]	3.3^{d}	3.5^{cd}	4.4^{bc}	0.46	5.5^{b}	5.0^{b}	4.8^{b}	0.58	14.9^{a}	16.5^{a}	12.1^{a}	1.92
TC[2,3]	7.5^{d}	8.4^{cd}	9.3^{c}	0.80	8.3^{cd}	7.4^{cd}	6.7^{d}	0.64	17.5^{ab}	19.0^{a}	14.3^{bc}	1.79
TCM[2,4]	2.35^{cd}	2.79^{ab}	2.81^{ab}	0.21	2.01^{de}	1.89^{e}	1.60^{f}	1.21	3.23^{ab}	3.45^{a}	2.72^{bc}	0.29

[1]Acetylcholine (ACho), betaine (Bet), free choline (Cho), glycerophosphocholine (GPCho), lysophophatidylcholine (LPC), not detected (nd), phosphatidylcholine (PC), phosphocholine (PCho), sphingomyelin (SM), total choline (TC), total choline moiety (TCM).

[2]Variables were Log10 transformed to obtain equal variance. Means and SEM are back-transformed.

[3]TC is the sum of ACho, Cho, LPC, PC, PCho, SM and GPCho.

[4]TCM is the sum of choline moiety originating from ACho, Cho, LPC, PC, PCho, SM and GPCho.

[abcd] Means within a row with different superscripts differ ($P < 0.001$).

Table 3.7 Concentration of lysophophatidylcholine (LPC) and phosphatidylcholine (PC) with a particular fatty acyl chain in milk of dairy cows during lactation.

	Stage and week of lactation											
	Early				Mid				Late			
	1	2	3	SEM	7	10	13	SEM	31	34	37	SEM
Metabolite concentration[1], μmol/L												
LPC												
16:0	0.81^{bc}	0.73^{c}	0.88^{b}	0.11	0.87^{bc}	0.84^{bc}	0.90^{bc}	0.11	1.49^{a}	1.71^{a}	1.72^{a}	0.25
18:0	0.32	0.29	0.39	0.08	0.31	0.42	0.41	0.07	0.59	0.69	0.68	0.08
18:1	0.64^{ab}	0.79^{ab}	0.90^{ab}	0.16	0.56^{bc}	0.37^{cd}	0.33^{d}	0.10	0.67^{ab}	0.77^{ab}	0.97^{a}	0.19
18:2	0.27	0.37	0.44	0.09	0.36	0.22	0.23	0.08	0.34	0.28	0.43	0.10
PC												
16:0/16:0	20^{c}	15^{d}	21^{c}	3.4	30^{b}	26^{bc}	28^{bc}	5.0	148^{a}	150^{a}	108^{a}	27.5
16:0/16:1	5.1^{d}	4.2^{d}	5.5^{d}	0.67	8.6^{c}	7.5^{c}	8.9^{c}	1.08	50.7^{ab}	55.8^{a}	38.9^{b}	7.23
16:0/18:1	48^{cd}	39^{d}	50^{c}	7.6	65^{b}	50^{cd}	56^{cd}	10.2	294^{a}	316^{a}	205^{a}	55.3
16:0/18:2	18^{b}	14^{b}	20^{b}	3.2	27^{b}	22^{bc}	27^{b}	4.6	113^{a}	113^{a}	75^{a}	21.0
16:0/20:3	14.7^{cd}	12.3^{d}	15.9^{c}	2.6	19.2^{c}	14.1^{cd}	16.1^{cd}	3.1	58.2^{a}	57.0^{a}	37.5^{b}	10.3
16:0/20:4	4.2^{c}	3.3^{d}	4.5^{c}	0.74	5.5^{c}	4.0^{cd}	4.7^{cd}	0.90	17.2^{ab}	17.6^{a}	11.3^{b}	3.19
16:0/20:5,16:1/20:4[2]	1.5^{b}	1.5^{b}	1.8^{b}	0.02	1.6^{b}	1.5^{b}	1.6^{b}	0.02	5.2^{a}	6.0^{a}	4.4^{b}	0.76
16:0/22:6,18:1/20:5, 18:2/20:4[2]	0.61^{b}	0.39^{c}	0.53^{b}	0.09	0.51^{bc}	0.43^{bc}	0.52^{bc}	0.08	2.20^{a}	1.28^{a}	1.54^{a}	0.39
18:0/18:1	17.0^{b}	14.0^{b}	17.0^{b}	2.8	19.7^{b}	14.7^{b}	15.6^{b}	3.2	62.6^{a}	64.7^{a}	43.7^{a}	11.7
18:0/18:2,18:1/18:1[2]	31^{c}	27^{c}	33^{c}	5.3	37^{c}	27^{c}	29^{c}	6.0	112^{ab}	111^{a}	72^{b}	20.1
18:0/20:3	0.84^{b}	0.50^{c}	0.69^{bc}	0.14	0.97^{b}	0.80^{b}	0.94^{b}	0.16	3.70^{a}	3.80^{a}	2.77^{a}	0.69
18:0/20:4	1.74^{b}	1.07^{c}	1.44^{bc}	0.24	1.79^{b}	1.43^{bc}	1.63^{bc}	0.27	6.71^{a}	6.82^{a}	4.81^{a}	1.25
18:0/22:5	0.68^{a}	0.45^{bc}	0.63^{a}	0.13	0.36^{c}	0.30^{c}	0.34^{c}	0.06	0.92^{a}	0.90^{a}	0.64^{bc}	0.19
18:1/20:4,18:0/20:5, 16:0/22:5[2]	2.89^{bc}	1.93^{d}	2.84^{bc}	0.46	2.06^{cd}	1.69^{d}	1.97^{cd}	0.33	6.75^{a}	6.52^{a}	4.63^{ab}	1.19
18:1/22:6	0.26^{bc}	0.21^{bc}	0.25^{bc}	0.05	0.25^{bc}	0.19^{c}	0.25^{bc}	0.05	0.75^{a}	0.67^{a}	0.39^{b}	0.17
18:0/22:6,18:1/22:5[2]	0.55^{bc}	0.46^{bc}	0.50^{bc}	0.11	0.40^{bc}	0.34^{c}	0.43^{bc}	0.08	1.14^{a}	1.10^{a}	0.70^{ab}	0.33
PEMT ratio[3]	0.009^{a}	0.009^{a}	0.008^{b}	0.0006	0.006^{cde}	0.007^{bc}	0.005^{de}	0.0006	0.005^{de}	0.004^{e}	0.004^{e}	0.0007

[1]All variables, except for LPC 18:0 and phosphatidylethanolamine N-methyltransferase (PEMT) ratio, were Log10 transformed to obtain equal variance. Means and SEM represents non-transformed data.
[2]Two or three possible combination of fatty acids were identified by the same product ions from HILIC LC-MS/MS
[3]PEMT ratio= (16:0/22:6,18:1/20:5,18:2/20:4 + 18:1/22:6 + 18:0/22:6,18:1/22:5)/ (16:0/16:1 + 16:0/20:5, 16:1/20:4 + 16:0/16:0 + 16:0/18:2 + 16:0/18:1 + 16:0/20:4 + 18:0/20:4 + 18:0/20:3 + 18:0/22:5 + 18:0/18:2, 18:1/18:1 + 18:0/18:1+ 18:1/20:4,18:0/20:5,16:0/22:5)
[abcde] Means within a row with different superscripts differ ($P < 0.001$).

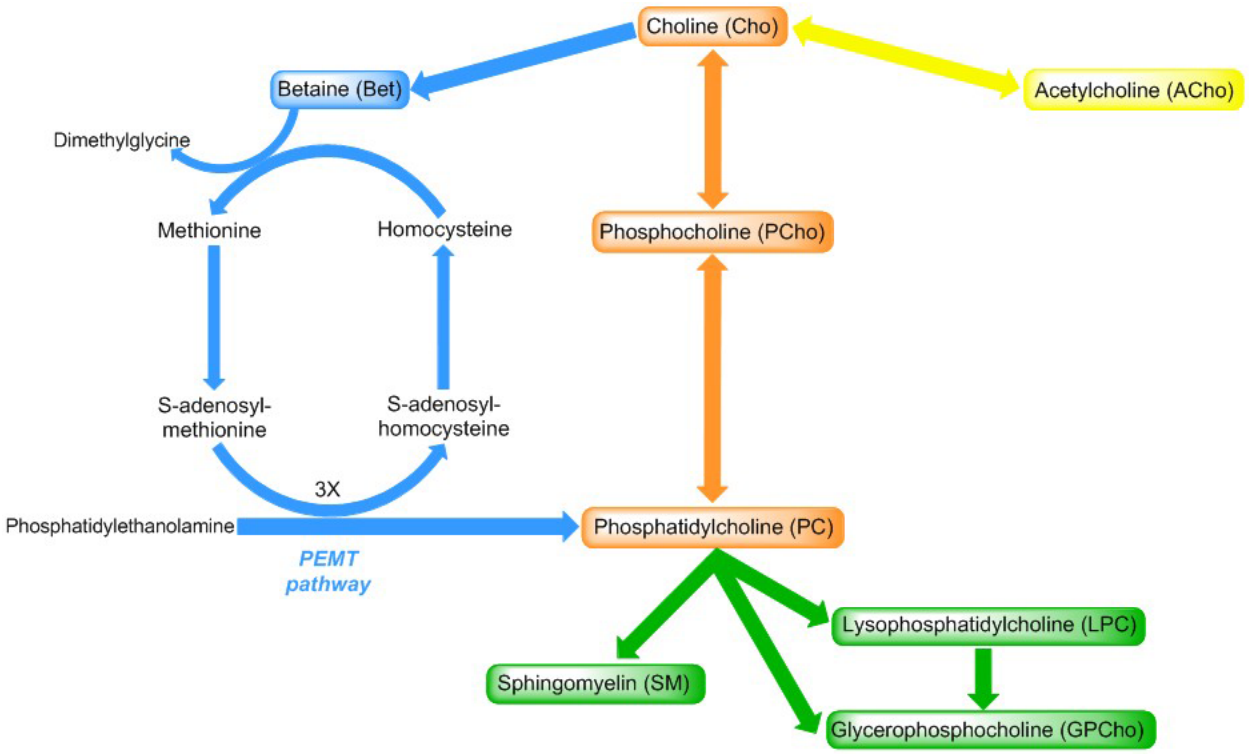


Figure 3.1 Metabolism of choline and its metabolites. The compounds shown in boxes were assayed in milk and plasma in the current study. Phosphocholine and phosphatidylcholine are formed from choline via the cytidine diphosphate (CDP) choline pathway. The formation of betaine from choline is irreversible. Betaine when oxidized will provide a methyl group to homocysteine to form methionine. Methionine is converted to S-adenosylmethionine, which is an important methyl donor. Phosphatidylcholine can be formed endogenously by methylating phosphatidylethanolamine in a three step process involving S-adenosylmethionine via the phosphatidylethanolamine N-methyltransferase (PEMT) pathway.

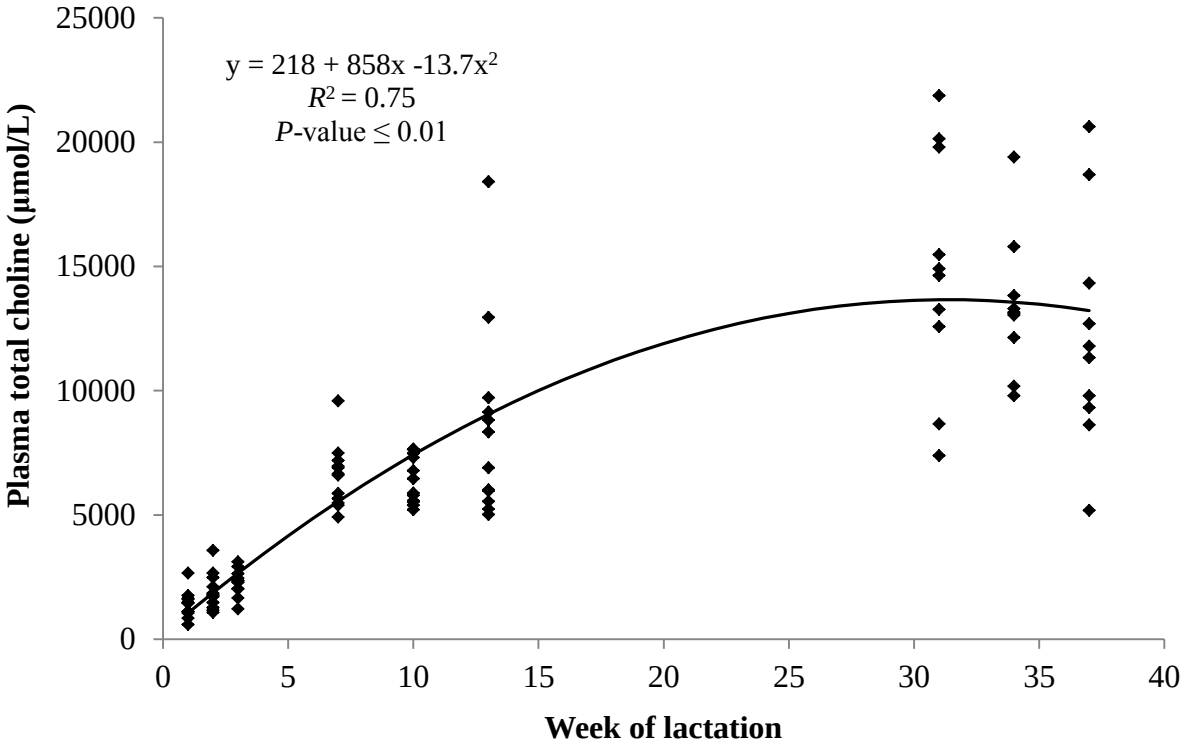


Figure 3.2 Relationship between total choline concentration in plasma and week of lactation. The standard errors for the intercept, linear and quadratic term were 564, 97.0, and 2.42, respectively.

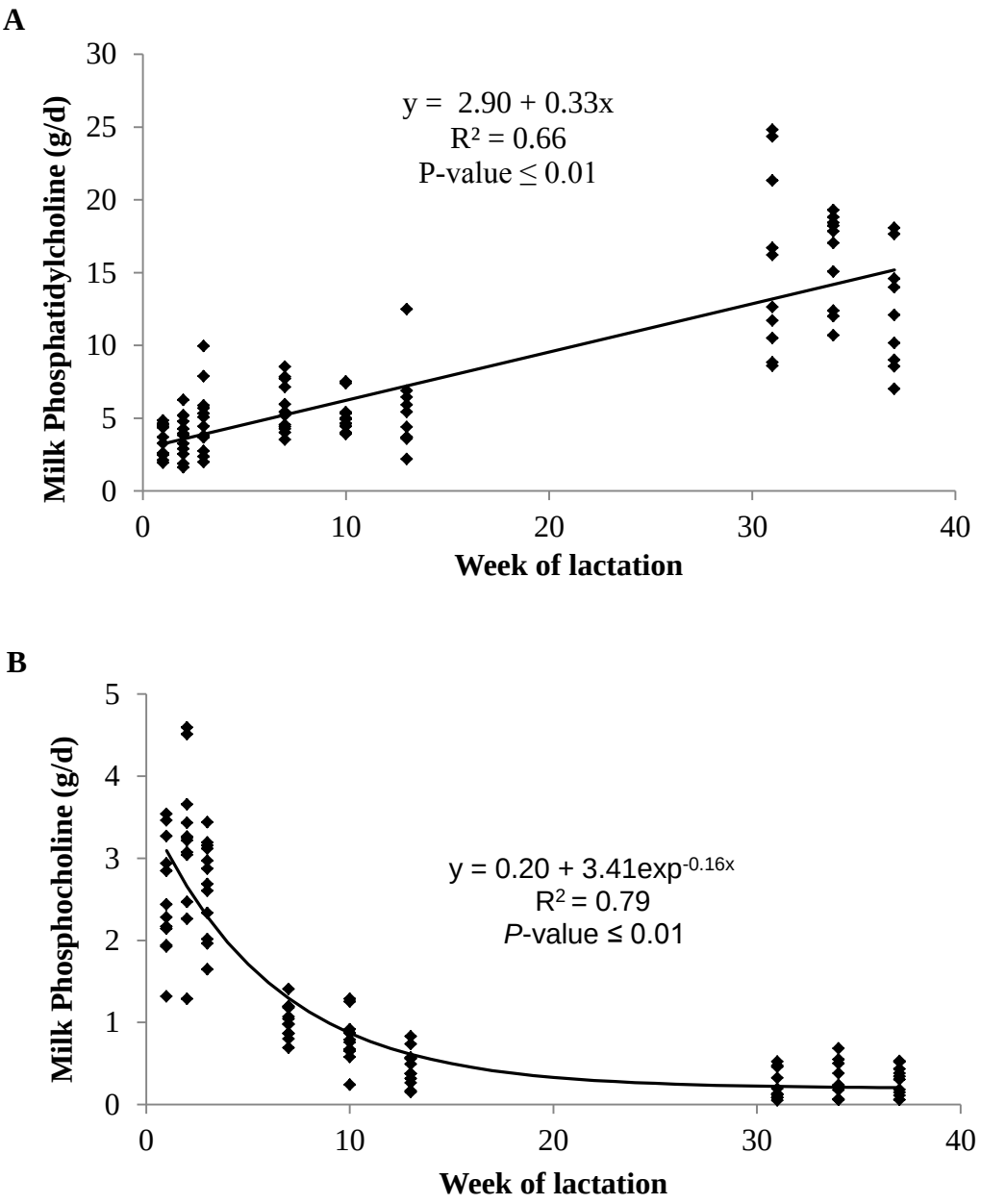
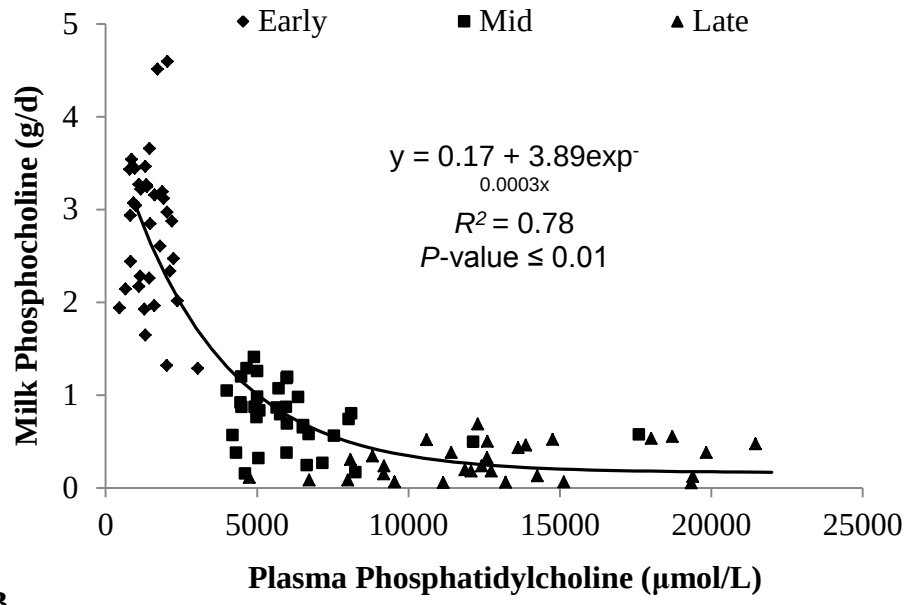


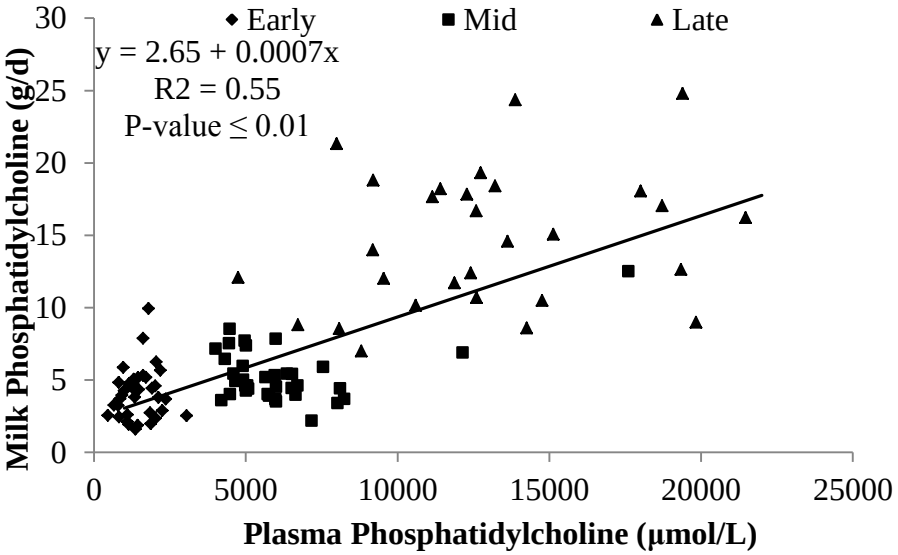
Figure 3.3 Relationship between week of lactation and milk yields of phosphatidylcholine (A), phosphocholine (B). The standard errors for the intercept and linear terms in (A) were 0.46 and 0.02, respectively. The equation was y = a + b exp-cx with the standards errors for the fitted parameters in (B) were 0.106, 0.190, and 0.022 for a, b and c, respectively.

Figure 3.4 Relationship between plasma phosphatidylcholine concentration and either yield of phosphatidylcholine (A) or phosphocholine (B). Each symbol represents either early (♦), mid (■) and late (▲) lactation. The standards errors for the intercept and linear terms in (A) were 0.58 and 0.00007. The equation was $y = a + b \exp^{-cx}$ with the standard errors for the fitted parameters in (B) were 0.133, 0.230, and 0.000046 for a, b and c, respectively.

A


Early
Mid
Late
y = 0.17 + 3.89exp-0.0003x
R2 = 0.78
P-value ≤ 0.01
Milk Phosphocholine (g/d)
Plasma Phosphatidylcholine (µmol/L)
0
1
2
3
4
5
0
5000
10000
15000
20000
25000

B


Early
Mid
Late
y = 2.65 + 0.0007x
R2 = 0.55
P-value ≤ 0.01
Milk Phosphatidylcholine (g/d)
Plasma Phosphatidylcholine (µmol/L)
0
5
10
15
20
25
30
0
5000
10000
15000
20000
25000

CHAPTER 4: EFFECT OF POST-RUMINAL INFUSION AND RUMEN-PROTECTED DELIVERY OF CHOLINE ON BLOOD AND MILK CHOLINE METABOLITES IN THE LACTATING DAIRY COW

This chapter is a lightly revised manuscript by Artegoitia V. M., Girard C. L.; Lapierre H.; Campagna S. R., Harte F., de Veth M. J. The use of "our" in this chapter refers to my co-authors and I. My primary contributions to this paper include (1) the sample preparation, (2) the collection and analysis of data, (3) the gathering and interpretation of literature, and (4) the manuscript writing.

Abstract

The majority of choline in feed ingredients is degraded in the rumen (NRC, 2001). Thus, rumen protected (RP) products have been developed to supply choline to the small intestine. However, the amount of choline absorbed at the small intestine (i.e. bioavailability) and its metabolism remain unknown. The objective of this study was to measure the effect of post-ruminal infusion and rumen protected choline (RPC) delivery of choline across the artery and portal and hepatic veins as well as in milk choline metabolites. Five lactating Holstein cows (206 ± 25 DIM) were used in a 5x5 Latin square design, with 5-d treatment periods and a 2-d interval between periods. Treatments were 1) control (0 g/d choline), 2) RPC - low dose (RPC-L), 3) RPC - high dose (RPC-H), 4) post-ruminal infusion (ABO) – low dose (ABO-L), 5) ABO - high dose (ABO-H). The low and high doses of RPC (Reashure, Balchem Corporation) and ABO each supplied 12.5 and 25 g choline/d, respectively. On the last day of each period, milk samples were taken and 9 blood samples were collected simultaneously from an artery, portal and hepatic veins at 30-min intervals Plasma, milk and feed ingredients were analyzed for acetylcholine (ACho), betaine (Bet), free choline (Cho), glycerophosphocholine, lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphocholine (PCho) and sphingomyelin (SM) by hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC LC-MS/MS). The plasma concentrations of total choline and lipid soluble choline metabolites (LPC, PC and SM) were not affected by treatment at arterial and portal and hepatic veins. The plasma arterial concentrations

of the water soluble choline metabolites, Cho, Bet and PCho, were increased with ABO-L and ABO-H while PCho also increased RPC-H. The difference for Cho arterial concentrations increased almost 3 times with ABO-H compared to the control ($P<0.01$), while ABO-L or RPC treatments were not changed. There was a low level of total choline transferred into milk, 2.36% RPC-H and 5.12% ABO-H. For the increases in total choline moiety in milk, Cho and PCho represented 40% and 5%, respectively, for RPC-H and 60% and 24%, respectively for ABO-H. A multiple regression analysis indicated that yields of Bet and PCho in milk were associated ($R^2=0.76$; $P<0.01$) with the total amount of choline post-ruminal infused. The association we found in this study, although developed based on a small sample set of cows at the end of the lactation, suggest that the prediction of choline supply may be possible based on Bet and PCho yields in milk.

Keywords: Choline, bioavailability, dairy cow

Introduction

Choline is an essential nutrient routinely supplemented in monogastric (poultry and swine) to support growth, feed efficiency and reproduction (Sewell et al., 1966; Emmert et al. 1997); however there is no choline requirement established for dairy cows. Choline has two known functions: as choline *per se*, when the choline moiety is required, and as a methyl donor (Pinotti, 2002). Choline moiety is a constituent cell membrane as phospholipids, phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and sphingomyelin (SM). Particularly, PC is essential for the synthesis of VLDL which plays a major role in lipid transport and prevents fatty liver disease (Yao and Vance, 1988). The function of choline as a methyl donor is

from the oxidation to betaine, which supplies a methyl group for the synthesis of methionine (Pinotti et al., 2002)

Ruminants differ from monogastric as the majority of choline in feed ingredients and supplements like choline chloride are degraded in the rumen (NRC, 2001), therefore dietary choline make almost no contribution to the choline body pool. In the dairy cow, despite low choline dietary availability, the output of choline compounds in milk are high and precursors from the *de novo* pathway may be limiting, especially at the onset of lactation (Baldi and Pinotti, 2006). Indeed, more than 50% of high producing cows experience moderate to severe fatty liver disease at the onset of lactation (Bobe et al., 2004), which is a classic symptom of choline deficiency (Garrow, 2007) . Therefore, microencapsulated technology has been developed to protect choline from degradation in the rumen and deliver it to the small intestine. Commercially available rumen protected choline, containing 28–50% choline chloride, protects ca. 75% of the choline from rumen degradation in vitro (Deuchler et l., 1998; Elek et al., 2008; Brüsemeister and Suedekum, 2006). Studies of the effect of rumen protected choline (RPC) supplementation on the performance in dairy cows of during the transition period reduced triacylglycerol accumulation in the liver and increase milk yield and milk fat concentration (Pinotti et al., 2010, Zom et al., 2011). In addition, a study conducted in dairy cows from 25 days prepartum to 80 days postpartum reported that cows fed with RPC showed reduced incidence of clinical ketosis, mastitis, and morbidity (Lima et al., 2012). This is a specific role during the transition cow; however, cows have requirement for choline through lactation, particularly for lipid transport and milk fat secretion (Pinnotti et al., 2002)

Although RPC supplementation improvements on the performance have been well described in the ruminants, little is known about the effectiveness of RPC products. Evaluating

rumen protection can be relatively easily accomplished through incubating products in rumen fluid, and has been successfully applied to evaluate protection of various RPC products (Kung Jr et al., 2003, Elek et al., 2008) this approach will not give an indication of the bioavailability of choline in the animal. Deuchler et al. (1998) provides evidence that choline supplemented in rumen-protected form or post-ruminal supply was available for absorption and secreted as total choline in milk, however graded doses of choline post-ruminal infused resulted in a curvilinear response. Recently, a study in supplemented (750 mg choline/d) breast-feeding women, founde that the total intake of choline influenced the concentrations of choline metabolites in the breast milk and blood of lactating women (Fischer et al., 2010). Therefore, choline metabolites might be a better indicator of choline supply than the concentration of total choline in blood and milk.

The objectives of the current study were twofold. Firstly, we sought to determine how graded levels of choline chloride post-ruminal infused were absorbed across the intestine and metabolized by the liver. This is important as there is not a clear understanding of absorption of freely available choline in the small intestine of ruminants. Secondly, we sought to evaluate whether a marker for choline absorption can be obtained from easily available sampling approaches (i.e. choline metabolites in blood and milk). If a biomarker for absorbed choline can be established it will more readily allow bioavailability trials to be conducted to assess delivery efficacy of RPC products. In an attempt to shed light on choline absorption in dairy cows we determined the bioavailability of RPC in the intestine, and choline metabolism across the liver at different levels of choline supplementation.

Materials and Methods

Animals were cared for according to the recommended Code of Practice for the Care and Handling of Dairy Cattle and the guidelines of the Canadian Council on Animal Care (2009). Five lactating Holstein cows (206 ± 25 DIM and averaging 708 kg ± 89 kg of BW, mean ± SE, respectively) equipped with a rumen cannula and chronic indwelling catheters in the portal, hepatic and 2 mesenteric veins as well as in a mesenteric artery (Huntington et al., 1989) at least five months before the start of the experiment were used. The catheters were exteriorized individually over the paralumbar shelf and spine. The right carotid artery was surgically raised to a subcutaneous position to allow access to arterial blood if the catheter placed in a mesenteric artery failed. In instances where both arterial catheters failed, an auricular artery was used. The cows were randomly assigned to treatment in a 5×5 Latin square design, with 5-d treatment periods and a 2-d interval between periods. Treatments were 1) control (0 g/d choline), 2) RPC - low dose (RPC-L), 3) RPC - high dose (RPC-H), 4) post-ruminal infusion (ABO) – low dose (ABO-L), 5) ABO - high dose (ABO-H). The low and high doses of RPC (Reashure, Balchem Corporation, New Hampton, NY; containing 21.6% of choline) and ABO each supplied 12.5 and 25 g choline/d, respectively. Choline (aqueous choline chloride 70%; Balchem Corporation, New Hampton, NY; containing 52.3% of choline) was infused post-ruminally continuously over the 5 d, whilst the RPC treatments were fed 12x/d as a top dress. The choline chloride was dissolved in tap water and was infused continuously through a peristaltic pump in a daily volume of 6 L/cow (260 mL/h) for the duration of each experimental period.

Cows were housed in a tie-stall barn with free access to water under 16 h of light per day (0500 to 2200 h) and were fed in the morning 1 kg of long hay per day and a TMR (Table 4.1) served in 12 equal meals per day (2-h intervals) by automatic feeders (Ankom, Fairport, NY) at

95% of their voluntary feed intake measured the week before the start of the study. Feed intake and refusals, when present, were weighed and recorded daily. Cows were milked twice a daily (0700 and 1900 h) and yields at each milking were recorded. At the last day of each experimental period milk was collected at 2 consecutive milkings (pm, am) to determine fat and protein content (DHI agency, Valacta, St-Anne-de Bellevue, QC, Canada).

On the last day of each 5 d-period, blood samples (n = 9) were taken simultaneously from the 4 catheters every 30 min during 4 consecutive hours starting at the 0900 h meal. Immediately after collection, blood samples were put in Vacutainer tubes with EDTA (Becton Dickinson Inc., Franklin Lakes, NJ), the tubes were then placed on ice until centrifuged (2000 × g for 10 min at -4°C). Plasma was collected and stored at –80 °C until sample analysis.

Milk samples from the last day of each period were collected at 2 consecutive milkings (pm, am) and were composited proportional to milk yield at each milking prior to extraction for choline analysis.

Feed ingredients were sampled weekly and frozen at -20°C for chemical composition analyses (Table 4.1) and choline metabolites (Table 4.2). Samples were dried, ground and analyzed for DM, CP, ash, ether extract, ADF, NDF, and acid detergent lignin (Ankom200 fiber analyzer, Ankom Technology Corp., Fairport, NY) and cobalt (Agri-Food Laboratories, Guelph, ON, Canada).

Milk, plasma and feed samples were extracted (Bligh and Dyer, 1959) and analyzed for ACho, Bet, Cho, GPCho, LPC, PC, PCho and SM, using HILIC LC-MS/MS based on methods outlined by Zhao et al. (2011) as described by Artegoitia et al. (2014). The following standards were used: Cho chloride-trimethyl-d9 (Cho-d9, Cambridge Isotope Laboratories DLM 549-1, Tewksbury, MA), Bet (Bet-d11 Cambridge Isotope Laboratories DLM 407, Tewksbury, MA),

PC chloride (PCh-d9,C/D/N Isotopes ICD D-2135, QC, Canada) PtC-d62 (Cambridge Isotope Laboratories DLM 606-0.1, Tewksbury, MA), SM-d13-c13 (Ricerca-custom made, Painesville, OH) AC-d13(C/D/N Isotopes ICN D-1780, QC, Canada), LPC-D31 (Avanti polar lipids, 860397, Alabaster, AL).

Statistical analyses for arterial, portal and hepatic concentrations, veno-arterial differences were conducted on the average for the 9 sampling times. One cow did not have a catheter in hepatic vein throughout the experiment. In the last experimental period one cow lost the portal catheter (RPC-L). Average DMI and milk production during the last 2 d of each experimental period as well as milk composition were analyzed. All variables were analyzed using mixed procedure of SAS (SAS version 9.3 Institute Inc., Cary, NC, USA) according to the following model:

$$Y_{ijk} = \mu + C_i + P_j + S_k + \varepsilon_{ijk},$$

where Y_{ijk} is the studied variable, μ is the overall mean, C_i is the cow effect, P_j is the period effect, S_k is the choline treatment effect, and ε_{ijk} is the residual error. Period and choline treatment effects were analyzed as fixed effects and cow as a random effect. Means are reported as least squares means with their respective pooled standard errors and considered to differ when $P \leq 0.05$.

Variable selection of choline metabolites in milk was performed using the all possible methods approach and relationships between selected variables were ranked by R^2 using PROC REG. Finally, multiple regression analysis was conducted using PROC REG to assess the relationship between milk choline metabolites and ABO-treatment dose response.

Results

The total amount of choline in the TMR was 92.4 mg/100g calculated from the proportion of individual ingredients (Table 4.1 and Table 4.2). Dry matter intake also was not different across treatments and the total amount of choline fed was 19.46 ± 2.22 g/d.

Milk yield and milk composition were not affected by choline either supplemented or post-ruminal infused (Table 4.3, $P > 0.2$).

Plasma choline metabolite concentrations on artery and differences between portal and artery, and differences between hepatic and artery concentrations are reported in Table 4.4. The arterial concentrations and differences across portal-artery and hepatic-artery for total choline and the lipid soluble choline metabolites, LPC, PC and SM were not affected by treatment ($P \geq 0.06$) The arterial concentrations of water soluble choline metabolites, Cho, Bet and PCho were increased with ABO-L and ABO-H ($P<0.01$), while arterial PCho was also increased for RPC-H ($P<0.01$). The portal-arterial difference for Cho increased almost 3 times with ABO-H compared to the control ($P<0.01$), while ABO-L and RPC treatments were not different.

Although total choline yield in milk was not affected by treatment (Table 4.5), the yield of the choline moiety in milk increased by 0.59 g/d for RPC-H and 1.28 g/d for ABO-H, representing an increase of 15% and 33%, respectively ($p<0.01$; Table 4.6). Cho and Bet yield increased ($P < 0.01$) in a dose dependent manner with post-ruminal infusion by 74% and 171%, , with ABO-L and 146% and 278% with ABO-H, respectively. The amounts of Cho and Bet secreted in milk increased from 0.54 to 0.94 and 1.33 g/d and from 0.14 to 0.38 and 0.53 g/d, respectively for the low and high doses. An increase in the yields of ACho and PC were also observed ($P = 0.04$), although only with ABO-H. The other metabolites were not changed by ABO ($P > 0.12$), nor were any changes observed in secretion of any of the individual choline

metabolites with RPC. Multiple regression analysis found that the choline post-ruminal infusion could be explained to a large degree by milk PC and Bet yield ($P < 0.01$; $R^2 = 0.76$). The model obtained for ABO dose of choline in milk (γ; g/d) was

$$\gamma = -5.986 + 42.735 Betaine + 7.472 Phosphocholine$$

Where Betaine and Phosphocholine are the yield in milk (g/d); SEE 7.48 and 3.67, respectively

Discussion

In dairy cows, choline has been proposed as a limiting nutrient, primarily during the transition period (Erdman and Sharma, 1991, Pinotti et al., 2002). Higher choline availability (by feeding RPC) can have a favorable effect on the production performance at the onset of the lactation (Erdman and Sharma, 1991, Pinotti et al., 2003, Zom et al., 2011). Although evaluating rumen protection can be relatively easily accomplished through incubating products in rumen fluid, and has been successfully applied to evaluate protection of various products (Kung Jr et al., 2003, Elek et al., 2008), this approach will not give an indication of the bioavailability of choline in the animal. Indeed, low degradation rate in the rumen of RPC (~20%) does not guarantee post ruminal bioavailability. Therefore, characterizing the net flux of choline metabolites across the intestine and liver as the supply of choline is increased may form the basis to understand choline absorption and metabolism and improving the efficacy of the level of supplementation of RPC during lactation in dairy cows.

In plasma PC was the main choline metabolite (95%), similar to previous studies in ruminant (Robinson et al., 1984; Artegoitia et al., 2014) and non-ruminants (da Costa et al., 2005, Fischer et al., 2007, Fischer et al., 2010). Although PC concentration in plasma was not

affected by choline supplementation or infusion in our study, Cho and Bet arterial concentrations increased in plasma as the amount of Cho infused increased as well as PCho increased with RPC. The increase of plasma Cho concentrations on arterial as post-ruminal Cho infusion dose increased. Similar to our results, in breast feeding woman, dietary choline supplementation (750 mg/d) increased the concentrations Cho and Bet in plasma but not the concentrations of PC, SM while PCho was not measured (Fischer et al., 2010). Cho is transported by the portal blood to be metabolized mainly by the liver for biosynthesis of phospholipid or conversion to the methyl donor betaine (Zeisel et al., 1991). Upon entry into a cell, Cho is rapidly phosphorylated to PCho by choline kinase, a cytosolic enzyme that catalyzes the initial step of CDP-choline pathway. As intracellular concentrations of Cho increase, choline kinase becomes saturated and the majority of choline enters the mitochondria where it is converted to betaine. Thus, choline oxidation is considered as a "spillover" pathway under high choline supply (Caudill et al., 2012). Once formed, betaine may be used as a methyl donor for the conversion of homocysteine to methionine, which is the first limiting amino acid required for transmethylation reactions and milk protein synthesis in dairy ruminants (Lobley et al., 1996).

The main choline metabolites in milk in our study were PC (58%) and Cho (22%) across all treatments, respectively. This is consistent with previous research which reported similar proportions in bovine milk (Holmes-McNary et al., 1996, Artegoitia et al., 2014). All the concentrations and yields in milk of the water soluble choline metabolites and total choline in our results increased as choline dose infusion increased. In addition, the yield of total choline moiety increased 0.59 g/d with RPC-H and 1.28 g/d with ABO-H. Based on the increase in the choline moiety in with RPC-H and ABO-H versus the choline amount supplemented, the recovery of choline in milk was approximately 2.36% and 5.12 %, respectively. For the increase

in total choline moiety in milk, Cho and Pcho represented 40% and 5%, respectively, for RPC-H and 60% and 24%, respectively for ABO-H. Deuchler et al. (1998) measured total choline in cows' milk in mid-lactation; they found that in animals receiving post-ruminal choline infusion with 0, 19, 37, 56 g/d, the choline content of milk was linearly increased and the estimated transfer was 5.37%, 3.13% and 2.25% for post-ruminal infusion, respectively; however the supplementation of RPC did not change the total choline yield in milk. In addition, Pinotti et al. (2003) reported an increase of total choline in milk when cows were supplemented with 15 g/d of choline in RPC during -14d to 30 d relative to calving (control 2.53 g/d vs 3.81 g/d RPC), representing 8.5% transfer into milk. The basis for these differences in transfer of choline into milk is not clear, however, may related to choline dose, lactation stage, length of supplementation and effectiveness of delivery. A multiple regression analysis indicated that concentrations of Bet and Pcho in milk were moderate associated with the total amount of choline post-ruminal infused. The association found in this study, although developed based on a small sample set of cows at the end of the lactation, suggests that the prediction of choline supply may be possible based on Bet and Pcho yields in milk.

In conclusion, the present study measured the changes of choline metabolites across intestine and metabolized by the liver with graded levels of choline chloride post-ruminal infused or RPC supplemented. The concentrations of total choline or lipid soluble choline metabolites, LPC, PC and SM were not affected by treatment at arterial, or across portal or hepatic veins. The concentrations of water soluble choline metabolites, Cho and Bet arterial concentrations were increased with ABO-L and ABO-H while PCho arterial concentrations increased with both ABO-L and ABO-H as well as and RPC-H dose treatment. The portal-arterial difference for Cho increased almost 3 times with ABO-H compared with control ($P<0.01$), while ABO-L or RPC

treatments were not changed. Although lipid soluble metabolites or total choline in plasma was not affected by treatment, a low amount of total choline was transferred into milk with RPC-H (2.36%) and with ABO-H (5.12%). Moreover, multiple regression analysis may suggest that concentrations of Bet and Pcho (R^2=0.72, P<0.01) in milk are more sensitive biomarkers for predicting absorption of choline.

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Appendix

Table 4.1 Ingredients and chemical composition of the total mixed ration[1]

Ingredient, % DM basis	Value
Soybean hulls	5.8
Corn silage[2]	29.9
Grass silage[3]	18.2
Corn	18.1
Canola meal	21.9
Beet pulp	2.0
Mineral and vitamin supplement[4]	1.8
Calcium Carbonate	0.3
Urea	0.2
Megalac®[5]	1.7
Nutrient composition	
DM, %	50.0
CP, % of DM	15.1
RDP, % of CP	49.4
Soluble protein, % of CP	31.1
ADF, % of DM	18.9
NDF, % of DM	32.6
NE_L, Mcal/d	1.59
Total choline mg/100g	92.4

[1]All cows were served daily 1 kg of long hay (85% DM) on a DM basis, 10.5% CP; 2.5% soluble protein; 30.6% ADF; 54.4% NDF
[2]8.6% CP; 3.4% soluble protein; 21.7% ADF; 40.0% NDF
[3]16.7% CP; 7.7% soluble protein; 31.2% ADF; 47.6% NDF
[4] Calcium salts of long-chain fatty acids 85%

Table 4.2 Choline and choline metabolite concentrations in feed ingredients in the total mixed ration.

Metabolite[1], mg/100 g	Feed ingredient							
	Beet	Canola	Corn	Hay grass	Megalac	Corn silage	Grass silage	Soybean hulls
ACho	0.0003	0.012	0.0036	0.098	0.796	0.006	0.003	0.003
Bet	43.5	3.5	1.3	11.9	6.3	19.3	7.7	4.7
Cho	3.1	72.2	8.9	38.5	92.9	37.4	55.7	16.8
LPC	8.8	42.5	2.7	0.5	10.6	0.5	0.4	2.6
PC	102.6	98.5	28.9	2.6	91.2	0.2	0.7	22.3
PCho	0.9	0.9	0.5	0.3	nd	0.1	0.2	0.6
SM	0.04	0.02	0.01	0.02	1.6	0.01	0.03	0.1
GPCho	0.5	24.4	10.6	1.0	0.1	0.6	0.1	7.1
TC[2]	115.9	238.6	51.6	43.0	197.1	38.9	57.1	49.5

[1] Acetylcholine (Acho) Betaine (Bet), free choline (Cho), phosphocholine (PC), glycerophosphocholine (GPCho), lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylcholine (PC)

[2] Total choline (TC) is the sum of Cho, LPC, PCho, PC, SM and GPC.

Table 4.3 Milk yield and composition and DMI of dairy cows during experimental period.

	Treatments*						
	CONTROL	RPC-L	RPC-H	ABO-L	ABO-H	SEM	*P*
DMI, kg/d[1]	20.6	21.8	20.9	20.8	21.2	1.2	0.50
Milk, kg/d	32.3	33.3	31.3	32.7	33.7	1.16	0.46
Milk protein							
%	3.46	3.47	3.42	3.42	3.39	0.09	0.20
g/d	1118	1150	1070	1122	1140	46	0.49
Milk fat							
%	4.04	4.03	4.05	3.99	3.96	0.10	0.58
g/d	1306	1338	1268	1326	1334	53	0.68

* Treatments: RPC-L= rumen protected choline supplemented at 12.5g/d choline; RPC-H= rumen protected choline supplemented at 25g/d choline; ABO-L=abomasal infused at 12.5g/d choline; ABO-H=abomasal infused at 25g/d choline;

[1]Dry matter intake

Table 4.4 Plasma choline metabolites (μmol/l) when cows were abomasal infused unprotected choline or supplemented rumen protected choline at different levels[1]

	Treatments*						
	CONTROL	RPC-L	RPC-H	ABO-L	ABO-H	SEM	*P*
Cho							
Artery	4.38^{c}	4.86^{bc}	5.64^{bc}	7.58^{b}	13.0^{a}	0.92	<0.01
Portal-Artery[3]	1.82^{b}	1.83^{b}	1.40^{b}	2.96^{b}	5.24^{a}	0.75	0.01
Hepatic-Artery[4]	-0.95	-0.91	-0.50	1.05	-0.81	0.70	0.31
Bet							
Artery	30.57^{c}	42.39^{c}	37.40^{c}	118.3^{b}	152.2^{a}	7.23	<0.01
Portal-Artery[3]	0.10	-1.49	-1.22	-0.72	-1.38	1.86	0.95
Hepatic-Artery[4]	0.27	0.14	-0.67	8.97	4.31	3.57	0.33
LPC							
Artery	170.4	209.0	217.6	203.5	182.0	15.15	0.11
Portal-Artery	-7.76	-24.5	-8.35	4.78	-13.31	12.17	0.59
Hepatic-Artery	8.34	-13.7	12.5	3.19	3.71	11.3	0.57
PCho							
Artery	3.49^{c}	3.74^{bc}	4.16^{b}	4.30^{b}	4.89^{a}	0.25	<0.01
Portal-Artery[3]	-0.23	0.84	-0.006	-0.02	-0.03	0.28	0.15
Hepatic-Artery[4]	0.17	-0.07	0.19	-0.30	0.14	0.25	0.61
PC							
Artery	7831	9139	9775	8507	7280	668	0.06
Portal-Artery[3]	889.0	-1867	-161.4	-678.5	-547.4	1005	0.48
Hepatic-Artery[4]	1766	-521	-757	-1055	904	1123	0.29
SM							
Artery	247.6	241.9	288.5	250.5	266.4	15.6	0.28
Portal-Artery[3]	1.94	-12.22	-19.55	-3.81	-17.26	12.53	0.74
Hepatic-Artery[4]	25.1	20.89	1.39	-4.81	-14.49	13.89	0.30
TC[2]							
Artery	8228	9606	10291	8959	7747	677	0.06
Portal-Artery[3]	896.8	-1906	-187.9	-680.2	-573.8	1013	0.47
Hepatic-Artery[4]	1823	-517	-738	-1058	890	1137	0.40

* Treatments: RPC-L= rumen protected choline supplemented at 12.5g/d choline; RPC-H= rumen protected choline supplemented at 25g/d choline; ABO-L=abomasal infused at 12.5g/d choline; ABO-H=abomasal infused at 25g/d choline;

[1] Data are presented as LSM ± SEM of Betaine (Bet), free choline (Cho), phosphocholine (PCho), lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylcholine (PC)

[2] Total choline (TC) is the sum of Cho, LPC, PtC, PC and SM

[3] Portal-Artery in plasma is calculated as the differences between the concentration of the choline metabolites in portal and artery

[4] Hepatic-Artery in plasma is calculated as the differences between the concentration of the choline metabolites in hepatic and artery

Table 4.5 Milk choline metabolite yields (g/d) when cows were abomasal infused unprotected choline or supplemented rumen protected choline at different levels

Metabolite[1] (g/d)	Treatments*					SEM	*P*
	CONTROL	RPC-L	RPC-H	ABO-L	ABO-H		
ACho	0.01^{b}	0.01^{b}	0.02^{ab}	0.02^{ab}	0.02^{a}	0.002	0.04
Bet	0.14^{c}	0.16^{c}	0.25^{c}	0.38^{b}	0.53^{a}	0.05	<0.01
Cho	0.54^{c}	0.62^{bc}	0.79^{bc}	0.94^{b}	1.33^{a}	0.14	<0.01
LPC	0.11	0.09	0.13	0.11	0.12	0.01	0.54
PC	17.05	15.95	18.36	15.95	17.55	1.19	0.22
PCho	0.34^{b}	0.24^{b}	0.37^{b}	0.36^{b}	0.65^{a}	0.16	0.04
SM	0.93	0.87	0.99	0.83	0.89	0.08	0.21
GPCho	0.94	1.11	1.11	1.30	1.09	0.22	0.22
TC[2]	19.94	18.89	21.76	19.57	21.64	1.33	0.15
TCM[3]	3.84^{c}	3.78^{c}	4.43^{b}	4.33^{bc}	5.12^{a}	0.23	<0.01

*Treatments: RPC-L= rumen protected choline supplemented at 12.5g/d choline; RPC-H= rumen protected choline supplemented at 25g/d choline; ABO-L=abomasal infused at 12.5g/d choline; ABO-H=abomasal infused at 25g/d choline;

[1] Data are presented as LSM ± SEM. of Acetylcholine (Acho) Betaine (Bet), free choline (Cho), phosphocholine (PCho), glycerophosphocholine (GPC), lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylcholine (PC)

[2] Total choline (TC) is the sum of Cho, LPC, PCho, PC, SM and GPCho.

[3] TCM= Total choline moiety (g/d)

Table 4.6 Milk choline metabolite concentrations (µmol/l) when cows were abomasal infused unprotected choline or supplemented rumen protected choline at different levels

	Treatments*						
Metabolite[1]	CONTROL	RPC-L	RPC-H	ABO-L	ABO-H	SEM	*P*
ACho	3.20^{bc}	3.15^{c}	3.55^{abc}	4.00^{ab}	4.32^{a}	0.35	0.04
Bet	38.86^{c}	42.32^{c}	60.73^{c}	100.14^{b}	137.15^{a}	13.40	<0.01
Cho	159.97^{c}	188.66^{bc}	222.75^{bc}	279.28^{b}	388.01^{a}	37.69	<0.01
LPC	6.36	5.96	7.29	6.56	7.12	0.77	0.72
PC	696.97	669.27	667.34	654.26	697.92	47.28	0.84
PCho	54.84^{b}	41.25^{b}	59.14^{b}	62.47^{b}	106.94^{a}	25.59	0.03
SM	40.95	39.64	42.00	36.98	38.98	2.97	0.30
GPCho	116.87	140.22	130.20	161.72	128.65	27.65	0.12
TC[2]	1079.16^{c}	1088.15^{c}	1132.27^{bc}	1205.57^{b}	1371.94^{a}	53.34	<0.01

* Treatments: RPC-L= rumen protected choline supplemented at 12.5g/d choline; RPC-H= rumen protected choline supplemented at 25g/d choline; ABO-L=abomasal infused at 12.5g/d choline; ABO-H=abomasal infused at 25g/d choline;

[1] Data are presented as LSM ± SEM. of Acetylcholine (Acho) Betaine (Bet), free choline (Cho), phosphocholine (PCho), glycerophosphocholine (GPC), lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylcholine (PC)

[2] Total choline (TC) is the sum of Cho, LPC, PCho, PC, SM and GPCho.

CHAPTER 5: CONCLUSIONS

Improving the absorption and utilization of vitamin B_{12} and choline with different delivery methods, ultimately can enhance the productive performance in the lactating dairy cows.

The overall goal of this research was to gain information on choline absorption and improve the efficacy of the level of choline and vitamin B_{12} supplementation during lactation in dairy cow. In the present research, I studied the absorption and utilization of different delivery systems for choline and vitamin B_{12} for the lactating dairy cow as well as the physiological variation of choline metabolites during lactation. *In vivo* measurements with multi-catheterized cows provided the net fluxes of choline and vitamin B_{12} across the small intestine and liver. Therefore, this information allowed determining absorption of choline and vitamin B_{12} in the intestine with different delivery systems as well as the metabolism across the liver at different level of choline supplementation.

The dietary supplementation of vitamin B_{12} is costly whereas systematic use of intramuscular injections of the vitamin is not practical under commercial conditions. Casein and whey proteins as delivery methods for cyanocobalamin (CN-CBL) in solution to increase intestinal absorption of vitamin B12 were studied (Chapter 2). Only 0.74% of the total amount of vitamin B_{12} infused post-ruminally reached the portal blood within the first 45 min and there was no further release during the rest of the sampling period (24 h) after the bolus. The dose of 100 mg of CN-CBL infused post-ruminally might not be sufficient to reach similar net release of vitamin B_{12} across intestine The intestinal absorption of CN-CBL was greater when the vitamin was given in solution with casein (4 μg/h) compared with CN-CBL given alone or with whey protein (-25 μg/h and -19 μg/h, respectively). The present results suggest that CN-CBL given with casein increased vitamin B_{12} absorption as compared to CN-CBL given alone. Therefore, dietary formulation of CN-CBL with casein may improve CN-CBL absorption in dairy cows.

Milk and dairy products are an important source of choline, an essential nutrient for human health. In addition, choline is found in various water- and lipid-soluble forms in milk, which are different in their absorption, transport and metabolism. Currently, there is scarce information about the factors that influence the concentrations of choline metabolites in milk. Therefore, choline and its metabolites in blood and milk through the first 37 weeks of lactation in the dairy cow were characterized (Chapter 3). The total choline in plasma increased over 10-fold from early to late lactation, with the majority of the increase coming from elevated PC levels which might be associated with the changes in types and levels of lipoproteins in plasma that occur during lactation. The concentration of the choline moiety in milk decreased from early (8 mg/100 g) to mid-lactation (5 mg/100 g) before rising again in late lactation (11 mg/100g); however, throughout lactation milk choline remained below the levels reported previously in commercial milk (16 mg/100 g). This large difference in choline levels in milk needs further evaluation to ensure that choline intake meets requirements to the human population. In milk phosphocholine was the main metabolite in early lactation and decreased rapidly over the first weeks of lactation. In contrast, phosphatidylcholine in milk was lowest in early lactation and increased steadily through lactation, with this change positively correlated with phosphatidylcholine in plasma. Also, we found that production variables (milk yield and milk fat and protein content) along with week of lactation were correlated with total milk choline yield, suggesting that it may be possible to predict milk choline output from easily measureable variables on the farm.

In dairy cows, supplementation of rumen protected choline (RPC) can improve the performance during the transition period. Although evaluating rumen protection can be relatively easily accomplished through incubating products in rumen fluid, this approach will not give an

indication of the bioavailability of choline in the animal. Therefore, choline and its metabolites were measured across the intestine and liver and ultimate in the milk, when low (L) and high (H) graded levels of choline were post-ruminal infused (ABO) or supplemented with RPC (Chapter 4). Notwithstanding plasma arterial total choline and lipid soluble choline metabolite concentrations were not affected by treatment, choline, betaine and phosphocholine concentrations increased with choline post-ruminal infusion as well as phosphocholine with RPC. The total choline moiety increased 0.59 g/d with RPC-H and 1.28 g/d with ABO-H. Based on the increase in milk total choline with RPC-H and ABO-H, the transfer of choline into milk was approximately 2.36 % and 5.12 %, respectively. For the increase in total choline moiety in milk, choline and phosphocholine represented 40% and 5%, respectively, for RPC-H and 60% and 24%, respectively, for ABO-H. The low amount of choline transfer into milk are not well understand and might be related to choline dose, lactation stage, length of supplementation and effectiveness of delivery. A multiple regression analysis indicated that concentrations of betaine and phosphocholine in milk were moderate associated with the total amount of choline post-ruminal infused. The correlation found in this study, although developed based on a small sample set of cows at the end of the lactation, suggest that the prediction of the level of choline supplementation may be possible based on betaine and phosphocholine yields in milk.

This information can be utilized by the feed industry to develop improved formulation to increase absorption of choline and vitamin B12, which ultimately can be used to nutritionist for management to improve the performance in dairy cows.

VITA

Virginia M. Artegoitia was born in Montevideo, Uruguay. She enrolled the Veterinary School in 1998 at the University of Uruguay. During her first year at the University she stayed with families of dairy farmers and she learned the importance of efficient animal production. She was interested in several academic areas, but dairy production was her main interest, and she attended 150 hrs. of technical practice on dairy farms. She began performing voluntary work as a veterinary assistant for dairy farmers for four years. Furthermore, she started teaching in theoretical-practical courses of Dairy Production. These activities helped her to develop many key skills such as organization, leadership and teamwork. She developed her Veterinary thesis degree on "Teat condition and morphology in relation to udder health" which was published in the Journal of Veterinary Medicine Society in Uruguay. She feels research was the aspect of her profession that provides her with the most stimulation and personal satisfaction. For this reason, after she graduated, she began to work with two research teams "Animal Welfare in the productivity of dairy cattle" and "Milk Quality" at the University of Uruguay. She received a scholarship support to develop her MSc degree at Uruguay. In 2008 she obtained an internship for six month at the University of Minnesota, and worked in the laboratory of Dairy Nutrition and Physiology of Animal Science. Therefore, she learned different techniques to measure milk casein and fatty acid fraction and had the opportunity to work on Biochemistry and Nutrition Lab at Agronomy School, and in Technological Laboratory of Uruguay. As a result of this experience, she graduated with honors and published on peer-reviewed articles. She participated in conferences and presented her work on dairy physiology and nutrition to improve cow's health and welfare considering the importance of quality of the milk for human consumption. In 2011

she came at the University of Tennessee as a visiting scholar and began to work with Dr. Federico Harte. After six month later she enrolled as a PhD student. During the course of her PhD, she approached Dr. Michael de Veth, who guided her in a collaborative research (multidisciplinary and cross institutional settings) with Department of Animal Science and Department of Chemistry as well as Agriculture-Agri Food Canada. She very much enjoys this multidisciplinary approach as she believes adds relevance and meaning of her work (personally and professionally). She hope in the near future to advise graduate students following the legacy of her mentors, she is looking forward to participating in and establishing a community of scholars.