Effect of Betaine Supplementation on Total Tract Digestibility, Ruminal Fermentation Measures, and Production Performance of Dairy Cows

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Hao-Che Hung

Major Professor: Pedram Rezamand, Ph.D.

Committee Members: Gwinyai Chibisa, Ph.D.; Mireille Chahine, Ph.D.; Kris Johnson, Ph.D.

Department Administrator: Amin Ahmadzadeh, Ph.D.

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Authorization to Submit Thesis

This thesis of Hao-Che Hung, submitted for the degree of Master of Science with a major in Animal Science and titled "Effect of Betaine Supplementation on Total Tract Digestibility, Ruminal Fermentation Measures, and Production Performance of Dairy Cows," has been reviewed in final form. Permission, as indicated by the signatures and dates given below, is now granted to submit final copies to the College of Graduate Studies for approval.

Major Professor:_____ Date:_____

Pedram Rezamand, Ph.D.

Committee

Members: _____ Date:_____

Gwinyai Chibisa, Ph.D.

_____ Date:_____

Mireille Chahine, Ph.D.

_____ Date:_____

Kris Johnson, Ph.D.

Department

Administrator: _____ Date:_____

Amin Ahmadzadeh, Ph.D.

Abstract

Betaine, also called trimethylglycine, is either produced endogenously by choline oxidation or found naturally in wheat or sugar beets. We hypothesized that betaine supplementation improves the total tract nutrient digestibility, production performance, and changes the ruminal fermentation measures in mid-lactation dairy cows. In experiment I, there were twenty-one mid-lactation dairy cows assigned to a 3 × 3 Latin square design with three periods of 28 d each and three treatments of betaine (0, 100, and 200 g/d). Milk yield and feed intake for each cow was recorded daily. During d 21 to 28, cows were fed with chromic oxide (15 g/d per cow). On d 26 to 28, fecal samples were collected and analyzed for chromic oxide by inductively coupled plasma – atomic emission spectrometry to determine digestibility. Milk samples were collected on d 21 and d 28 for components analysis and fatty acids profile via gas chromatography. In experiment II, three ruminal-cannulated Holstein dairy cows were used in a 3 × 3 Latin square design with three periods, 8 d each (1 d for in situ bags introduction and ruminal fluid collection, and 7 d for wash-out), and three treatments of betaine at 0, 100, and 200 g placed into Dacron bags and double sealed. Ruminal fluid was collected, and large mesh bags containing Dacron bags were introduced into the rumen at different time points (0, 1, 1.5, 2, 3, 6, 12, and 24 h). At each time point, three bags of 100 g and three bags of 200 g betaine were prepared. Dry and organic matter degradation was determined on dried, post in situ sample bags. Ruminal fluid samples from different time-points were analyzed for VFA profile using gas chromatography. Data were analyzed using the Proc Mixed of SAS with significance declared at $P \leq 0.05$ and trends at $0.05 < P < 0.1$. In experiment I, the apparent total tract digestibility of dry matter tended to increase by supplementing 100 g betaine as compared

with that with no dietary betaine (0.61 vs 0.58 ± 0.01; $P = 0.1$). Milk fat percent (3.16 vs 3.36 ± 0.08%, for 0 g and 200 g betaine/d, respectively) and C20:1 (0.005 vs 0.004 ± 0.0006%, for 100 g and 200 g betaine/d, respectively) tended ($P = 0.1$) to differ among treatments. Dry matter intake (25.4, 25.4, 25.4 ± 0.11 kg/d), milk yield (29.7, 29.3, and 30.0 ± 0.7 kg/d), and energy-corrected milk yield (28.5, 28.1, and 29.0 ± 0.8 kg/d) did not differ among treatments (0, 100, and 200g betaine/d per cow, respectively). In experiment II, there were increases in the ratio of acetate to propionate with betaine supplementation (1.7, 1.9, and 1.8 ± 0.03, for 0, 100, and 200 g betaine, respectively; $P < 0.0001$) and molar proportion of isovalerate with betaine supplementation ($P < 0.005$). There were decreases ($P < 0.001$) in pH (6.4, 6.3, and 6.2 for 0, 100, and 200 g betaine, respectively) and molar proportions of propionate, butyrate, and isobutyrate with betaine supplementation. There were no detectable changes in the molar proportion of acetate (0.42, 0.43, and 0.42 ± 0.006) or valerate (0.05, 0.04, and 0.05 ± 0.005). Overall, the result showed that betaine supplementation tended to affect the total tract digestibility of dry matter and milk fat of mid-lactation dairy cows whereas no effect was observed in production measures tested. Furthermore, changes in volatile fatty acid profile observed suggest the possibility of an effect of betaine supplementation on ruminal microbial populations, which warrants further investigation.

Key words: betaine, production performance, ruminal fermentation measures, digestibility

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Dedication

This thesis is dedicated to the memory of my father,

Bill Shou-Pen Hung

who gave me the cosmic courage to face countless challenges

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Introduction

The vast majority of income for a dairy farm is from milk produced, and milk fat is one of the factors determining the unit price of milk. The main cost of dairy operation is feed, which has a significant effect on milk yield and milk fat. It is crucial to improve milk production and diminish feed expenditure to maximize profit for dairy practice. Furthermore, utilizing feeds that can increase the efficiency of productivity is crucial for dairy farm management.

There are several feed ingredients that can increase the efficiency of productivity. For example, betaine, also called trimethylglycine (de Zwart et al., 2003), can be either found in wheat and sugar beets or produced endogenously by choline oxidation. Various functions of betaine have been described including osmolyte, methyl donor (Eklund et al., 2005), being fermented to acetate in the rumen (Mitchell et al., 1979) and being used possibly for *de novo* fatty acid synthesis (Fernandez et al., 2004a).

In meat production, 'carcass modifier' is extensively used to describe betaine because of its lipotropic and growth-promoting effects. In dairy production, supplementing betaine bears multiple benefits under thermoneutral environment (Hall et al., 2016). These include increased milk production (Wang et al., 2010; Peterson et al., 2012), increased volatile fatty acids (VFA) production, and greater fat-corrected milk (FCM) yield (Wang et al., 2010). Nonetheless, Davidson et al. (2008) observed no beneficial effect of rumen-protected betaine supplementation. Ruminal microbial utilization and degradation may block betaine's access to the ruminal epithelium, other nondigestive tract cell types, and the duodenum. However, Nakai et al. (2013) stated that dietary betaine was found in the duodenum, indicating that some betaine escapes ruminal degradation.

Collectively, betaine supplementation may increase milk production, milk fat, and VFA. Further studies need to determine the effects of betaine on the ruminal environment.

Chapter 1: Literature Review

1.1 Dairy Industry

Dairy farming is a crucial agriculture industry. Nonetheless, milk production of the dairy farm has always been facing various challenges including economic, social, and environmental. Hence, dairy farmers have constant need to improve their management practices to meet the rising demands of milk supply (Risco and Melendez et al., 2011). One area that dairy farmers focus on is increasing milk yield while keeping cows healthy. If farmers only pursue greater milk yield and neglect health condition of the animals, animals may experience disorders that are associated with imbalanced energy metabolism, such as lameness, abomasum displacement, and ketosis. Ultimately, the diseases may lead to failure of dairy farm management.

There are two main aspects that farmers need to investigate, which consist of the housing system and animal nutrition management. Animal housing systems should be designed based on the natural behavior of dairy cows to make them comfortable. The major factor is animal nutrition, diets with a significant effect on milk production, which determines the income of dairy farm. Consequently, one of the most important tasks for dairy farmers is implementating feeding practices that can keep animals healthy and gain the maximum profit with the minimum feeding input. The mechanism of milk synthesis and potential feeds that can increase nutrient utilization are two aspects that need to be discussed to enhance most milk yield.

1.2 Milk Synthesis

Synthesizing milk is crucial for mammals because it enables the newborn to survive. Utilizing nutrients to produce milk creates a significant burden on the dam. Hence,

there are a tremendous amount of physiological modifications and alterations in the metabolism of many tissues when the dam is preparing for lactation. Milk synthesis involves many complex mechanisms.

1.2.1 Mammary gland cytology

The basic unit secreting milk in the mammary gland is the alveolus, which is lined by epithelial cells. Epithelial cells are responsible for taking nutrients from the blood to synthesize milk; therefore, the number of epithelial cell in the alveolus, and the volume of blood supply are the two factors that determine the amount of milk synthesized. The epithelial cell synthesizes lactose, protein, and lipid into milk via various organelles inside the epithelial cell (Akers, 2002).

Mitochondria plays a key role in the cellular activity, and there is an increase in mitochondrial size, activity, and numbers during parturition. One of the functions of the mitochondria includes oxidative phosphorylation generating adenosine triphosphate as an energy source. The endoplasmic reticulum (ER) also involves in cellular function, which is a cell membrane component interacting with a nucleus in the cell. Endoplasmic reticulum can synthesize membrane, protein, caseins, and enzyme for lipid and lactose synthesis (Akers, 2002).

Golgi apparatus is a cellular membrane component communicating with ER and the location of lactose synthesis. Golgi apparatus, rough endoplasmic reticulum (RER), and mitochondria are more metabolically active during lactation than during the dry period. There may be an interaction between RER and Golgi apparatus (Kuhn, and White, 1975) to synthesize milk because of lactalbumin, which is synthesized in RER and lactose synthetase in the Golgi apparatus (Akers, 2002).

1.2.2 Mammary gland metabolism

The mammary gland creates a massive physiological demand on the animal. Furthermore, there is a high priority of utilizing nutrients for the mammary gland, even at the cost of the health of the dam. Failure to accommodate the physiological demand will lead to metabolic disorders, such as milk fever and ketosis.

There are a plethora of metabolites used by the mammary gland including glucose (from propionate), acetate, butyrate, triglycerides (TAG), and amino acids. Acetate, propionate, and butyrate are VFA formed from the microbial fermentation of carbohydrates in the rumen. Metabolites are absorbed through ruminal blood vassel to the liver, if the metabolites pass through rumen, they will be absorbed via the abomasums and intestine (Akers, 2002).

When propionate is produced in the rumen, it is transferred to the liver where it will be used to synthesize glucose (gluconeogenesis), which will be utilized mainly for lactose synthesis in the mammary gland. In the liver, glucose will be transformed into glycogen (glycogenesis), amino acids, and TAG. Between 60 and 85 % of blood glucose is taken up by the mammary gland for lactose synthesis (Schwendel et al., 2015). Thus, to have high milk yield, high blood glucose is necessary. Part of glucose will be transformed into uridine diphosphate galactose then lactose synthetase located in the Golgi apparatus will combine uridine diphosphate galactose and glucose to synthesize lactose. The vast majority of milk protein is synthesized from free amino acids. The main type of milk proteins include caseins, β-lactoglobulin, and α-lactalbumin (Schwendel et al., 2015).

The plasma solutes can affect water transferring from plasma to milk. The four major milk solutes are lactose, proteins (caseins, blood proteins), ions (sodium, potassium,

chlorine), and minerals (calcium, phosphorus). Lactose, calcium, and phosphorus do not diffuse into milk directly; they are transported into milk by the cell membrane and Golgi apparatus. Lactose is the main osmolyte that drives water into milk (Schwendel et al., 2015), so when lactose is packaged by Golgi apparatus to form vacuole, which transport nutrient to the alveolar lumen, the ions will follow the concentration gradient into the vacuole or alveolar lumen (Akers, 2002).

1.3 Milk Fat Synthesis

The milk fat content of a dairy cow is approximately 4% (Ma and Cori, 2012). The major form of milk fat is TAG (95%). *De novo* synthesis and free fatty acids (FA) from the diet or body fat lipolysis are the two major sources of fat that ends up in milk (Ma and Cori, 2012).

Approximately 70% of FA in milk fat are saturated because of hydrogenation of dietary polyunsaturated FA by the ruminal microorganisms. The most abundant saturated FA is palmitic acid (16:0) accounting for around 30% of the total FA, followed by myristic acid (14:0) and stearic acid (18:0) (MacGibbon and Taylor, 2006). Aside from saturated FA, there are also unsaturated FA containing one or multiple double bonds in milk fat. Among monounsaturated FA accounting for nearly 25% of the milk, the most abundant is oleic acid (18:1). Within polyunsaturated FA, the most abundant are linoleic acid (18:2) and α-linolenic acid (18:3) (Ma and Cori, 2012). The *trans*-FA containing one or more *trans* double bonds comprise around 2.7% of the FA in milk, and vaccenic acid (*trans*-11 18:1) is the major *trans* 18:1 isomer (MacGibbon and Taylor, 2006; Precht and Molkentin, 1995). Among conjugated linoleic acids in milk, the *cis*-9, *trans*-11 isomer is the primary form (75 to 90%; Precht and Molkentin, 1995). The major source of short-chain (4 to 8 carbons) and

medium-chain (10-14 carbons) FA are *de novo* synthesized in the mammary gland, whereas the origin of long-chain FA (> 16 carbons) is the uptake from circulation. Both *de novo* synthesis in the mammary gland and uptake from circulation are the sources of FA with 16 carbons (Bauman and Griinari, 2003).

1.3.1 *De novo* synthesis

In ruminants, acetate and β-hydroxybutyrate are the two precursors used for *de novo* FA synthesis. Acetate originates from ruminal carbohydrates fermentation and β-hydroxybutyrate is a product of butyrate metabolism by ruminal epithelial cells. The mammary epithelial cells synthesize short and medium chain FA. Inside the mammary epithelial cell, acetate is converted to acetyl-CoA, then acetyl-CoA will be converted to malonyl-CoA. The step that acetyl-CoA is converted into malonyl-CoA is a rate-limiting step (Kim et al., 1997) catalyzed by acetyl-CoA carboxylase. Once malonyl-CoA is formed, the two carbons originating from malonyl-CoA are added to a growing fatty acyl chain (Smith, 1994). Two molecules of reducing equivalents (nicotinamide adenine dinucleotide phosphate, NADPH) are required for each cycle (Neville and Piccanio, 1997). The termination of fatty acyl synthesis up to 16 carbons needs the involvement of transacylase (Knudsen and Grunnet, 1982).

1.3.2 Preformed fatty acid

The main sources of fat in milk is circulating FA originating from absorption of dietary lipids and the mobilization of body fat. In ruminants, dietary and microbial FA absorbed from the digestive tract are the primary origin of preformed FA in milk fat taken up from circulation. Normally, the FA in milk fat that are from the lipolysis of body fat are less than 10%. However, the percentage of mobilized FA will increase when cows are in a

negative energy balance (NEB; Bauman and Griinari, 2003). Because dietary TAG are hydrophobic, they have to be packaged in lipoproteins to be transported in blood. Lipoprotein lipase, which is on the capillary wall, can breakdown TAG and chylomicrons into non-esterified FA (NEFA) and glycerol. How FA cross the capillary endothelium and interstitial space is not known (Abumrad et al., 1998). The membrane transfer of FA may be a facilitated procedure (Abumrad et al., 1998). It is possible that cluster of differentiation 36 and FA binding protein 3 are involved in FA transportation because the most abundant FA binding protein isomer in bovine mammary gland is FA binding protein 3 and cluster of differentiation 36 is the homolog of FA translocator (Bionaz and Loor, 2008). In the mammary epithelial cell, FA are activated by CoA esters and directed to TAG synthesis.

1.3.3 Triglyceride synthesis

Triglycerides synthesis occurs in the ER in mammary epithelial cells. FA are esterified to the sn-1, -2, and -3 position of the glycerol backbone to produce TAG. The pathway that FA are esterified to form TAG is called glycerol-3-pathway (Bauman and Griinari, 2003). TAG droplets in the cytoplasm are secreted from the apical surface into the alveolar lumen covered with protein and cellular membrane (Keenan and Mather, 2006).

1.4 Fatty Acids Metabolism in the Liver

Non-esterified FA, from lipolysis of adipose tissue and the uptake of remnant lipoprotein particles, is the major source of FA entering the liver. Esterification to CoA activates the FA taken up into the hepatocytes. Entering the mitochondria for oxidation or being a substrate for glycerolipid synthesis (triacylglycerol and phospholipids) are the two pathways for the long-chain acyl-CoA in the liver. The enzyme carnitine palmitoyl transferase 1 (CPT 1), expressed in the outer mitochondrial can control FA entering the

mitochondria. Hence it is a key regulation that CPT 1 can determine the pathway of the long-chain acyl-CoA in the liver (Drackley et al., 2006).

Cytosolic Malonyl-CoA (an intermediate in FA synthesis) can hinder CPT 1 effectively. High blood insulin concentration with glucose readily available result in a greater degree of FA synthesis rather than oxidation. Thus the concentration of Malonyl-CoA is high. During NEB, insulin levels are low, so FA oxidation is favored (Frayn et al., 2006). The cytosolic triacylglycerol pool within the hepatocyte is the precursor for very low density lipoprotein (VLDL)-triacylglycerol. Lipolysis of this cytosolic pool is necessary to generate FA that are then esterified to make new triacylglycerol within the ER, where VLDL particles are assembled (Frayn et al., 2006). Very low density lipoprotein is the main way to export triacylglycerol. Packaging triacylglycerol into VLDL requires phospholipids.

1.5 Metabolic Disorder of Fatty Acid

Negative energy balance is a challenge that occurs around calving and the onset of lactation. To deal with NEB, the cow will use its stored fat to produce energy; this process is called lipid mobilization, which leads to an elevated concentration of NEFA. Excess lipid mobilization will cause many problems such as metabolic and reproductive disorders. Hence monitoring NEB via concentration of NEFA in blood might be a feasible way to prevent cows from various disorders. During the periparturient period, there will be a certain degree of NEB because of the decrease in dry matter intake (DMI) during the dry period and dramatic elevation of energy requirement resulting from the onset of lactation. To compensate for the NEB status, the cow will utilize its body fat as an energy source via a process known as lipid mobilization (Contreras et al., 2010). Lipid mobilization leads to release of FA in the blood; therefore, causing the elevated concentration of NEFA. The

increase in the concentration on NEFA in the blood can supply energy to the body. However, an overload of NEFA might be harmful to the animal. In the liver, NEFA can be metabolized into TAG. The bovine liver has limited ability to oxidize or export TAG as VLDL because of a finite number of apolipoproteins. When the limit is reached, the TAG accumulates in the liver, and acetyl CoA (resulting from oxidation of FA) that is not utilized in the tricarboxylic acid cycle is converted into ketone bodies (Adewuyi et al., 2005). The over-accumulation of TAG in the liver will lead to FA syndrome and this undermines the normal function of liver. In addition to ketosis and fatty liver, there are other metabolic disorders such as milk fever, uteritis, abomasal displacement, and mastitis that may occur when plasma NEFA concentration increase during the periparturient period (Contreras et al., 2010). Elevated blood NEFA can cause the impairment of the immune function hence making the cows more vulnerable to various infections and decreasing ovarian function (DeBie et al., 2016).

1.5.1 Inflammation and immune dysfunction

The high concentration of NEFA can invoke low-grade inflammation and affect immune function (Scholte et al., 2016). Similarly, dairy cows with the immune function disorder are vulnerable to disease during the transition period when blood NEFA elevates dramatically. During the transition period, the concentration of saturated FA such as palmitic acid increase within the blood. Leukocytes can be activated by the high concentration of saturated FA (Contreras et al., 2010).

The concentration of other FA such as mono- and polyunsaturated FA decrease when saturated FA increase in blood. These FA consist of arachidonic acid (an omega-6 FA), eicosapentaenoic acid (EPA), and docosahexaenoic (DHA, both omega-3 FA). Because their

products affect various steps of the inflammatory pathway, these FA are crucial factors for immune function. Immune dysfunction may happen easily in dairy cows if the availability of these necessary FA decreases.

Leukocyte function is affected directly by varying the concentration and composition of plasma NEFA (Scholte et al., 2016). Changing the composition of the cellular membrane of blood cells might be a means to alter immune function. Phospholipids, which are components of the cell membrane originate from various types of FA such as saturated, monounsaturated and polyunsaturated FA. The bilayer membrane that surrounds and protects the cells is formed by FA together with proteins. The composition of lipids in blood especially NEFA could influence the FA composition of the cellular membrane. Hence the phospholipid membrane of leukocytes can be affected if there is any change in the content of blood NEFA (Contreras et al., 2010). Switching the internal signals of leukocytes is another approach to alter immune function. The leukocytes could be more likely to induce inflammation because of saturated FA. Therefore, altering the way a cow responds to infectious and metabolic diseases may be facilitated through changing the composition of FA within the cellular membrane of leukocytes during the transition period (Contreras et al., 2010).

1.5.2 Reproductive failure

The relationship between energy balance and follicular dynamics within the ovary during the early postpartum period in dairy cows has been examined in some studies (Lucy et al., 1991 and DeBie et al., 2016). Lucy et al. (1991) evaluated follicular development in dairy cows using ultrasonography. With a more positive energy balance before day 25 postpartum, the number of class 3 (10 to 15 mm) follicles increased, whereas the number of

class 1 (3 to 5 mm) and class 2 (6 to 9 mm) follicles decreased. Lucy et al. (1991) suggested that the movement of smaller follicles into larger size classes is enhanced in cows with improving energy balance.

With dietary energy restriction, the growth of dominant follicles is limited. After the energy balance improved from its nadir, dominant follicle diameter and plasma oestradiol increased in early postpartum cows (Beam and Butler, 1999). There are differences in dominant follicle development between lactating and nonlactating dairy cows; smaller dominant follicles are observed in nonlactating compared to lactating cows during the first follicular wave of a synchronized estrous cycle (De La Sota et al., 1993). In the same study, compared with lactating cows, plasma oestradiol concentrations during the preovulatory period were several-fold greater in nonlactating cows. The increased serum NEFA concentration are reflected in the follicular fluid, in which the oocyte matures, undermining granulosa cell viability, oocyte developmental competence, and embryo quality, and hence dairy cow fertility (DeBie et al., 2016). Energy balance during the transition period has effects on the interval to first ovulation. However, energy status that over 6 weeks to 12 weeks postpartum period usually has no significant effect on the recovery of ovarian cycles. Apart from the influence of NEB during the early postpartum period, recovery of daily energy balance from its nadir value likely provides a signal to initiate cyclic ovarian activity. The number of days for the energy balance nadir has a positive effect on the number of days to first ovulation. (Beam and Butler, 1999).

Overall, it is crucial to find a feed ingredient to alleviate the metabolic burden of liver and therefore reduce the incidence of various metabolic disorders.

1.6 Betaine

Betaine, also called trimethylglycine (de Zwart et al., 2003), derives from the amino acid glycine with three methyl groups, and has been described as methylamine because of the three chemically reactive methyl groups (Craig, 2004). There are various dietary sources of betaine such as wheat and sugar beets. The byproduct of sugar beets, molasses solubles contain betaine. Betaine can also be produced endogenously by choline oxidation. The three most common types of betaine are betaine anhydrous, betaine monophosphate, and betaine hydrochloride. Multiple functions of betaine are osmolyte, methyl donor (Eklund et al., 2005), being fermented to acetate in the rumen (Mitchell et al., 1979) and being used possibly for *de novo* FA synthesis (Fernandez et al., 2004a). The reason that betaine can act as an osmolyte is that it is a dipolar zwitterion and is highly soluble in water (Eklund et al., 2005). In the tropical areas, the osmolyte protective activity of betaine is the reason for the use of molasses solubles as a protective measures to against heat stress. The three methyl groups can be used for trimethylation reaction. Hence betaine has a connection with protein and energy metabolism (Eklund et al., 2005).

1.7 Physiological and Nutritional Functions of Betaine

Betaine plays two major roles consisting of an organic osmolyte, which protects the cell against hypertonic stress and methyl donor that can be used for many biochemical pathways in FA and amino acid metabolism. As an osmolyte, betaine can increase water retention in cells and protect the enzyme inside cells from malfunction (Eklund et al., 2005). Methyl groups play a key role in multiple cellular functions such as deoxyribonucleic (DNA) methylation, phosphatidylcholine synthesis, and protein synthesis. The main organs for methyl group metabolism are liver and muscles (Obeid, 2013).

Betaine has been utilized widely in animal nutrition. Farmed fish are fed with betaine as an osmolyte to prevent hypertonic stress when they are moved from water with low to high salinity. In the poultry industry, betaine can be used to protect numerous intestinal microorganisms against osmotic fluctuation and hence improve microbial fermentation activity (Saeed et al., 2017). Betaine prevents poultry from coccidia infection, mitigate clinical signs, and enhance performance (Saeed et al., 2017). Furthermore, betaine can improve growth, increase nutrient utilization efficiency, and reduce fat deposition in swine and poultry (Saeed et al., 2017). In dairy cows, betaine may improve milk yield (Peterson et al., 2012), VFA production, FCM yield (Wang et al., 2010), and the performance of dairy cows under heat stress (Hall et al., 2016).

1.8 Methyl Donor

The main methyl donor in the cell is S-adenosylmethionine (SAM) connecting with various cellular reactions such as DNA methylation, synthesis of phosphatidylcholine, and reactions with numerous biochemicals (e.g., neurotransmitters, creatine, carnitine, and antioxidants such as glutathione and taurine). The key dietary sources of methyl groups are methionine, betaine, choline, and 5-methyltetrahydrofolate.

Betaine is converted to dimethylglycine when it donates one methyl group to transfer homocysteine into methionine via betaine homocysteine methyltransferase. Then most dimethylglycine is converted into sarcosine and glycine. Glycine can be used to synthesize bile salts, glutathione, creatine, and protein such as collagen (Obeid, 2013). The high concentration of homocysteine in blood will cause injury to the endothelial cell and result in inflammation of blood vessels and then atherogenesis (Obeid, 2013). Methionine is the precursor of SAM, which can be used to synthesize phosphatidylcholine via the

phosphatidylethanolamine methyltransferase (PEMT) pathway. Phosphatidylcholine can be utilized to generate free choline or synthesize VLDL. Free choline can be used to synthesize phosphatidylcholine via the cytidine diphosphate (CDP)-choline pathway. Choline can also be oxidated to form betaine. Choline deficiency will lead to the fatty liver because VLDL cannot be generated to excrete TAG. Hence it results in the over-accumulation of TAG in the liver. Therefore betaine as a methyl donor can avoid the formation of fatty liver and may indirectly enhance liver function. Methylation of DNA is a vastly investigated process of epigenetic modification that methyl groups are added to the C5 position of the cytosine to form 5-methylcytosine. It occurs due to the one-carbon metabolism pathway that relies on several enzymes that require dietary-derived such as folate, choline, and betaine. DNA methylation is critical to silence retroviral elements, X chromosome inactivation, manipulating tissue-specific gene expression, and genomic imprinting (Robertson and Wolffe, 2000). The key part of DNA methylation is that it may result in different effects on gene activities in different genomic regions based on the underlying genetic sequence (Moore et al., 2013). The function of DNA methylation includes genome defense, structural integrity, and transcriptional repression (Robertson and Wolffe, 2000). The evidence advocating the genome-defense hypothesis originates indirectly from several sources (Robertson and Wolffe, 2000). DNA methyltransferase 1 homozygous knockout embryonic stem (ES) cells containing only 30% of normal methylation degree resulted in a tenfold augment of the mutation rate connecting gene rearrangements (Robertson and Wolffe, 2000). These cells also show more transcription from an endogenous transposable element than wild-type ES cells, and this massive augment of transcription may lead to the elevation of the unstable genome. Regarding genome integrity, masking or inhibiting homologous

recombination between DNA repeats could be utilized by DNA methylation to stabilize the genomes of organisms.

1.9 Osmolyte

The function of betaine as an osmolyte is important because the control of cellular hydration state and cell volume is critical for preserving cell function. Numerous kinds of osmolytes occur naturally including amino acids (glycine and proline), methylamines (betaine and trimethylamine-N-oxide), and polyols and sugars (sorbitol and sucrose, Khan et al., 2010). Two complementary mechanisms of organic osmolytes are known to protect cells exposed to hypertonicity. Although inorganic ions can act as an osmolyte, increasing the concentrations of these ions perturbs protein function (Khan et al., 2010). However, organic osmolytes have less protein perturbing effect. Furthermore, a high concentration of organic osmolytes can stabilize protein structure (Burg and Ferraris, 2008). Renal medullary cells bear the highest amount of organic osmolytes because of their role in concentrating the urine. When animals are under hypertonicity, betaine concentration increases via synthesis from choline in the kidney and liver and then enters the cell from the extracellular fluid. The degree of hypertonicity determines the number of betaine transporters, called betaine/ γ-aminobutric acid transporter 1 (BGT 1). Hypertonicity increase transcription of the BGT 1 gene. Thus betaine transporter activity is regulated by control of the BGT 1 gene. Also, BGT 1 is regulated via plasma membrane insertion. Under the normotonic circumstance, the relatively small quantity of BGT 1 exists in the cytoplasm. When a cell encounters hypertonicity condition, BGT 1 localizes to basolateral plasma membranes (Burg and Ferraris, 2008).

1.10 Metabolic Burden related to Betaine

1.10.1 Methyl-deficient diet

Disorders in protein synthesis in the liver, fatty liver, and muscle disturbances have been reported from various studies in which animals are fed methyl-deficient diets (Guerrerio et al., 2012). Through epigenetic methylation mechanisms (Cordero et al., 2013) or lipid-affiliated mechanisms, alcohol-affiliated or non-alcohol related liver malfunction can be reversed by betaine, choline, or folate (Obeid, 2013). Only one part of the metabolic burden of methyl group deficiency or nutrient supplementation can be mirrored via alteration of the homocysteine level. A meaningful factor in determining the fasting plasma concentration of homocysteine can be folate, betaine (Holm et al., 2005), or choline (Caudill et al., 2009). There was a negative relationship between intake of betaine and choline with homocysteine concentrations when the subject fasted and post-methionine load (Caudill et al., 2009). However, in populations ingesting foods rich in folic acid, this inverse correlation was no longer present (Lee et al., 2010). Betaine is probably more efficient as a methyl donor and as a homocysteine-decreasing nutrient in populations not on fortification programs when folic acid lowers plasma homocysteine (Lee et al., 2010). In women with low folate intake, alcohol consumption (≥15 g/day) was also a valuable indicator of the negative relationship between choline and homocysteine. In the liver, alcohol can elevate plasma homocysteine, decrease liver SAM, and lead to the formation of fatty liver because it is recognized to undermine methionine synthase function (Barak et al., 2002). Therefore, as an origin of SAM and indicator of homocysteine in alcoholism, the betaine homocysteine methyltransferase pathway becomes more crucial (Barak et al., 2002). In healthy men from a population without required fortification with folic acid, phosphatidylcholine

supplementation for two weeks decreased mean plasma homocysteine by 18% and post-methionine load homocysteine by 29% compared to the placebo group (Olthof et al., 2005). There was an inverse correlation between the intake of major glycerophosphocholine acquired from milk and plasma homocysteine in women with low intakes of folate at <400 μg/day with mean homocysteine (Chiuve et al., 2007).

To sum up, the role of folate in decreasing fasting homocysteine can be reinforced by betaine and choline support. Betaine has a greater effect on diminishing post-methionine load homocysteine than its effect on fasting homocysteine. In the subjects not receiving folic acid, the homocysteine-lowering function of betaine is stronger. With the comparison of the plasma betaine, it appears that plasma folate could be a factor in determining the effect of betaine on plasma homocysteine (Allen et al., 1993).

1.10.2 Energy metabolism

Since the mitochondrial enzyme choline dehydrogenase and betaine aldehyde dehydrogenase expressed in the cytosol and mitochondria can oxidize choline to betaine, another source of betaine can be a choline-rich diet. There was a relationship between betaine and metabolism of protein (muscular tissue) and fat (Fernandez-Figares et al., 2002). Furthermore, the effect of betaine on sparing methionine and choline has been discussed in animal nutrition (Fernandez-Figares et al., 2002). The overall effects of methionine and choline-sparing by betaine are increased availability of methionine for protein synthesis, and choline for lipid metabolism (Obeid, 2013). Under the condition of low amino acid and energy intake, betaine is practical as a portion agent of metabolism in animals (Fernandez-Figares et al., 2002). Hence, to work out public health concerns related to surplus fat in meat products, betaine has the potential to deal with the issue. Various studies (Zabaras-Krick,

1997) reported that betaine could influence lipid metabolism as a lipotropic compound. It might help maintain the synthesis of carnitine, required for the transport of long-chain FA to the mitochondria where they are oxidized improving energy metabolism (Zabaras-Krick, 1997). Yao and Vance (1989) reported that betaine could correct VLDL production from hepatocytes grown in a choline-deficient environment. Löest *et al.* (2002) further reported that betaine might lower the requirement of choline methyl groups; therefore, choline availability for lipid metabolism can be enhanced.

1.11 Betaine in Digestive Tract

The osmolytic function of betaine could enhance intestinal function and growth (Siljander-Rasi et al., 2003). After gathering in cell organelles, betaine perform its osmoprotective effect by replacing inorganic ions; therefore, protecting enzymes and cell membranes. Betaine may diminish the energy required for ion-pumping in the intestine thus reducing the energy requirement and providing more energy for intestinal cell growth (Siljander-Rasi et al., 2003). In the poultry industry, betaine is used to protect intestinal cells against coccidia infection, which can lead to malabsorption and diarrhea caused by undermining the function of villus to mediate the exchange of water and various nutrients (Kettunen et al., 2001). Supplemental betaine can preserve the integrity of the gut epithelium of weaned pigs (Xu and Yu, 2000). Enhanced intestinal cell proliferation can lead to a larger surface area for nutrient absorption. The integrity of gut epithelium can influence nutrient absorption and digestion, so the osmotic ability of betaine may enhance nutrient digestibility. The increased digestibility of dry matter (DM) or organic matter (OM) represent the better digestibility of other nutrients such as crude protein (CP) in piglets (Xu and Yu, 2000).

1.12 Betaine in Rumen

There is also a positive effect of betaine on fermentation in ruminants by acting as a source of available ruminal nitrogen or methyl groups (Löest et al., 2002). Hence; it may enhance microbial fermentation rate and modify fermentation patterns. Wang et al. (2010) reported that the molar proportion of acetate was not affected; however, the molar proportion of propionate decreased linearly as a result of betaine supplementation. Therefore, the ratio of acetate to propionate (A:P) increased with increased betaine supplementations. The augmentation of the total concentration of VFA can originate from the increase in acetate and butyrate (Wang et al., 2010). Ruminal pH decreased because of the increased total VFA. However, there was no radioactivity found in propionate and butyrate (Mitchell et al., 1979). When betaine is converted to acetate in the rumen, it is transported to the mammary gland, and then it can be utilized for milk fat synthesis (Kim et al., 1997). Nakai et al. (2013) reported that after 12 h of ruminal incubation, the betaine level decreased by nearly 6%, and by around 15% at 24 h of incubation. This study showed that betaine did not break down easily; therefore, betaine could be found in the duodenum when it was administered orally. Also, betaine dissolves fast in ruminal fluid after dairy cows consume it as it is highly water-soluble. Nakai et al. (2013) suggested that more than 80% of betaine escaped from the rumen and reached the duodenum within 12 h. Because betaine is a potent osmolyte, microorganism in the digestive tract can also have the benefit from the osmotic function of betaine (Ratriyanto et al., 2009), hence betaine could support the growth of microbial populations that would enhance ammonia N utilization, particularly by the fibre-degrading populations (Wang et al., 2010).

1.13 Betaine Effect on Milk Production

Peterson et al. (2012) demonstrated that inclusion of dietary betaine at 100 g/d increased milk yield, decreased milk protein percentage and all levels of betaine supplementation (75 g/d and 100 g/d) slightly altered the milk FA profile. However, there was no significant change regarding milk fat or lactose percentage in mid-lactation dairy cows. Wang et al. (2010) reported that betaine supplementation could improve DM total tract digestibility and increase total ruminal VFA concentration, which might come from the enhanced growth of ruminal microbial population (especially cellulolytic bacteria) leading to the enhancement of ammonia nitrogen utilization (Wang et al., 2010). Therefore, the increase in milk yield could be explained by the increase in total VFA in the rumen and the improved total tract digestibility. Hall et al. (2016) also stated that betaine increased milk production in thermoneutral condition. Furthermore, betaine maintained the integrity of bovine mammary epithelial cells and enhanced the expression of heat shock protein in heat stress condition during an *in vitro* experiment (Hall et al., 2016). However, there was no effect of betaine on milk production in heat stress condition with high dose betaine (Hall et al., 2016).

1.14 Hypothesis

We hypothesize that the supplementation of betaine increases in milk production due to improved total tract digestibility of OM and change in VFA profile in the rumen.

1.15 Objectives

The objectives of this study are to determine the effect of supplemental betaine on total tract digestibility, productive performance, and ruminal fermentation measures in mid-lactation dairy cows.

Chapter 2: Effect of Betaine Supplementation on Total Tract Digestibility, Productive Performance, and Ruminal Fermentation Measures of Dairy Cows.

2.1 Materials and Methods

2.1.1 Total tract digestibility and productive performance

Animal and Treatment

The University of Idaho Animal Care and Use Committee approved (Protocol 2016-53) all the animal procedures used for the experiment. The study was conducted at the University of Idaho Dairy Center located in Moscow, Idaho. Twenty-one mid-lactation Holstein dairy cows (10 primiparous cows and 11 multiparous cows) were used in a 3 x 3 Latin square design with three periods and three treatments of rumen-unprotected betaine (Betaine anhydrous 97% purity; Amalgamated Sugar Co., Nampa, ID). The dietary treatments were: 1) A regular University of Idaho dairy lactation ration (Table 2.1, control), 2) control and 100 g/d betaine, and 3) control and 200 g/d betaine. Betaine was top-dressed on the regular University of Idaho dairy lactation ration in treatment 2) and 3). Each period lasted 28 d with 14 d for adaptation and 14 d for sampling.

Cows were housed in a tie-stall research barn and fed individually. Cows were milked twice a day (6:00 and 19:00) and fed right after milking. During the trial, there was a multiparous cow had the infection of acute clinical mastitis and it was removed from this trial. The other animals were healthy, had good udder conformation, and no clinical mastitis. Diet was formulated to meet or exceed the nutritional requirements according to the National Research Council (2001). Animal management, care and milking all abided by the University of Idaho dairy practices protocol. Cows had access to fresh, clean water at all times.

Sample Collection and Analysis

Feed intake was measured and recorded daily. Samples of total mixed ration (TMR) and refusals were collected each week and stored at -20℃ for DM and chemical analysis. Nutrient composition of TMR for each period was analyzed by near-infrared analysis (DairyOne, Ithaca, NY).

Milk yield was recorded daily. After d 14 of each period, milk samples collected weekly during two consecutive milkings and pooled in proportion to milk yield at each milking. The milk samples were sent to Washington DHIA (Burington, WA) for milk components analysis. Milk FA analysis was done by Agilent 7890A gas-chromatography system (Agilent Technologies, Santa Clara, CA).

Cows were fed with 7.5 g of chromic oxide (Cr_2O_3) twice a day on d 21 through d 28 of each period as a digestion marker. Grab fecal samples were collected for individual cows at different times (3 h intervals) on d 26, 27, and 28 of each period and pooled by the cow for each period. The samples were dried at 55°C for 48 h then ground to pass through a 1-mm sieve via Retsch SM200 (Retsch GmbH, Haan, Germany) and stored. After grinding, the samples were in the furnace at 600°C for 6 h and then digested in preparation of Inductively Coupled Plasma Emission Spectrometry (ICPES, Agilent Technologies, Santa Clara, CA) analysis to determine Cr_2O_3 quantity in the representative faecal sample used to analyze digestibility. The DM digestibility was calculated by the following equation (Guzman-Cedillo et al., 2016):

$$\text{DM digestibility} = (\text{DM intake} - \text{DM excretion}) \div \text{DM intake}$$

We assumed that the digestibility of chromic oxide was the same with DM digestibility so that the DM excretion could be determined by the following equation:

$[Cr_2O_3 \text{ intake} - (DMe \times M_{RS})] \div Cr_2O_3 \text{ intake} = (DM \text{ intake} - DMe) \div DM \text{ intake}$

where DMe is DM excretion and M_{RS} is the marker content in the representative faecal sample. Once DM excretion was determined, DM digestibility could be calculated.

Blood samples were obtained from the coccygeal vein into vacuum tubes on d 26, 27, and 28 of each period 2 h after the morning feeding for FA analysis. Spot urine samples were collected during fecal sampling. Body condition score measurement was performed before the beginning of the study and monthly afterward.

Fatty acids analysis for milk and serum was done by the gas-chromatography system with flame ionization detector and an Agilent HP-88 column (100m × 0.25mm with 0.2 μm film thickness, Agilent Technologies). After sample injection, the oven temperature was 120°C for 1 min and then increased to 175°C at a rate of 10°C /min and held for 10 min then increased to 210°C and held for 5 min, then increased 5°C /min to 230°C and held for 5 min then increased to 240°C a rate of 5°C /min and held for 5 min. Individual FA were identified by comparison to the standard mixture Supelco 37 FAME (Supelco, Bellefonte, PA).

Statistical Analysis

Data were analyzed in a 3 × 3 Latin square design assuming 3 treatments 0, 100, and 200 g of betaine and 3 periods. In each period, a total of 20 cows were used, leading to 2 squares with 7 cows and 1 squares with 6 cows. All statistical computations were conducted in MIXED procedures of SAS (version 9.2; SAS Institute Inc., Cary, NC). Treatment means were compared using pairwise comparisons with a Tukey adjustment to control the experiment-wise error rate. Significance was declared at $P < 0.05$ and trends at $0.05 < P < 0.1$.

2.1.2 Ruminal fermentation measures

Animal and Treatment

Once the study of digestibility was completed, three ruminally cannulated Holstein dairy cows from the University of Idaho Dairy Center were randomly assigned to a sequence of treatments in a 3 × 3 Latin square design with three periods and three treatments of rumen-unprotected betaine. While on trial, the cows were fed a lactation ration (Table 2.1). Rumen-unprotected betaine (betaine 97% purity; Amalgamated Sugar Co., Nampa, ID) at 0, 100, and 200 g was prepared, placed into Dacron bags (50μM pores, 10 x 20 cm, ANKOM Technology, Macedon, NY) and double sealed. These were loaded into large mesh bags and introduced into the rumen of three cannulated animals for all period of sampling. Each period had 8 days consisting of 1 day of betaine incubation in to rumen and 7 days of wash-out.

Sample Collection and Analysis

Degradation of OM was determined. During the ruminal degradation experiment, the three doses of betaine (0, 100, and 200 g) were incubated for 0, 1, 1.5, 2, 3, 6, 12, and 24 h, rinsed and dried as described by Stern et al. (1997). Organic matter degradation was performed on dried, post *in situ* sample bags (Van Soest et al., 1991). Milk samples were obtained twice a day and kept in a refrigerator. Each day of milk samples was pooled proportionately by yield. Total mixed lactation ration was sampled daily and kept in -20℃; samples were sent to Dairy One (Ithaca, NY) to be analyzed via near-infrared (NIR) analysis to determine nutrient composition.

Ruminal pH was measured immediately by an electric pH meter (Mettler Toledo, Columbus, OH). Volatile fatty acids in the rumen from "ruminal degradation" experiment

were determined using gas chromatography. Samples were filtered through two layers of cheesecloth and centrifuged at 2,400 × *g* at 4℃ for 20 min on a Sorvall ST 16R centrifuge (Thermo Scientific, Sunnyvale, CA). After centrifuging, the supernatant was frozen at -20° C. Samples were then thawed at room temperature, and centrifuged at 2,400 × *g* at 4° C for 30 min. Five mL of supernatant was transferred into a 15 mL conical tube and 1 ml of 25% metaphosphoric acid was added to remove any remaining proteins and placed in the -20℃ freezer overnight. After thawing at room temperature, the samples were centrifuged on a Sorvall Evolution RC centrifuge (Thermo Scientific, Sunnyvale, CA) at 1000 × *g* for 10 min at 23℃ and filtered through 11μm pore size filter papers(Whatman^{TM}, GE Healthcare, UK). Gas chromatography was conducted on a Hewlitt-Packard 6890 series GC using an Agilent DB-FFAP column (Hewlett-Packard, Avondale, PA). Oven temperature was initially 100℃, increased to 150℃ for 10 min, then 175℃ for 1.50 min, with a constant pressure of 2.00 psi and a 1:10 split. Hydrogen was used as the carrier gas. The standard solution used is Volatile Fatty Acid Mix (Sigma-Aldrich, St. Louis, MO).

Statistical Analysis

Data were analyzed in a 3 × 3 Latin square design assuming 3 treatments 0, 100, and 200 g of betaine and 3 periods. In each period, a total of 3 cows were used. Source of variation in the model included effects of treatment, time point, and treatment × time point interaction. All statistical computations were conducted in MIXED procedures of SAS (version 9.2; SAS Institute Inc., Cary, NC). Treatment means were compared using pairwise comparisons with a Tukey adjustment to control the experiment-wise error rate. Significance was declared at $P < 0.05$ and trends at $0.05 < P < 0.1$.

2.2 Results and Discussion

2.2.1 Dry matter intake and body condition score

Dry matter intake (25.4, 25.4, 25.4 ± 0.11 kg/d for treatments 0, 100, and 200g betaine/d per cow, respectively) and body condition score (BCS, 3.1, 3.2, 3.2 ± 0.05 for treatments 0, 100, and 200g betaine/d per cow, respectively) did not differ across treatments (Table 2.2). The two major dietary factors that could affect DMI are physical and chemical characteristic of feed ingredients (Allen, 2000). Fiber content, availability of hydrolysis, particle size, particle fragility, silage fermentation products, characteristics of fat, and the ruminal degradation of protein are the specific physical and chemical characteristic of diets that can affect DMI (Allen, 2000). Wang et al. (2010) also reported that the supplementation of rumen-unprotected betaine did not affect DMI. Additionally, Davidson et al. (2008) described that there was no effect of rumen-protected betaine on DMI. Conversely, Löest et al. (2002), reported that daily DMI was higher for steers fed betaine than for control steers. The different quality or forms of betaine might lead to the different palatability hence there were inconsistent results from various studies.

Wang et al. (2010) reported that betaine supplementation could significantly improve apparent total tract digestibility of DM, OM, CP, ether extract, neutral detergent fiber, and acid detergent fiber. Digestibility is one of the key factors that determine DMI. Oba and Allen (1999) noted that forage with increased NDF digestibility could significantly improve DMI and milk yield hence the increased digestibility could be the reason of the improved DMI in the study of Löest et al. (2002). Even so, the effect of increasing in digestibility might not end up improve DMI (Peterson et al., 2012; Wang et al., 2010).

2.2.2 Total tract digestibility of dry matter

There was no difference in the current study. However, the apparent total tract DM digestibility tended to increase by supplementing 100 g betaine as compared with that with no dietary betaine (0.61 vs. 0.58 ± 0.01; $P = 0.1$). Wang et al. (2010) reported that betaine supplementation could significantly increase apparent total tract digestibility of DM, OM, CP, ether extract, neutral detergent fiber, and acid detergent fiber. Furthermore, Wang et al. (2010) reported that the improved total tract digestibility is consistent with increased ruminal VFA concentration and milk fat content.

In weaned pigs, the total tract digestibility of DM and CP was improved by betaine supplementation (Eklund et al., 2005). In addition, the higher activity of proteolytic enzymes indicated the enhanced intestinal cell activity in weaned pigs with betaine supplementation (Xu and Yu, 2000). The betaine effect in improving total tract DM and CP digestibility resulted from the improved intestinal epithelium growth because of betaine osmolytic property, which enhances the absorption of Na^+ into cells (Eklund et al., 2005). Furthermore, the increase in ruminal VFA concentration might indicate that the betaine supplementation can improve ruminal microbial fermentation (Wang et al., 2010). Kettunen et al. (2001) also reported that betaine increased intestinal lactic acid and VFA concentration in broiler chicks.

2.2.3 Organic matter degradation

The product used in this study was anhydrous betaine (97% purity, Amalgamated, Nampa ID). In the present study, the significant in situ OM degradation ($P < 0.0001$) was observed when betaine retention time was longer than 12 h (Figure. 2.1). However, the OM to DM ratio remained at approximately 0.85 when retention time reached 24 hours.

Based on the study by Mitchell et al. (1979), betaine degradation is fast in the rumen. After incubation of betaine into the rumen, there were around half of the carboxyl and methyl carbons of betaine presumably reserved within the animal in 10 to 14 h. Even though betaine vanished from the rumen rapidly, it is possible that some of the betaine escaped ruminal degradation to be absorbed into the circulation and reach the liver to participate in methyl-transfer reactions. Löest et al. (2001) reported that percentages of betaine remaining after 24 h of incubation were 21% for the grain-based diet, and 47% for the forage-based diet, meaning that the rate of degradation was slower on the forage-based diet than the grain diet. Furthermore, Löest et al. (2001) suggested that betaine might escape from the rumen and be absorbed postruminally and have lipotropic effects on carcass characteristics. Also, Nakai et al. (2010) stated that betaine could be present in the duodenal digesta after administration via the esophagus. The ruminal fluid turnover rate for Holstein cows is 7% to 8% per h in vivo (Nakai et al., 2010). Nakai et al. (2010) suggested that betaine dissolves in the ruminal fluid right after dairy cows ingest it because betaine is highly water-soluble and that more than 80% of the betaine containing ruminal fluid escapes from the rumen then reaches the duodenum within 12 h. Overall, based on the present and previous studies regarding OM degradation, betaine in ruminal fluid could possibly reach the duodenum and act as a lipotropic agent to alleviate the lipid metabolic burden of the liver.

2.2.4 Milk yield

In the present study, milk yield (29.7, 29.3, and 30.0 ± 0.7 kg/d for treatments 0, 100, and 200g betaine/d per cow, respectively) did not differ across treatments (Table 2.2). This result is consistent with the study by Davidson et al. (2008). Conversely, Wang et al. (2010) and Peterson et al. (2012) reported that betaine supplementation increased milk yield.

Furthermore, Hall et al. (2016) stated that betaine supplementation increased milk yield when cows were in a thermoneutral environment. The effect of betaine in increasing total tract digestibility, total VFA concentration (Wang et al., 2010) and protecting mammary gland cell integrity (Hall et al., 2016) may help explain the increased milk yield in the studies by Wang et al. (2010) and Peterson et al. (2012). The possible reason to explain the different result of Davidson et al. (2008) however, may be the nutrient composition of TMR (limited mrthionine) and the different forms of betaine (rumen-protected betaine) in which case it cannot alter ruminal fermentation. Compared with the free-stall barn in Peterson et al. (2012), the tie-stall barn in the present study might in part result in the different consequence. The reason to explain the lack of significant difference in milk yield in the present study might in part be related to cow-to-cow variation in milk production.

2.2.5 Milk composition

No treatment differences ($P > 0.4$) were observed for milk protein, and lactose (percentage and total yield, Table 2.2). There was also no change observed for milk somatic cell count (SCC, $P = 0.75$). The result of the present study is in line with the studies by Davidson et al. (2008), Wang et al. (2010) and Fernandez et al. (2004b) who noted that betaine supplementation had no significant effect on milk protein and lactose. Peterson et al. (2012) reported however, that milk protein percentage decreased with the betaine supplementation ($P < 0.01$). The increased milk yield might in part result in the dilution of milk protein percentage (Peterson et al., 2012). Although Wang et al. (2010) reported that betaine supplementation could increase ruminal VFA concentration, there was no significant change observed in milk lactose, which originates from propionate in rumen.

In the present study, the SCC was not significantly different, which is consistent

with the report by Peterson et al. (2012). The SCC in the present study was, however, higher than 300,000 cells/ml, which means that several cows in this study might have had subclinical mastitis (Blowey and Edmondson, 2010).

There was no difference in milk fat yield (kg/d; $P = 0.86$), energy-corrected milk yield (ECM, kg/d; $P = 0.69$), or 3.5% FCM yield (kg/d; $P = 0.82$) across treatments. Milk fat percent (3.16 vs. 3.36 ± 0.08%, for 0 g betaine/d and 200 g betaine/d, respectively) however, tended to differ between treatments ($P = 0.1$) in the present study. Wang et al. (2010) reported that milk fat content and FCM was significantly greater with betaine supplementation than the control ($P < 0.03$). Conversely, Peterson et al. (2012) found that milk fat percentage and milk fat yield per day did not change among treatments ($P = 0.30$, and $P = 0.37$, respectively).

Mitchell et al. (1979) stated that betaine could increase the concentration of acetate in the rumen, which is used for *de novo* FA synthesis. Furthermore, once betaine reaches the intestine and enters into the circulation, it can go into PEMT pathway to help the liver to excrete TAG via the formation of VLDL (Eklund et al., 2005). Hence increased VLDL in the circulation can transport the TAG to the mammary gland and enhance milk fat synthesis (Drackley et al., 2006). Therefore this could explain the tendency for an increase in milk fat content in our study.

2.2.6 Milk fatty acid profile

There were no significant changes in milk saturated FA content (Table 2.3, $P = 0.9$ for the sum of C11:0 to C24:0) as well as the FA synthesized *de novo*, including C11:0 to C16:0 ($P = 0.90$) with betaine supplementation. Peterson et al. (2012) and Fernandez et al. (2004a) reported a similar result where the sum of C4:0 to C20:0 was not affected across

treatment. Fernandez et al. (2004b) reported however, an increase in C8:0, C10:0, C12:0, and C17:0 as well as in the sum of C6:0 to C20:0 as a result of betaine supplementation in lactating dairy goats. Within the rumen, betaine increased the concentration of acetate (Mitchell et al., 1979), which is then absorbed into the blood and utilized for the *de novo* synthesis of FA with 4 to 16 carbon atoms (Jensen, 2002). The precursors for *de novo* synthesis of FA arise from the microbial fermentation of cellulose and hemicelluloses in the rumen (Jensen, 2002).

There was no difference in milk mono-unsaturated FA (MUFA) content (Table 2.4, $P = 0.91$ for the sum of C14:1 to C24:1). Peterson et al. (2012) reported a similar finding where betaine had no effect on the sum of MUFA *trans* C10:1, C14:1, C15:1, C16:1, C17:1, C18:1 isomers, and *cis*-9 C18:1 ($P = 0.93$). In the present study however, C20:1 (0.005 vs 0.004 ± 0.0006%, for 100 g betaine/d and 200 g betaine/d, respectively) tended to differ between treatments ($P = 0.1$ for both). In addition, Peterson et al. (2012) found a difference in FA C15:1 ($P = 0.04$), which was not observed in the present study.

Milk poly-unsaturated FA (PUFA) content did not differ (Table 2.5) across treatments ($P = 0.77$ for the sum of C18:2, C18:3, C20:2, C20:3, C20:4, C22:2, C20:5, and C22:6) in the present study. Fernandez et al. (2009) reported a similar observation where betaine supplementation did not change the sum or individual PUFA in lactating dairy goats. Peterson et al. (2012) however, reported a decrease in the sum of PUFA ($P = 0.02$), with all dosages of betaine supplementation. Nonetheless, the individual PUFA were not affected by betaine supplementation in that study.

2.2.7 Serum fatty acid profile

Among saturated FA (Table 2.7) for cows fed betaine at 0, 100, or 200 g/d, there were decreases in the content of C11:0, C12:0, C15:0 and C17:0 ($P < 0.05$); C13:0 tended to decrease, but C18:0 tended to increase ($P < 0.1$). However, no change was observed in the content of total SFA with betaine supplementation (0.41, 0.40, and 0.40 ± 0.006 for 0, 100, and 200 g betaine, respectively; $P = 0.96$).

With regards to MUFA (Table 2.8) for cows fed betaine at 0, 100, or 200 g/d, there were decreases in the content of C14:1 and C22:1 ($P < 0.05$), tendencies of decrease were also observed in the content of C15:1, C17:1, C18:1 *trans*-9, and C20:1 ($P < 0.1$). The total MUFA content decreased (Figure 2.9) with betaine supplementation (0.16, 0.15, and 0.15 ± 0.0032 for 0, 100, and 200 g betaine, respectively; $P < 0.05$).

Regarding PUFA (Table 2.8) for cows fed betaine at 0, 100, or 200 g/d, there was a decreases in the content of C18:2 *all trans*- 9, 12 (Figure 2.10, $P < 0.05$), and a tendency for a decrease of C20:2 ($P < 0.1$) was observed. Increases in the molar proportion of C20:3n-6 and C20:3n-3 were observed ($P < 0.05$). In addition, there was a tendency to increase the content of C18:3n-3 ($P < 0.1$). The total omega-3 FA (n-3) consisting of C18:3n-3, C20:3n-3, C20:5, and C20:6 significantly increased (Figure 2.11, $P < 0.05$), which resulted in the ratio of omega-6 FA (n-6, the sum of C18:2 *all trans*-9,12, C18:2 *all cis*-9,12, C18:3n-6, C20:2, C20:3n-6, C20:4, and C22:2) to the n-3 FA decreased (Figure 2.12, $P < 0.05$) when betaine was added to the diet.

There have been various studies using choline supplementation to study the activity of PEMT and CDP-choline pathway, both of which produce phospholipid phosphatidylcholine. West et al. (2013) reported that the PEMT pathway would generate

phosphatidylcholine enriched in long-chain PUFAs such as DHA (22:6n−3) and arachidonic acid (ARA; 20:4n−6). However, the CDP–choline pathway produces phosphatidylcholine that is enriched in di- and monounsaturated FA such as linoleic acid (18:2n−6) and oleic acid (18:1n−9). In the present study, the PEMT pathway activity might have been enhanced based on the tendency for an increase in C18:3n-3 as well as the significant increase in C20:3n-6, C20:3n-3 and the total n-3 FA as increased phosphatidylcholine enriches in long-chain PUFAs in phospholipid fraction of serum lipid (da Costa et al., 2011). Leng et al. (2016) stated that betaine could lead to increased FA β-oxidation in the mitochondria because of the augmented expression of peroxisome proliferator-activated receptor alpha (PPAR α), CPT 1, and 3-hydroxy acyl-CoA dehydrogenase. PPAR α is the key transcriptional activator of genes involving mitochondrial β-oxidation; CPT 1 can facilitate the transfer of FA into the mitochondrial. 3-hydroxy acyl-CoA dehydrogenase is involved in β-oxidation. Therefore, the possibility of relatively low activity of CDP-choline pathway and enhanced β-oxidation originating from activation of PPAR α via PUFA might in part explain the tendency of decreases in C21:0, C15:1, C17:1, C18:1 *trans*-9, C20:1, and C20:2 as well as the significant decrease in the total MUFA and some SFA (C11:0, C12:0, C13:0, C15:0, and C17:0). Although there was a tendency for an increase in C18:0, total SFA however, did not significantly change, which might be explained by the lesser involvement with phosphatidylcholine synthesis than PUFA (Ingrid Richardson and Wurtman, 2007).

In addition, the tendency for a decrease in C18:1 *trans*-9 suggests that dietary betaine might improve cardiovascular health, and bear some level of anti-carcinogen effect, which might be beneficial for humans (Ip, 1997). Betaine is converted to dimethylglycine when it donates one methyl group to transfer homocysteine into methionine via betaine

homocysteine methyltransferase. The high concentration of homocysteine in blood will cause injury to the endothelial cell and result in inflammation of blood vessels and then atherogenesis (Obeid, 2013). Therefore, the tendency for a decrease in C18:1 *trans*-9, the tendency for an increase in C18:3n-3 as well as the increase in C20:3n-3 and the total n-3 FA are consistent with the effect of betaine in improving vasculitis and atherogenesis via reducing the concentration of homocysteine in the blood (Obeid, 2013).

Hence, under the condition of the present study, betaine supplementation may have augmented the PEMT pathway activity, β-oxidation, and to some degree of anti-inflammatory effects.

2.2.8 Ruminal volatile fatty acid profile and pH

There were increases in the ratio of acetate to propionate (Table 2.6; 1.7, 1.9, and 1.8 ± 0.03, for 0, 100, and 200 g betaine, respectively; $P < 0.0001$) and molar proportion of isovalerate with betaine supplementation ($P < 0.005$). The molar proportions of propionate, butyrate, and isobutyrate decreased ($P < 0.001$) with betaine supplementation (Figure 2.2, 2.3, and 2.4). A decrease in pH (Figure 2.7; 6.4, 6.3, and 6.2 for 0, 100, and 200 g betaine, respectively) was observed ($P < 0.001$). The ruminal retention time effect decreased in pH (Figure 2.8, $P < 0.001$) as well. There were no detectable changes in the molar proportion of acetate (0.42, 0.43, and 0.42 ± 0.006) or valerate (0.05, 0.04, and 0.05 ± 0.005).

The results regarding acetate, propionate, the acetate to propionate ratio, and ruminal pH are consistent with the report by Wang et al. (2010). Although Mitchell et al. (1979) and Nakai et al. (2013) noted that betaine in the rumen could be metabolized into acetate and increase the concentration of acetate, the molar proportion of acetate in the present study did not change. Nevertheless, the decreased in ruminal pH may imply an

elevated total VFA concentration (Wang et al., 2010). Furthermore, the increased VFA concentrations indicate a greater microbial fermentation rate in the present study. The significant increase of the acetate to propionate ratio may in part explain the tendency of increase in milk fat percent (3.16 vs. 3.36 ± 0.08%, for 0 g betaine/d and 200 g betaine/d, respectively, $P = 0.1$) in the present study.

Overall, the present study shows that betaine supplementation could modify the ruminal VFA profile and possibly increase in the total VFA concentration. The changes in VFA profile observed suggest the possibility of an effect of betaine supplementation on ruminal microbial populations.

2.3 Conclusion

Based on previous studies, betaine supplementation could improve milk yield and milk fat content. Therefore, it was hypothesized that the supplementation of betaine increases the milk production by improving total tract digestibility of OM and change in VFA profile in the rumen. However, under the conditions of the present study, betaine supplementation did not significantly affect DMI, apparent total tract digestibility, milk yield, milk components, milk fat content, FCM, and most of milk FA. The possible reason to explain the lack of significant difference in production performance in the present study might in part relate to cow-to-cow variation. Although the overall productive performance did not differ among treatments, there were changes observed in serum FA profile, ruminal VFA profile, and decreased ruminal pH. Our observations from serum FA profile of the present study indicate that betaine supplementation can potentially augment the PEMT pathway, β-oxidation, and some degree of anti-inflammatory effect because of a significant decline in the n-6 to n-3 ratio. In addition, it suggests that the concentration of phosphotidyl choline in serum and milk should be further investigated to determine the PEMT pathway activity for the human benefits of milk phosphotidyl choline. A significant increase in the ratio of acetate to propionate and the decrease in ruminal pH observed in the present study may suggest an alteration of ruminal microbial population, which may improve fermentation and microbial protein synthesis. Furthermore, the OM to DM ratio maintained at approximately 0.85 when retention time reached 24 hours, which indicates that there might be some betaine escaping from the rumen and potentially reaching the abomasum and small intestine.

To sum up, betaine supplementation in the present study improved rumen

fermentation and might have escaped from the rumen and absorbed in the intestine, which could have resulted in lipotropic effect on the animals. Future studies are warranted to focus on the effect of betaine supplementation on ruminal microbiome and the PEMT pathway activity related to milk phosphotidyl choline.

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Table 2.1: Feed ingredients and nutrient composition of the basal ration and analyzed nutrient composition of betaine[1]

Item, Ingredient, % of DM	Amount	SEM
Alfalfa hay	17.5	
Mixed grass	10.0	
Barley silage	17.0	
Alfalfa silage	12.0	
Rolled barley	12.0	
Canola meal	11.9	
Ground corn	3.5	
Distillers grain	9.5	
Calcium soaps of fatty acids	0.5	
Vitamin-mineral premix[2]	5.5	
Sodium bicarbonate	0.6	
Nutrient composition		
DM, %	54.1	2.28
CP, % of DM	17.7	0.29
Soluble protein, % of CP	40.7	0.33
ADF, % of DM	26.1	0.52
NDF, % of DM	39.0	0.68
NFC, % of DM[3]	29.7	0.46
Crude fat, % of DM	4.3	0.20
Ash, % of DM	9.4	0.10
TDN, % of DM	65.3	0.33
NEL, Mcal/kg[4]	1.52	0.01

[1] Betaine (95.1% of DM) used contained (on DM basis) 75% CP (100% soluble protein), 0.3% ADF, 0.4% NDF, 24.4% NFC, 1.1% starch, 0.2% crude fat, 91% TDN, 0.003% Ca, 0.03% K, and 2.2 Mcal/kg NEL.

[2] Vitamin-mineral premix contained 17.5% Ca, 0.17% P, 0.3% S, 3.1% Mg, 0.08% Fe, 0.28% Zn, 0.17% Mn, 0.001% Se, 275 kIU/kg vitamin A, 77 kIU/kg vitamin D, and 1,320 IU/kg vitamin E.

[3] NFC = 100 − (NDF + CP + fat + ash).

[4] NEL = 0.866 − [0.007 × ADF (% of DM)].

Table 2.2: Daily DMI, BCS, milk yield, composition, component yield, and feed efficiency from mid-lactating Holstein cows with different dosage of dietary betaine.

	Betaine, g/d				
Item	0	100	200	SEM	*P*-value
DMI, kg/d	25.4	25.4	25.4	0.10	0.98
BCS	3.10	3.20	3.20	0.05	0.76
Milk production, kg/d					
Milk yield	29.7	29.3	30.0	0.70	0.77
Milk fat	0.96	0.97	0.99	0.04	0.86
Milk protein	0.93	0.91	0.93	0.02	0.78
Milk lactose	1.39	1.32	1.37	0.04	0.45
Milk composition, %					
Milk fat	3.16	3.18	3.36	0.08	0.19
Milk protein	3.14	3.15	3.10	0.05	0.74
Milk lactose	4.58	4.51	4.55	0.06	0.65
SCC (× 100000 cells/ml)	5.28	7.27	5.51	2.02	0.75
ECM, kg/d[1]	28.5	28.1	29.0	0.75	0.69
3.5% FCM, kg/d[2]	28.4	28.4	29.0	0.84	0.82
ECM/DMI	1.13	1.11	1.14	0.03	0.71
FCM/DMI	1.12	1.12	1.14	0.03	0.86

[1] ECM (kg/d) = [milk yield (kg/d) × 0.327] + [milk fat (kg/d) × 12.86] + [milk true protein (kg/d) × 7.65] (Davidson et al., 2008).

[2] 3.5% FCM (kg/d) = [milk yield (kg/d) × 0.4324] + [milk fat (kg/d) × 16.2162] (Davidson et al., 2008).

Table 2.3: Saturated milk FA profile (molar proportion of total identified FA) from mid-lactating Holstein cows with different dosage of dietary betaine.

	Betaine, g/d				
FA, % of total FA identified	0	100	200	SEM	*P*-value
C11:0	0.07	0.08	0.07	0.01	0.75
C12:0	3.25	3.38	3.26	0.12	0.66
C13:0	0.13	0.13	0.13	0.01	0.84
C14:0	11.6	11.7	11.6	0.27	0.98
C15:0	1.20	1.21	1.18	0.34	0.79
C16:0	29.8	30.42	30.16	1.00	0.91
C17:0	0.63	0.63	0.64	0.01	0.80
C18:0	13.7	13.4	13.6	0.64	0.94
C20:0	0.31	0.30	0.30	0.01	0.90
C21:0	0.12	0.12	0.12	0.01	0.69
C22:0	0.16	0.15	0.16	0.01	0.91
C23:0	0.05	0.05	0.05	0.01	0.99
C24:0	0.08	0.09	0.08	0.01	0.40
de novo[1]	0.46	0.47	0.46	0.01	0.90
Σ Saturated[2]	61.1	61.6	61.4	0.80	0.90

[1] de novo = the sum of C11:0 to C16:0.

[2] Σ Saturated = the sum of C11:0 to C24:0.

Table 2.4: Monounsaturated milk FA profile (molar proportion of total identified FA) from mid-lactating Holstein cows with different dosage of dietary betaine.

	Betaine, g/d				
FA, % of total FA identified	0	100	200	SEM	*P*-value
C14:1	1.44	1.48	1.45	0.07	0.93
C15:1	0.39	0.38	0.39	0.01	0.78
C16:1	1.05	1.17	1.15	0.10	0.63
C17:1	0.30	0.32	0.32	0.01	0.31
C18:1 *trans*-9	1.85	1.94	1.92	0.12	0.86
C18:1 *cis*-9	30.2	29.4	29.9	0.83	0.78
C20:1	0.37	0.51	0.36	0.07	0.20
C22:1	0.14	0.13	0.13	0.01	0.85
C24:1	0.14	0.13	0.13	0.01	0.53
Σ Monounsaturated[1]	35.9	35.5	35.8	0.70	0.91

[1] Σ Monounsaturated = the sum of C11:1 to C24:1.

Table 2.5: Polyunsaturated milk FA (molar proportion of total identified FA) from mid-lactating Holstein cows with different dosage of dietary betaine.

	Betaine, g/d				
FA, % of total FA identified	0	100	200	SEM	*P*-value
C18:2 all *trans*-9, 12	0.41	0.37	0.38	0.02	0.50
C18:2 all *cis*-9, 12	1.27	1.25	1.22	0.09	0.92
C18:3:n-6	0.07	0.07	0.07	0.01	0.99
C18:3:n3	0.66	0.64	0.65	0.02	0.68
C20:2	0.14	0.15	0.14	0.01	0.93
C20:3n-6	0.11	0.10	0.10	0.01	0.77
C20:3n3	0.10	0.10	0.10	0.01	0.97
C20:4	0.03	0.03	0.03	0.01	0.99
C22:2	0.08	0.08	0.08	0.01	0.62
C20:5	0.04	0.04	0.04	0.01	0.94
C22:6	0.07	0.06	0.06	0.01	0.40
Σ PUFA[1]	3.00	2.90	2.80	0.10	0.77
Σ n-6[2]	2.10	2.10	2.00	0.10	0.83
Σ n-3[3]	0.88	0.85	0.85	0.02	0.64
n-6:n-3	2.36	2.40	2.39	0.11	0.95
SFA:UFA	1.58	1.63	1.62	0.06	0.95

[1] Σ PUFA included C18:2, C18:3, C20:2, C20:3, C20:4, C22:2, C20:5, and C22:6.

[2] n-6 included C18:2 *all trans*-9,12, C18:2 *all cis*-9,12, C18:3n-6, C20:2, C20:3n-6, C20:4, and C22:2.

[3] n-3 included C18:3n-3, C20:3n-3, C20:5, and C22:6.

Table 2.6: Ruminal pH and VFA profile (molar proportion of total identified VFA) from mid-lactating Holstein cows with different dosage of dietary betaine.

	Treatment				
Item	0	100	200	SEM	*P*-value
pH	6.42[a]	6.35[a]	6.18[b]	0.04	<0.001
VFA (% of total VFA identified)					
Acetate	42.1	42.8	42.4	0.60	0.720
Propionate	24.7[a]	22.2[b]	24.0[a]	0.40	<0.001
Butyrate	23.1[a]	22.8[a]	20.5[b]	0.40	<0.001
Isobutyrate	1.58[a]	1.67[a]	1.21[b]	0.07	0.005
Valerate	4.88	4.46	5.29	0.46	0.450
Isovalerate	3.75[a]	6.13[b]	6.71[b]	0.63	<0.001
Acetate:propionate	1.71[a]	1.94[b]	1.80[a]	0.03	<0.001

[a,b]Means within a row that do not share a common letter are significantly different ($P < 0.05$).

Table 2.7: Saturated serum FA profile (molar proportion of total FA identified) from mid-lactating Holstein cows with different dosage of dietary betaine.

	Betaine, g/d				
FA, % of total FA identified	0	100	200	SEM	*P*-value
C11:0	0.47[a]	0.31[b]	0.30[b]	0.05	0.03
C12:0	0.43[a]	0.29[b]	0.26[b]	0.34	< 0.01
C13:0	0.55	0.46	0.46	0.03	0.05
C14:0	1.21	1.11	1.04	0.05	0.11
C15:0	0.95[a]	0.81[b]	0.79[b]	0.03	< 0.01
C16:0	12.4	12.5	12.4	0.20	0.92
C17:0	1.42[a]	1.28[b]	1.24[b]	0.03	< 0.01
C18:0	20.4	21.3	21.5	0.36	0.09
C20:0	0.36	0.34	0.30	0.03	0.33
C21:0	0.36	0.27	0.30	0.03	0.10
C22:0	0.56	0.44	0.45	0.12	0.75
C23:0	0.68	0.63	0.61	0.03	0.26
C24:0	0.74	0.66	0.65	0.07	0.65
Σ Saturated[1]	40.5	40.4	40.3	0.60	0.96

[1] Σ Saturated = the sum of C11:0 to C24:0.

[a, b] Means within a row that do not share a common letter are significantly different ($P < 0.05$).

Table 2.8: Unsaturated serum FA profile (molar proportion of total FA identified) from mid-lactating Holstein cows with different dosage of dietary betaine.

	Betaine, g/d				
FA, % of total FA identified	0	100	200	SEM	*P*-value
C14:1	1.18[a]	1.03[b]	1.03[b]	0.05	0.04
C15:1	0.67	0.52	0.60	0.05	0.08
C16:1	0.96	0.83	0.80	0.08	0.33
C17:1	0.72	0.54	0.57	0.01	0.10
C18:1 *trans*-9	0.67	0.52	0.54	0.05	0.10
C18:1 *cis*-9	9.77	9.86	9.66	0.21	0.80
C20:1	0.37	0.32	0.30	0.02	0.09
C22:1	1.26[a]	0.97[b]	0.80[b]	0.08	< 0.01
C24:1	0.63	0.60	0.57	0.04	0.60
Σ Monounsaturated[1]	16.2[a]	15.2[b]	14.9[b]	0.32	0.01
C18:2 all *trans*-9, 12	0.76[a]	0.61[b]	0.51[c]	0.04	< 0.01
C18:2 all *cis*-9, 12	32.9	33.6	33.8	0.64	0.57
C18:3:n-6	0.92	0.87	0.85	0.04	0.44
C18:3:n-3	3.51	3.57	3.81	0.09	0.06
C20:2	0.50	0.40	0.39	0.04	0.07
C20:3n-6	1.82[a]	2.64[b]	2.59[b]	0.19	< 0.01
C20:3n-3	1.28[a]	1.27[a]	1.54[b]	0.09	< 0.01
C20:4	0.17	0.17	0.16	0.02	0.84
C22:2	0.60	0.58	0.58	0.03	0.82
C20:5	0.48	0.42	0.44	0.05	0.65
C22:6	0.30	0.28	0.24	0.03	0.21
Σ PUFA[2]	42.7	43.9	44.3	0.83	0.36
Σ Unsaturated[3]	58.9	59.0	59.2	0.60	0.95
Σ n-6[4]	37.7	38.9	38.8	0.77	0.46
Σ n-3[5]	5.57[a]	5.54[a]	6.02[b]	0.13	0.02
n-6:n-3	6.80[ab]	7.07[a]	6.50[b]	0.16	0.04
SFA:UFA	0.69	0.69	0.68	0.02	0.94

[1] Σ Monounsaturated = the sum of C11:1 to C24:1.

[2] Σ PUFA included C18:2, C18:3, C20:2, C20:3, C20:4, C22:2, C20:5, and C22:6.

[3] Σ Unsaturated consist of Σ Monounsaturated and Σ PUFA

[4] n-6 included C18:2 *all trans*-9, 12, C18:2 *all cis*-9, 12, C18:3n-6, C20:2, C20:3n-6, C20:4, and C22:2.

[5] n-3 included C18:3n-3, C20:3n-3, C20:5, and C22:6.

[a, b, c] Means within a row that do not share a common letter are significantly different ($P < 0.05$).

Figure 2.1: Organic matter degradation from mid-lactating Holstein cows with different dosage of dietary betaine. Betaine degraded significantly when ruminal retention time was longer than 12 hours ($P < 0.001$). Vertical bars represent the standard error of the mean.

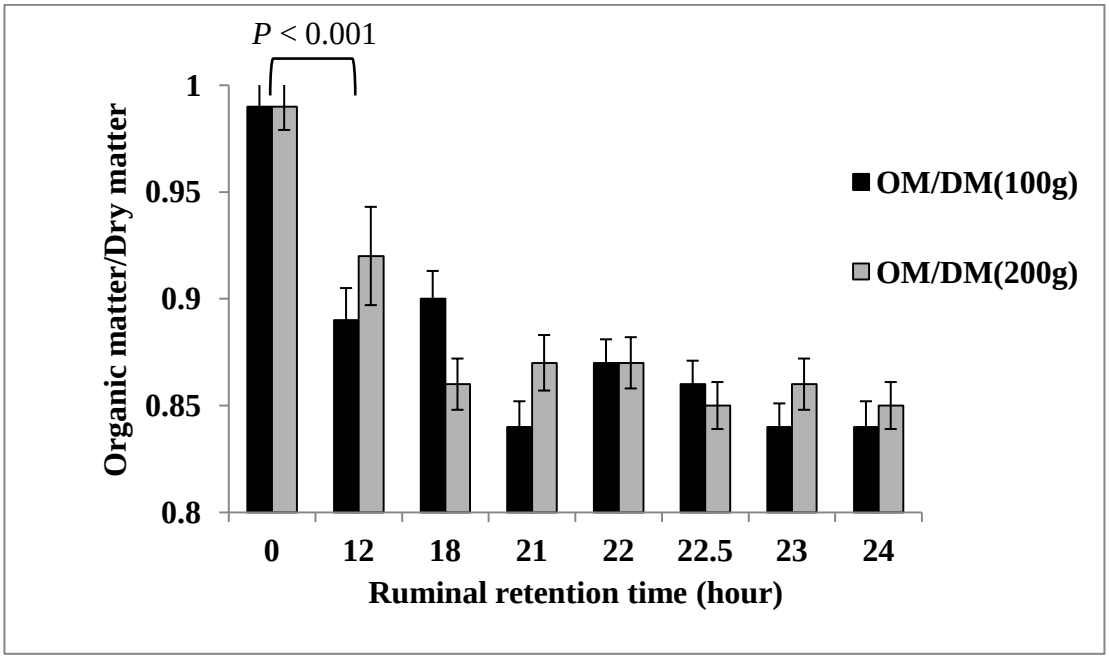


Figure 2.2: The molar proportion of propionate in total identified ruminal VFA (%) from mid-lactating Holstein cows with different dosage of dietary betaine. Significant differences were observed among treatments (columns not sharing a common letter, $P < 0.001$). Vertical bars represent the standard error of the mean.

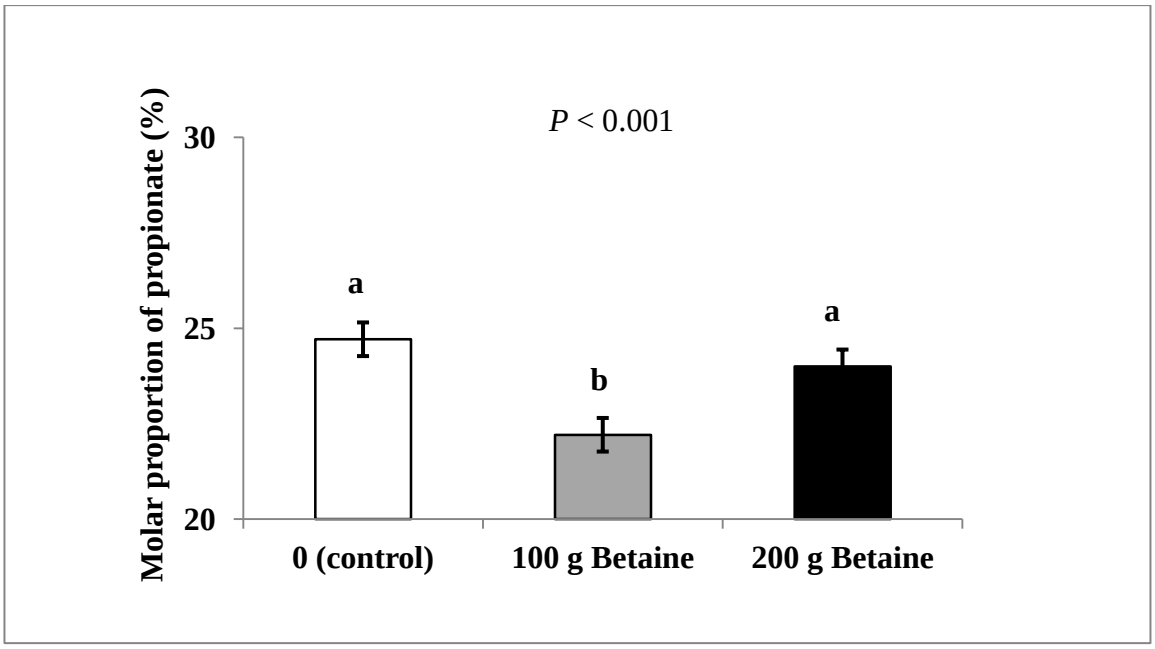


Figure 2.3: The molar proportion of butyrate in total identified ruminal VFA (%) from mid-lactating Holstein cows with different dosage of dietary betaine. Significant differences were observed among treatments (columns not sharing a common letter, $P < 0.001$). Vertical bars represent the standard error of the mean.

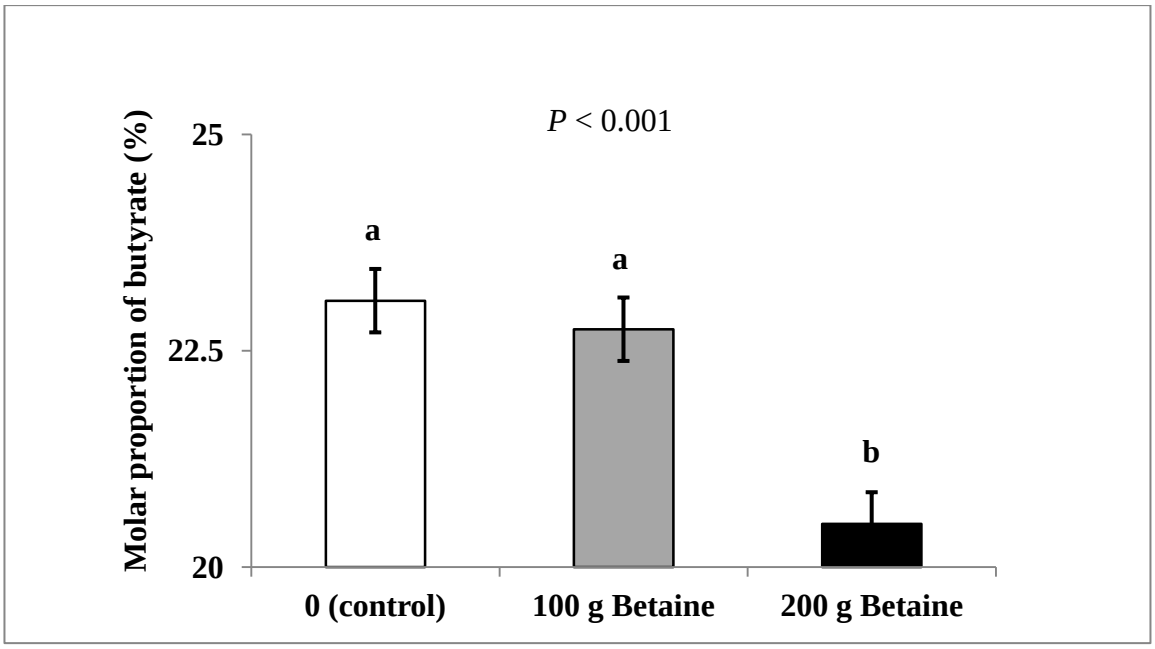


Figure 2.4: The molar proportion of isobutyrate in total identified ruminal VFA (%) from mid-lactating Holstein cows with different dosage of dietary betaine. Significant differences were observed among treatments (columns not sharing a common letter, $P < 0.001$). Vertical bars represent the standard error of the mean.

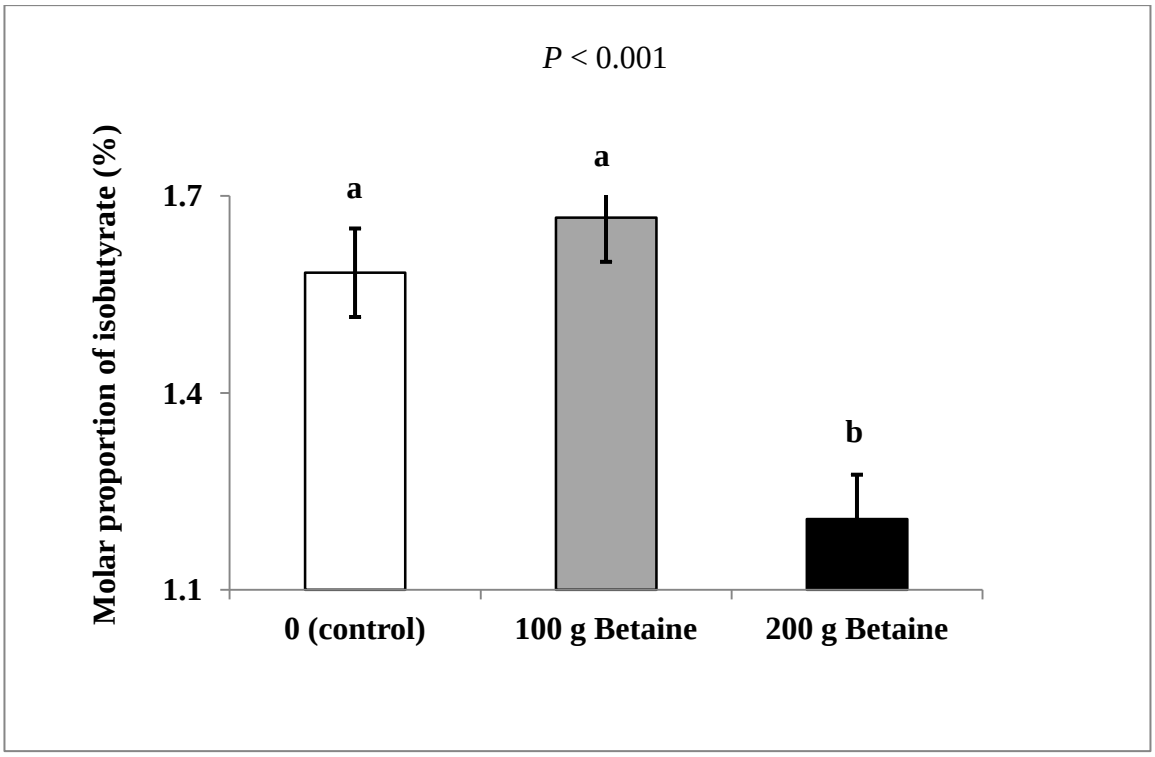


Figure 2.5: The molar proportion of isovalerate in total identified ruminal VFA (%) from mid-lactating Holstein cows with different dosage of dietary betaine. Significant differences were observed among treatments (columns not sharing a common letter, $P = 0.005$). Vertical bars represent the standard error of the mean.

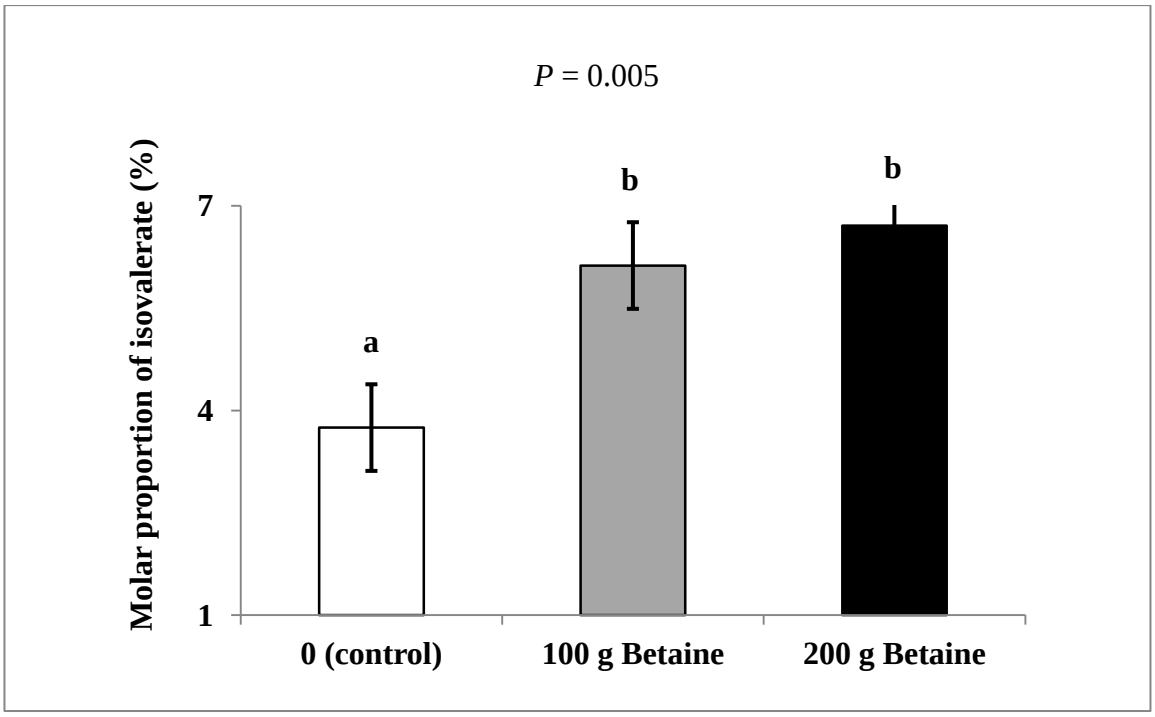


Figure 2.6: The acetate to propionate ratio from mid-lactating Holstein cows with different dosage of dietary betaine. Significant differences were observed among treatments (columns not sharing a common letter, $P < 0.001$). Vertical bars represent the standard error of the mean.

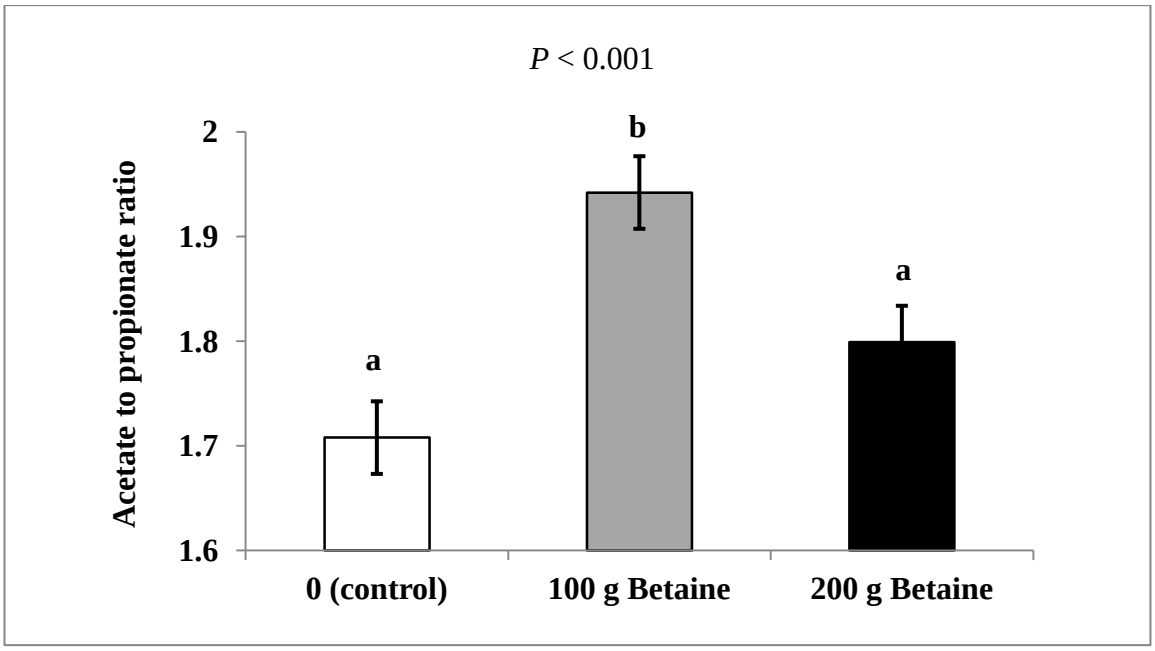


Figure 2.7: The ruminal pH from mid-lactating Holstein cows with different dosage of dietary betaine. Significant differences were observed among treatments (columns not sharing a common letter, $P < 0.001$). Vertical bars represent the standard error of the mean.

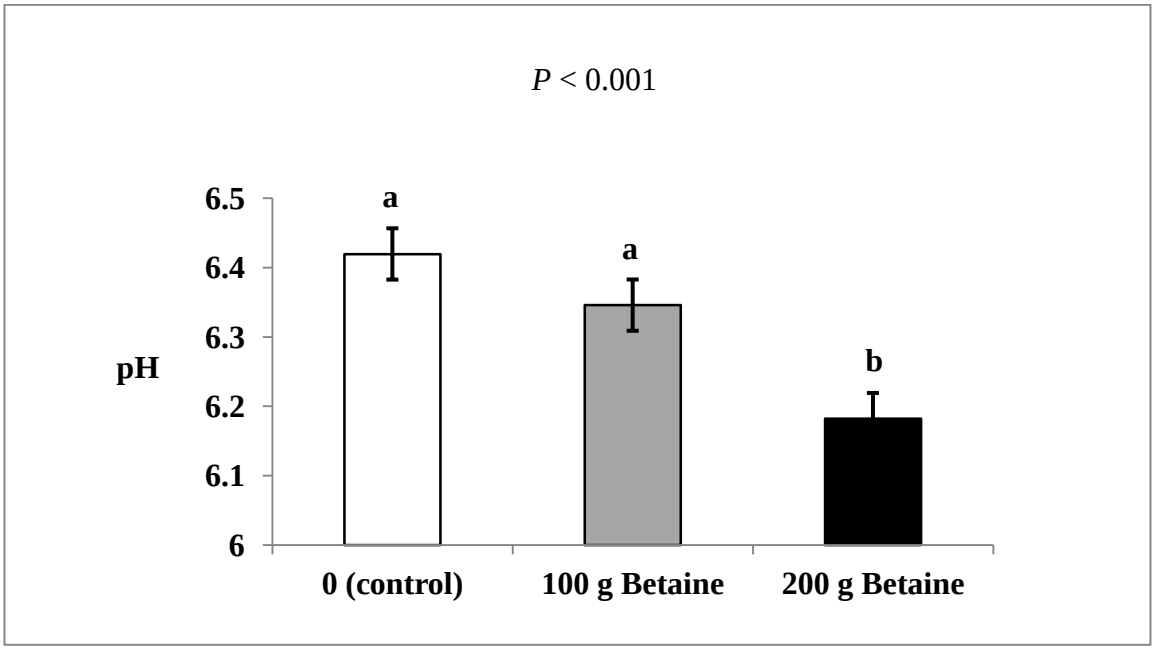


Figure 2.8: The ruminal pH from mid-lactating Holstein cows with different dosage of dietary betaine at different time points. Significant differences were observed across time points (overall time points $P < 0.001$). Vertical bars represent the standard error of the mean.

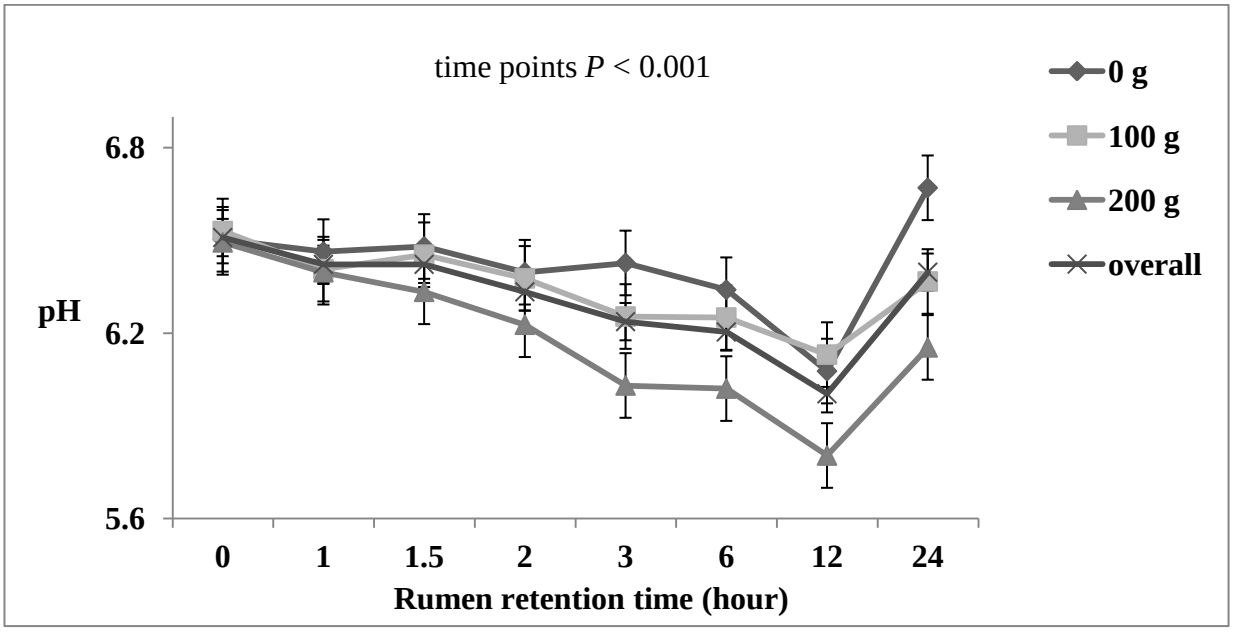


Figure 2.9: The serum MUFA (molar proportion of total FA identified) from mid-lactating Holstein cows with different dosage of dietary betaine. Significant differences were observed across treatments (columns not sharing a common letter, $P = 0.01$). Vertical bars represent the standard error of the mean.

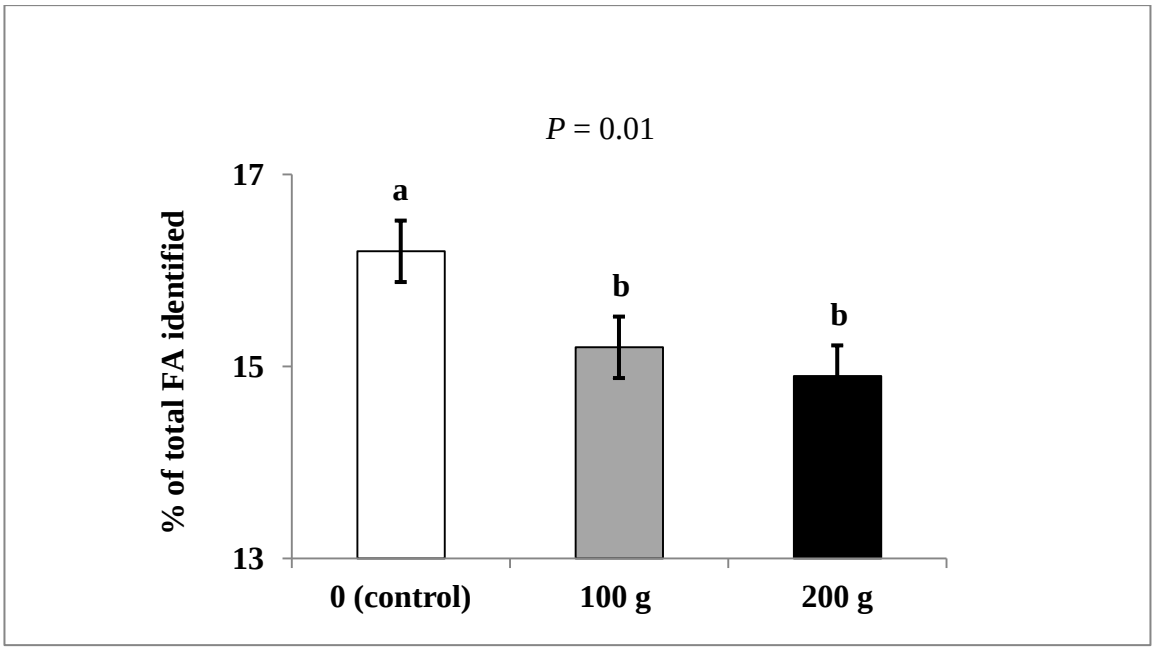


Figure 2.10: The serum C18:2 *all trans*-9, 12 (molar proportion of total FA identified) from mid-lactating Holstein cows with different dosage of dietary betaine. Significant differences were observed across treatments (columns not sharing a common letter, $P < 0.01$). Vertical bars represent the standard error of the mean.

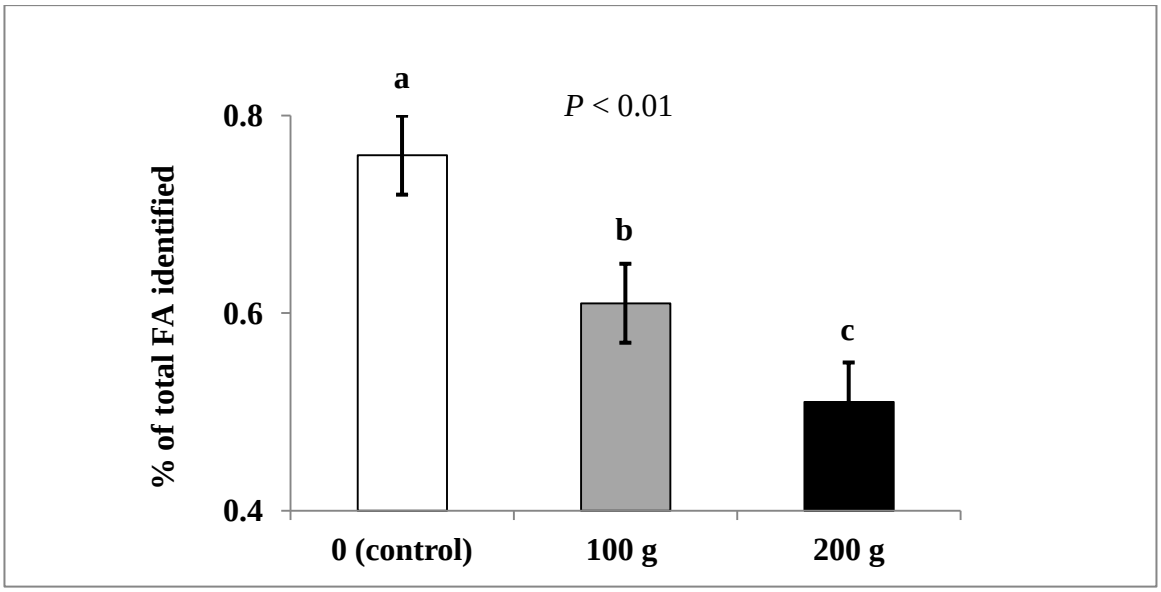


Figure 2.11: The serum total n-3 FA (molar proportion of total FA identified) from mid-lactating Holstein cows with different dosage of dietary betaine. Significant differences were observed across treatments (columns not sharing a common letter, $P = 0.02$). Vertical bars represent the standard error of the mean.

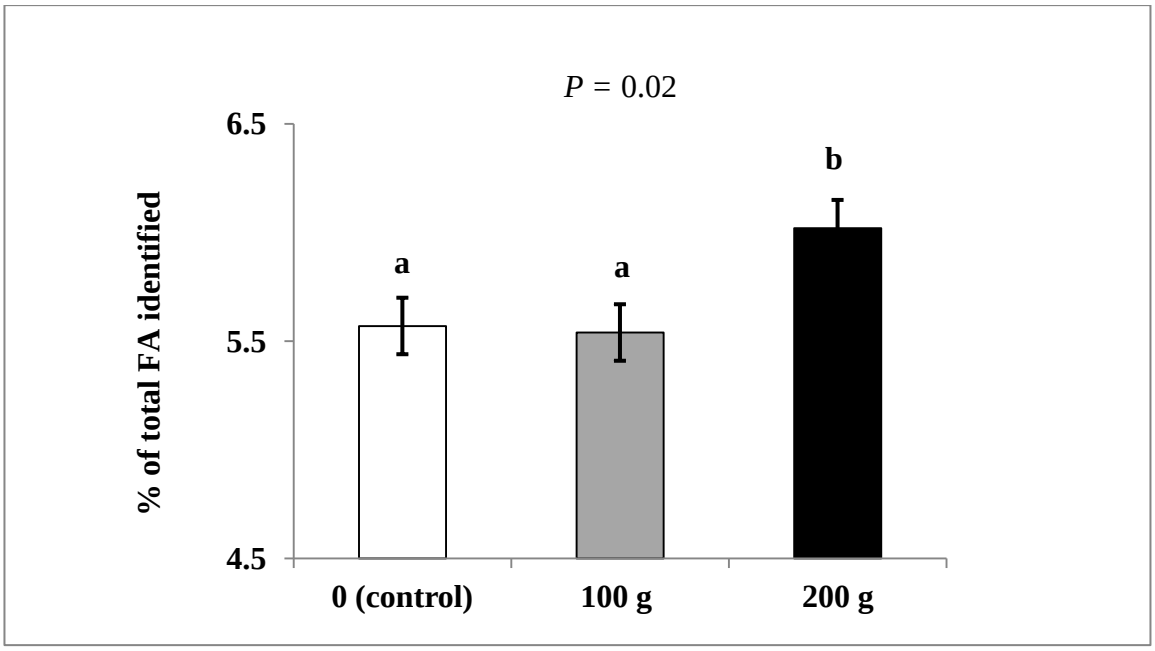
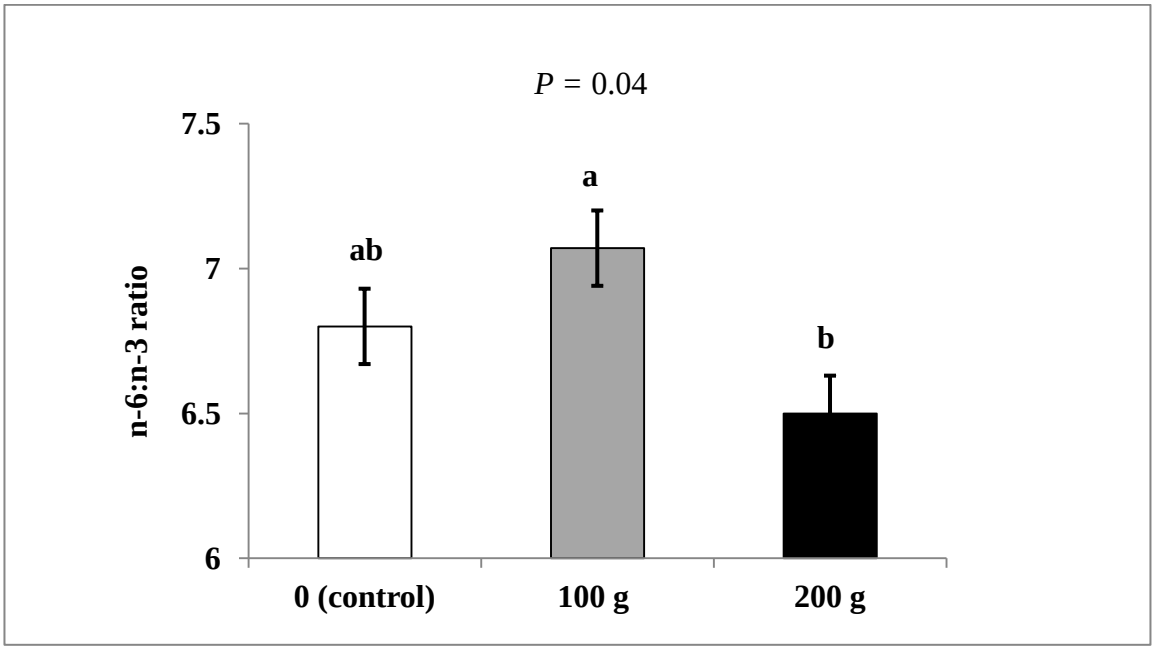


Figure 2.12: The serum n-6: n-3 from mid-lactating Holstein cows with different dosage of dietary betaine. Significant differences were observed across treatments (columns not sharing a common letter, $P = 0.04$). Vertical bars represent the standard error of the mean.



Appendix 1.

University of Idaho
Institutional Animal Care and Use Committee

Date: Tuesday, October 4, 2016

To: Pedram Rezamand

From: University of Idaho
Institutional Animal Care and Use Committee

Re: Protocol 2016-53
Effect of Betaine supplementation on total tract digestibility, rumen fermentation, and rumen microbiome of dairy cows

Your animal care and use protocol for the project shown above was reviewed and approved by the Institutional Animal Care and Use Committee on Tuesday, October 4, 2016.

This protocol was originally submitted for review on: Wednesday, September 7, 2016
The original approval date for this protocol is: Tuesday, October 4, 2016
This approval will remain in affect until: Wednesday, October 4, 2017
The protocol may be continued by annual updates until: Friday, October 4, 2019

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Craig McGowan,