Bioactive nutrients for improved metabolic function of dairy cattle

by

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Abstract

Dairy cows undergo many homeorhetic adaptations during the transition to lactation. Although many of the physiological processes - including increased lipolysis and postpartum inflammation - are adaptive, exaggerated responses can contribute to metabolic disease and reduced milk production. L-carnitine has been shown to increase hepatic oxidation of fatty acids and reduce hepatic lipid accumulation in early lactation cows; however, L-carnitine is degraded in the rumen. An experiment using 4 ruminally-cannulated Holstein heifers in a split plot design demonstrated that the relative bioavailability of L-carnitine was greater when delivered abomasally than ruminally. There was a dose × route interaction and a route effect for increases in plasma carnitine above baseline, with increases above baseline being greater across all dose levels (1, 3, and 6 g L-carnitine/d) when infused abomasally compared to ruminally. A second experiment used 56 lactating Holstein cows in a randomized complete block design to evaluate 2 rumen-protected products (40COAT and 60COAT) compared to crystalline L-carnitine at doses targeting 3 and 6 g/d carnitine. Although crystalline and 40COAT were effective in linearly increasing carnitine concentrations, only subtle responses were seen for the 60COAT, which were less than that for crystalline carnitine in plasma, milk, and urine. Ineffectiveness of rumen-protected products to increase carnitine concentrations beyond crystalline may have been due to over-encapsulation that hindered liberation of the carnitine and its absorption in the small intestine. Although L-carnitine has the potential to reduce postpartum hepatic lipidosis, effective rumen protection of L-carnitine while maintaining intestinal availability needs further investigation. Plant polyphenols have anti-inflammatory properties and when administered during the transition period, have been shown to increase milk production. An experiment used 122 multiparous Holsten cows in a randomized block design to determine the effect of short term

(5-d; **SBE5**) and long term (60-d; **SBE60**) administration of *Scutellaria baicalensis* extract (**SBE**)on whole-lactation milk yield, 120-d milk component yield, and early lactation milk markers of inflammation. Whole-lactation milk yield was increased for SBE60 compared to control, but was not different for SBE5 compared to control. Greater total pellet intake, milk lactose yield, and reduced SCC during wk 1-9 for SBE60 compared to control, all could have contributed to the observed sustained increase in milk yield. Milk production parameters were not different for SBE5 compared to control. No treatment effects were observed for BCS or milk markers of inflammation (haptoglobin) and metabolic function (β-hydroxybutyrate). Overall, long term administration of *S. baicalensis* effectively increased milk production, however the mechanism by which this was achieved is unknown. Although routes of administration to effectively achieve their physiological responses were different between L-carnitine (abomasal delivery) and SBE (feeding), both bioactive nutrients can improve the metabolic function of early lactation dairy cows.

Table of Contents

List of Figures

List of Tables

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Chapter 1 - Literature review

Plant Polyphenols in Ruminants

Plant polyphenols have been used throughout history for their medicinal properties. Recently, progressive research has begun investigation of their use in different areas of ruminant health and nutrition to enhance overall productivity of ruminants. Antibiotics have long been used for both therapeutic and sub-therapeutic purposes, including disease treatment, disease prevention, and growth promotion (Allen et al., 2013). Links between antibiotic use in food animals and antibiotic-resistant bacteria in humans and recent trends in consumer beliefs have driven government and corporate policies aimed at elimination of sub-therapeutic use of antibiotics and heavier restriction on therapeutic use (Greathead, 2003; Landers et al., 2012). Legislature regulating antibiotic use began in Europe with Regulation 1831/2003 in which antibiotics other than coccidiostats and histomonostats are no longer classified as feed additives and require veterinary prescription (Castanon, 2007). Similarly, the United States has developed the Veterinary Feed Directive, effective as of 2017, requiring a written directive from a licensed veterinarian to use medicated feed (Greathead, 2003; U.S. FDA, 2015). Since the ban in Europe, several programs have been developed to investigate the use of phytochemicals and plant bioactives as an alternative approach to enhancing animal health and efficiency (Rochfort et al., 2008).

In addition to the primary compounds considered to be the principle nutrients (fat, carbohydrates, protein), plants contain secondary metabolites that function in signaling interactions between the plant and environment, give plants their color and taste, contribute to defense strategies, and other roles (Boudet, 2007; Diaz-Sanchez et al., 2014). Polyphenols are plant secondary metabolites that have been associated with health benefits including antioxidant and anti-inflammatory activity (Balasundram et al., 2006; Middleton et al., 2000). Polyphenols

can be further classified into flavonoids and non-flavonoids based on the number of phenol rings (Rodriguez-Ramiro et al., 2016). Flavonoids consist of fifteen carbons that are arranged in a C6-C3-C6 configuration with two aromatic rings (A and B rings) joined by a 3-carbon bridge that is usually a hetercyclic ring (C ring). Variations in the hydroxylation pattern of the C ring separate the following major flavonoid classes: i) flavonols (e.g., quercetin, kaempferol, myricetin), ii.) flavones (luteolin, bacalein), iii.) flavanones (naringerin, hesperitin), iv). flavanols (catechin), v.) isoflavones (genistein), vi.) flavanonols (tavifolin), and vii.) anthocyanidins (cyanidin) (Balasundram et al., 2006; Rodriguez-Ramiro et al., 2016). It is important to understand the structure of the different polyphenol classification as many of their associated health benefits depend on structure (i.e. higher degree of hydroxylation is associated with increased antioxidant activity; Balasundram et al., 2006).

The beneficial health effects of flavonoids have been extensively reviewed in human health and have more recently been studied in animals (Di Carlo et al., 1999; Middleton et al., 2000; Keservani and Sharma; 2014). Application of polyphenols, especially flavonoids, to improve animal health and productivity is a growing area of research. The objective of this review is to discuss the use of flavonoids in calf health, efficiency of rumen fermentation, animal growth, milk production, and use during times of inflammation such as the transition period and clinical mastitis in dairy cows.

Bioavailability

Preruminant calf

Systemic availability of polyphenols, their metabolites, or both is required for direct post absorptive effects. Bioavailability of flavonoids change as a ruminant develops, due to changes in intestinal permeability, absorptive capacity, microbe establishment, and development of the

rumen. To maximize effectiveness of polyphenols, bioavailability and alterations in bioavailablity in response to the developing ruminant gastrointestinal tract must be considered. For example, the most studied flavonoid, quercetin, is readily available to the preruminant calf but is known to be degraded by rumen microbes (Berger et al., 2015; Maciej et al., 2015). The effect of reticulorumen maturation was clearly seen, as the area under curve (**AUC**) for plasma total flavonol concentration decreased for calves dosed with quercetin at 4 weeks of age compared to neonates (Maciej et al., 2015). The point at which degradation affects quercetin has been identified as 3 d up to 12 d of life (Gruse et al., 2016; Maciej et al., 2016). Quercetin supplementation increased plasma flavonol concentrations in 7 d old calves; however calves fed only formula exhibited increased plasma total flavonol concentration and greater AUC compared to calves receiving colostrum (Gruse et al., 2015). Authors attributed lesser bioavailability of quercetin in colostrum-fed calves to the fact that colostrum stimulates intestinal maturation (and therefore reduced permeability) and contains beneficial bacteria that are known to degrade quercetin. In contrast, a subsequent study by Gruse and others (2016) reported similar quercetin absorption for colostrum or formula-fed calves. There appears to be a threshold for saturation as a 5-fold larger dose (50 vs. 10 mg/kg BW) did not result in greater plasma flavonol concentrations (Gruse et al., 2015).

The form of quercetin administered, quercetin aglycone (**QA**) or its glucorhamnoside, rutin (**RU**), impacts the kinetics of absorption. Based on greater maximal plasma flavonol concentration and shorter time to maximal plasma flavonol concentration, Maciej and others (2015) identified QA as the more bioavailable quercetin source. The shorter time to maximal plasma flavonol concentration for QA suggests either altered gut kinetics or increased absorption in the proximal intestine relative to RU. The sugar moiety of flavonoids determines site of

absorption, and the more complex glycoside rutin is absorbed in the distal small intestine and colon, whereas QA is readily absorbed in the small intestine (Day et al., 1998; Hollman et al., 1999). In a subsequent study by Maciej and others (2016), calves receiving quercetin from d 2 to 26 of life reached maximal plasma flavonol concentration at 12 d for QA but plasma flavonol concentration was still increasing at study cessation (d 26) in calves receiving RU (Maciej et al., 2016). The sustained increasing plasma flavonol concentrations in response to RU in calves with a developing rumen supports the potential for RU to serve as a better source of quercetin as the rumen develops and microbial degradation becomes a larger factor.

In additional to QA, quercetin monoglucosides are efficiently absorbed by humans. The β-glycosidic bonds can be cleaved by intracellular β-glucosidases or the brush border membrane enzyme lactase-phlorizin hydrolase, making them available for small intestinal absorption. Steric hindrance of the glyosidic bond in more complex glycosides present in RU require cleavage by microflora, resulting in absorption in the distal portion of intestines in monogastrics, but also providing advantages to developed ruminants (Lesser et al., 2006). In addition to QA and RU, Maciej and others (2016) also investigated the green tea extract supplementation in newborn calves; however, plasma concentrations of individual catechins were below the detection limit. In general, catechins are less well absorbed than quercetin and are not typically glycosylated (Scholz and Williamson, 2006).

Ruminal degradation effect on bioavailability

Ruminal degradation of polyphenols has negative consequences for systemic bioavailability of some monomeric polyphenols, although microbial activity provides the opportunity for effective use of polymeric polyphenols. Polyphenols are absorbed by monogastrics and preruminant calves in the small intestine, but the monomeric form is required

for absorption (Felgines et al., 2000; Cermak et al., 2003). Compared to the neonatal calf, in which QA bioavailablity was greater than that of RU, the relative bioavailability for total flavonols from RU was nearly 8-fold greater compared with QA in adult cows. (Berger et al., 2012). Poor systemic availability of QA is attributed to rapid and extensive microbial degradation of quercetin. During an in vitro simulation, 90% of QA was degraded within the first 5 hours of incubation (Berger et al., 2015). When ruminal effects were bypassed through intraduodenal administration of QA and RU, QA clearly increased plasma flavonol concentrations over baseline while RU had no effect (Gohlke et al., 2013). As typically seen in the large intestine of monogastrics, rumen microbes can cleave the glycosidic fractions of polymeric polyphenols, thus making them available for intestinal absorption (Gladine et al., 2007). When administered intraduodenally, polymeric or glycosylated forms of polyphenols such as RU are unable to be absorbed in the small intestine, whereas QA is in a readily absorbed form.

Microbial hydrolysis of glycosidic fractions has also proven effective with supplementation of grape seed and peel extracts (**GSP**) high in proanthocyanidins. Catechin and epicatechin are the main monomeric forms of proanthocyanidines in GSP. Intraruminal administration of GSP resulted in detectable plasma concentrations of five different phenolic compounds, including epicatechin. The relatively high concentration of epicatechin and other phenolics was likely the result of microbial degradation of the polymeric proanthocyanidins. Microbial effects on the major catechins in green tea extracts (**GTE**; gallocatechin, epigallocatechin, catechin, epicatechin, epigallocatechin-gallate, epicatechin-gallate) are extensive, with catechin the only form detected at low levels in plasma after intraruminal administration in dairy cows. Intraduodenal administration of GTE increased plasma concentrations of epicatechin, epigallocatechin, and epigallocatechin gallate in a dose dependent

6

manner (Wein et al., 2016). Feeding citrus fruit extract containing more than 95% naringenin-7-rhamnoglucoside to sheep resulted in abundant naringenin in plasma (Gladine et al., 2007). Overall, like monogastrics the monomeric form of polyphenols are most bioavailable for preruminant calves, but as the rumen develops, the polymeric or glycosylated forms of polyphenols offer protection from ruminal degradation and increased bioavailability.

Polyphenols for Young Stock

Prophylactic antibiotics are commonly used in animal production beginning as early the first days of life. Medicated milk replacers are used on 49.9% of heifers raised, spanning across 57.5% of operations, to combat scours, diarrhea, and other digestive diseases that largely contribute to the 7.8% preweaned heifer death loss in 2006 (USDA, 2010). Calf health is adversely affected by weather events, inadequate nutrition, infectious agents, and the calf's passive immune system (Berge et al., 2005). Failure of passive transfer affects one in five heifer calves and a high proportion of 1 d old calves received by calf ranches have failure of passive transfer (USDA 2010). Eliminating medicated milk replacer without increasing death loss would require measurement of passive transfer and treatment with therapeutic antibiotics in cases of inadequate immunity, unless nonantibiotics alternatives are identified (Berge et al., 2005). Drackley (2008) has reviewed potential alternatives to medicated milk replacer, including use of mannan oligosaccharides, galactosyl-lactose, and a blend of fructooligosaccharides, alllicin, and gut-active microbes. Kehoe and Carlson (2015) compared a blend of animal plasma, yeast cell wall extracts, ascorbic acid, inulin, and direct-fed microbials to medicated milk replacer to assess effects on calf health and growth. Both the medicated and nonmedicated additives increased ADG and reduced fecal score compared to control calves, indicating some nonmedicated

alternatives could be beneficial to calf health and growth. To our knowledge, polyphenols and antibiotics have not been directly compared in milk replacer.

Polyphenol impacts on calf metabolism

Multiple studies have examined the effects of flavonoids on calf metabolism with the main effect involving glucose metabolism. Flavonoid supplementation in calves during the first month of life has not affected plasma non-esterified fatty acids (**NEFA**), β-hydroxybutyrate (**BHBA**), lactate, urea, albumin, triglycerides, cholesterol, insulin, glucagon, or cortisol (Gruse et al., 2016; Maciej et al., 2015; Maciej et al., 2016; Oliveira et al., 2010). Despite biochemical studies showing inhibitory effects of quercetin on intestinal glucose absorption, several in vivo calf studies have observed no effect of QA or RU on plasma glucose concentration (Gruse et al., 2016; Maciej et al., 2015; Maciej et al., 2016). Gruse and others (2015) more intensively investigated the effects of quercetin on glucose metabolism in calves receiving colostrum or not. Colostrum feeding clearly influenced glucose absorption, with higher glucose concentrations compared to colostrum-deprived calves. Although colostrum-deprived calves supplemented with quercetin exhibited increased plasma xylose concentration after xylose feeding and had higher peak labeled glucose concentration than their colostrum-deprived unsupplemented counterparts, quercetin supplementation did not fully compensate for the developmental advantages of colostrum. Quercetin also did not influence mRNA abundance of hepatic gluconeogenic or glycogenolytic enzymes independent of colostrum feeding.

Calf Health

General measures of calf health including rectal temperature, heart and respiratory rate, respiratory sounds, nasal discharge, and attitude score were unaffected by milk replacer supplemented with quercetin, green tea extract (GTE), or pomegranate extract (**POMx**; Maciej et

al., 2016; Oliveira et al., 2010). Hepatic expression of the antioxidative enzymes (catalase, glutathione peroxidase, superoxide dismutase) and oxidative stress markers (Trolox equivalent antioxidative capacity [**TEAC**], thiobarbituric acid reactive substances [**TBARS**], and ferric reducing ability of plasma) also did not differ in calves receiving quercetin (Gruse et al., 2016; Maciej et al., 2016). These measures are increased in sick or stressed calves, and calves on these studies received colostrum and were relatively healthy, so the lack of a response might be because feeding flavonoids is most beneficial for sick or stressed calves (Ahmed and Hassan, 2007; Maciej et al., 2016). Calves receiving GTE had lower incidence of respiratory and digestive disease compared to controls after being transported to a heifer rearing facility at 10 d of life (Ishihara et al., 2001).

Evidence of decreased risk of disease in response to polyphenol supplementation supports the concept that these nutrients may enhance resilience during periods of stress. Feeding fermented green tea probiotics (**FGTP**) and mixed additives (FGTP, illite, and licorice) to beef cattle from birth through 60 d postweaning did not alter white blood cell counts or leukocyte differential in either pre- or post-weaning phases (Sarker et al., 2010). On the contrary, in vitro culture of blood mononuclear cells from calves fed POMx produced more lymphocyte-derived cytokines (INF-γ and IL-4), however phagocytic killing capacity of isolated neutrophils against *Escherichia coli* was not altered. When challenged with ovalbumin (**OVA**) vaccination, calves receiving POMx produced increased amounts of anti-OVA total immunoglobulin G (**IgG**) compared to control calves. Thus there is potential for POMx to improve humoral and cell-mediated immunity (Oliveira et al., 2010). Under normal growing conditions, feeding of flavonoids extracted from propolis from 1 week of age through weaning was effective at moderating levels of circulating IgG in a dose and time dependent manner. During the first

month of age a low dose of flavonoid (7.3×10^{-5} g/kg BW) was effective at decreasing

concentration of circulating IgG. At 8 wk of age the medium dose (7.3×10^{-4} g/kg BW) most

effectively moderated circulating IgG concentration indicating a greater dose is required with

rumen development; however, there is a dose threshold as IgG concentration was increased for

calves on the high (7.3×10^{-3} g/kg BW) compared to medium dose. Authors suggested the

immune response observed could be linked to the microbiological impacts of flavonoids. By

limiting certain types of bacteria, flavonoids contribute to maintaining the integrity of the gut,

and thereby, to moderating the circulating levels of IgG (Yaghoubi et al., 2008). Green tea

extract decreased total number of intestinal bacteria, however the magnitude of reduction was

species-specific. Beneficial bacteria, *Bifidobacterium spp*. and *lactobacillus spp.* decreased

slowly whereas *C. perfringens* decreased more quickly, thus improving overall intestinal

microbial balance (Ishichara et al., 2001). As non-pathogenic diarrhea is caused by an imbalance

in intestinal microflora, the improved balance elicited by GTE resulted in reduced diarrhea

frequency.

The impact of flavonoids on severity and duration of diarrhea is greatest during the first

weeks of life and most successfully delivered in milk or by oral drench. Quercetin fed in milk

replacer for the first week of life improved fecal scores whereas POMx top-dressed on grain

starter had no effect (Oliviera et al., 2010; Maciej et al., 2016). As diarrhea incidence is most

prevalent when milk is the main source of nutrients, the mode of delivery is crucial. Following

the additional stress of transportation to a heifer rearing facility, calves receiving GTE in their

milk replacer exhibited improved fecal scores and reduced frequency of diarrhea (Ishihara et al.,

2001). When calves were colostrum-deprived and experimentally induced with enterotoxigenic

E. coli diarrhea, short-term treatment with an enteric-coated formulation of crofelemer extract

(**ECROF**) effectively decreased diarrheal duration and incidence relative to control (15.8% vs. 57.9%; Teixeira et al., 2015). In a farm with a particularly high incidence of diarrhea morbidity, calves supplemented with either high or low doses of concentrated pomegranate extract (**CPE**) (10 and 5% polyphenols as % DM) exhibited reduced diarrheal intensity and duration compared to controls. Fecal oocyte count for *Cryptosporidium parvum*, a protozoal parasite that commonly infects young dairy calves, causing enteric infection and diarrhea, was reduced by the high dose of CPE. Authors attributed this to the reduced duration and intensity of diarrhea and the possible reduction of oxidative stress by CPE (Weyl-Feinstein et al., 2014). Similarly, in lambs experimentally infected with *Haemonchus contortus,* green tea polyphenols were effective in reducing fecal egg counts and parasite burden with resultant improvements in growth (Zhong et al., 2015).

Growth

Advantageous pre-weaning effects of polyphenols on growth performance have been unusual and are mostly reported in calves struggling with health challenges. Under *E. coli*-induced diarrhea calves receiving ECROF had greater ADG during the first 10 d of life (Teixeira et al., 2015) and a reduction in *Cryptosporidium parvum* load by CPE resulted in calves with greater BW gain during the first 2 weeks of life than controls (Weyl-Feinstein et al., 2014). Caution is warranted in interpreting these results, as in both studies, flavonoids reduced diarrheal incidence and intensity. Therefore, differences in BW could be attributed to large amounts of water loss in feces of calves in other groups. Quercetin supplementation in relatively healthy calves via oral drench or in milk replacer did not influence BW, ADG, or starter intake during the first month of life (Maciej et al., 2015, 2016). When flavonoids were top-dressed or incorporated in starter pellets, no effects on pre-weaning growth measures were detected

11

(Oliveira et al., 2010; Sarker et al., 2010). This can be attributed to the majority of the calf's nutrition coming from their liquid diet, and the amount of starter and therefore polyphenol consumed was limited. When weaning was determined by a threshold of daily starter intake, high $(7.3 \times 10^{-3}$ g/kg BW) flavonoid (propolis) supplemented calves reached weaning sooner and has greater BW than control or low $(7.3 \times 10^{-5}$ g/kg BW) calves, but the medium $(7.3 \times 10^{-4}$ g/kg BW) group was not different from either high or low and control. Despite increased BW gain at 6 wk of life in the high group, BW was not different between groups at 120 d of age. These data suggest greater flavonoid supplementation eases the transition to dry feed, decreasing time on milk replacer while achieving growth at 4 mo of age (Yaghoubi et al., 2008).

Post-weaning growth performance was enhanced by flavonoid supplementation when intake was not compromised. Calves on pelleted feed including fermented green tea probiotics (**FGTP**) or mixed additives (FCTP, illite, and licorice) showed greater BW gain and ADG than illite alone and control groups (Sarker et al., 2010). Addition of POMx to starter grain resulted in decreased intake and BW with increasing doses of POMx. Both high tannin content and poor palatability of POMx could contribute to the observed reduced feed intake. As dry matter digestibility was unaffected, the reduced feed intake was the likely driver behind reduced body weight gain (Oliveira et al., 2010). Despite some alteration in rumen fermentation, young Holstein bulls on a high concentrate diet supplemented with a plant extract blend (cyanrin, gingsen, and fenugreek; Biostar, Phystosynthese, France) did not show improved BW gain compared to control bulls or those receiving monensin (Devant et al., 2007).

Fermentation

Alterations in rumen fermentation can increase overall efficiency of the animal by increasing energy digestibility and metabolizability. Similar to ionophores, some flavonoids have

antibacterial effects, selecting against gram positive bacteria (Cushnie and Lamb, 2005). Polyphenols have also been investigated for their ability to reduce methane production, either indirectly or by direct action against methanogens. As methane production accounts for 2-12% of gross energy intake losses, and selection against gram positive bacteria increases the production of propionate relative to acetate, these manipulations can both contribute to increased efficiency of the ruminant animal.

In vitro

In vitro screening of individual flavonoids, plant extracts consisting of several flavonoids, and blends of plant extracts high in flavonoids and other classes of secondary plant metabolites have been studied. When 5 flavonoid-rich plant extracts were investigated, no difference in dry matter degradability was detected until 72 h, at which point DM degradability (**DMD**) was increased for POMx but lower for *Betula schmidtii* (birch) and *Camellia japonica* (camellia) relative to control. Despite similar total flavonoid and polyphenol concentrations detected for POMx and birch extract, differences in DMD might highlight flavonoid profile differences between the two (Kim et al., 2015). Individual flavonoids quercetin and naringin showed no effect on DMD relative to control (87.9%), however DMD was reduced by 5-7% for flavone, myricetin, catechin, rutin, and kaempferol. Reduced DMD and decreased total VFA concentration by flavone, myricetin, and kaempferol were attributed to strong antibacterial effects of these individual flavonoids. Incubations with naringin, catechin, rutin, and quercetin all produced total VFA concentrations comparable to control. The lack of decrease in VFA concentration that would be expected with a decrease in DMD by catechin and rutin led authors to believe catechin and rutin are fermented and thus provide microbes with an alternate carbon source (Oskoueian et al., 2013). Bioflavex, a citrus extract high in flavonoids, and a pure form of

each of the individual flavonoid components did not alter total VFA concentrations (Seradj et al., 2014). At lesser concentrations (200-250 mg/L) quercetin had no effect on VFA concentrations, but VFA concentrations were increased by 12% with a greater dose of quercetin (500 mg/L; Lourenco et al., 2008; Berger et al., 2015). Similarly, total VFA concentrations were 11 and 13% greater when incubated with *Solidago vigaurea* extract (16.2% of DM rutin) and *P. oleracea* (kaempferol, apigenin, myricetin, quercetin, and luteolin; Broudiscou et al., 2000; Wan et al., 2013).

Due to differences in cell membrane structure of Gram positive and negative bacteria, flavonoids select against Gram positive bacteria. Incubation with flavonoids decreased populations of *Ruminococcus albus* and *Ruminococcus flavefaciens*, both Gram positive bacteria (Kim et al., 2015). As Gram negative bacteria produce more propionate, the reduction in Gram positive bacteria leads to the decrease in acetate:propionate ratio seen with flavonoids (Oskoueian et al., 2013; Seradj et al., 2014; Wang et al., 2013). Although naringin and quercetin did not affect general bacteria or fungi populations, populations of total protozoa and methanogens were decreased. Flavone, myricetin, and kaempferol decreased populations of total bacteria, fungi, protozoa, and methanogens compared to control, and catechin decreased the total population of protozoa compared to control. Flavone, myricetin, catechin, rutin, and kempferol all decreased DMD. All the flavonoids evaluated decreased methane production compared to control and extent of inhibitory action was ranked in descending order as follows: myricetin>kaempferol>flavone>quercetin>naringin>rutin>catechin (Oskoueian et al., 2013). *Equisetum arvense* extract (isoquercetin) and *Salvia officinlis* extract (luteolin-7-glucoside) decreased methane by 14.2 and 8.2%, respectively (Broudiscou et al., 2000). Similar effects were seen for *P. oleracea* and monensin as methane emission as proportion of total gas production

was decreased from 21.02% to 9.44 and 12.27%, respectively, accompanied by large population decreases for methanogens and protozoa (Wang et al., 2013). The methanogen population was reduced by different flavonoid types when incubated in inoculum from high forage, high concentrate, and a 60:40 forage:concentrate mixture (Oskoueian et al., 2013; Seradj et al., 2014, and Wang et al., 2013). The relationship between methanogens and protozoa is symbiotic, however, when analyzed by Pearson correlation coefficient, effects of plant extracts on total protozoa numbers and methane emission only equaled 0.24. Additionally, the increase in lactate-consuming *Megasphaera elsdenii* population in rumen fluid from a high concentrate diet incubated with citrus extract (Bioflavex) suggests potential preventative implications for lactic acidosis (Seradj et al., 2014). As pH was either unaffected (Kim et al., 20151; Oskoueian et al., 2013, Seradj et al., 2014) or decreased (Wang et al., 2013), observed effects on microbial population can be largely attributed to the antibacterial nature of flavonoids.

Overall, *in vitro* screening suggests the individual flavonoids myricetin, flavone, and kaempferol produce excessively severe antimicrobial effects with negative effects on fermentation; catechin and rutin have little effect, and quercetin and naringin exhibit positive effects on fermentation. Differences in antimicrobial effects can be attributed to structures of each polyphenol (Broudiscou et al., 2000; Seradj et al., 2014). Combinations of polyphenols present together in plant extracts mostly exhibited positive effects. As seen with Broudiscou's research (2000), certain plant extracts or individual flavonoids are better suited for certain outcomes than others. For example, *L. officinalis* and *S. virgaurea* were best suited for promoting fermentation while *E. arvense* and *S. officinalis* were most effective in mitigating methane production. Evidence suggesting no negative effects on fermentation is equally important, as it allows for confident use of quercetin to treat or combat health issues. In vitro results provide

valuable initial data on polyphenol interactions with rumen fermentation; however it is important to consider dosage and type of rumen inoculum used when interpreting results and designing in vivo experiments.

In vivo

Effects of flavonoids on fermentation and methane production were much less pronounced during *in vivo* studies and were influenced by diet. Berger and others (2015) detected no change to total ruminal VFA concentrations when dry cows were intraruminally administered quercetin aglycone or rutin, but lactating dairy cows supplemented rutin exhibited greater ruminal VFA concentrations (Cui et al., 2015). Similarly, Ma and others (2017) observed an increase in VFA concentration but no change in acetate to propionate ratio in sheep consuming mulberry leaf flavonoids. The increase in VFA concentrations was assumed to be either due to greater digestibility or degradation of flavonoids. Digestibility of dry matter or specific nutrients (CP, EE, NDF, total carbohydrates, non-fibrous carbohydrates) was not altered in feedlot lambs or dairy cows receiving propolis extract (Stelzer et al., 2009; Silva et al., 2014). Propolis addition to roughage based diets (72.5% roughage) for growing cattle reduced total DM digestibility and rumen pH (Prado et al., 2010). As quercetin is rapidly degraded in the rumen, degradation of flavonoids has been suggested to act as an alternative carbon source for metabolism of rumen microbes, potentially accounting for higher total VFA concentrations (Oskoueian et al., 2013; Berger et al., 2015; Ma et al., 2017). When interpreting VFA concentrations, it must be considered that in addition to changes in production, altered absorption or passage rates can also contribute to observed changes in VFA concentrations.

Methanogen and protozoan populations decreased in response to mulberry leaf flavonoids which coincided with a 12% decrease in methane emissions (Ma et al., 2017), similar to the 17 to

20% drop in methane by grape marc meal in dairy cows (Moate et al., 2014). In contrast, rutin supplementation did not alter methane emissions even under metabolic challenge induced via intake restriction (Stoldt et al., 2016). Authors attributed the lack of effect in their study to insufficient residence time to suppress methanogens while also cautioning that the relative flavonoid doses used in many in vitro studies were much higher than they used and not feasible for animal studies.

Incorporation of flavonoids in high concentrate finishing diets can improve rumen fermentation and potentially reduce risk of acidosis, however these effects did not translate to improved growth. Similar to the ionophore antibiotic monensin, supplementation of bulls on a high concentrate diet with Biostar (**BPE**; a blend of three plant extracts containing luteolin and cyanrosid) reduced rumen pH and decreased acetate to propionate ratio, however the additional growth of monensin-fed bulls was not seen with BPE (Devant et al., 2007). Heifers fed another commercial blend of flavonoids mainly composed of naringinen (Bioflex; **PEX**) while on a high concentrate diet exhibited increased VFA concentration with decreased A:P, increased pH, and a greater population of *Megasphaera elsdenii*. Thus the increased population of lactate-consuming bacteria could account for the lessened drop in pH and tendency for decreased lactate concentration in PEX heifers when acidosis was experimentally induced (Seradj et al., 2014). Similarly, AntaPE (**AntaPE**; AntaPhyt RU, Dr. Eckel, Niederzissen, Germany) limited the drop in rumen pH and decreased time spent under pH 5.6 in heifers experimentally induced with subacute ruminal acidosis). Although rumen concentrations of LPS were not different, AntaPE heifers had decreased concentrations of acute phase proteins (serum amyloid A and LPS binding protein) and decreased blood concentration of neutrophils (De Nardi et al., 2014). The reduced

17

inflammatory state in heifers challenged with subacute ruminal acidosis shows the health-promoting effects of polyphenols under challenging conditions.

Transition Cows

The transition period, defined as three weeks before and three weeks following calving, is a time of incredible metabolic stress (Drackley, 1999). Many homeorhetic adaptations occur to meet the sudden large nutrient demand for lactogenesis including increased gluconeogenesis, lipolysis, and insulin resistance. The trauma of calving induces some degree of inflammation in the days following calving, however the magnitude and persistence varies by cow and more dramatic responses have been linked to decreased production. Bradford and others (2015) recently reviewed the contributing factors, complex and integrated metabolic signaling pathways, and approaches to mitigate postpartum inflammation.

Feed intake and milk production

It has been clearly demonstrated that cows experiencing greater degrees of subacute systemic inflammation have reduced milk production and reproductive performance (Huzzey et al., 2012). Cows in the lowest quartile of liver activity index (highest degree of inflammation) produced 20% less milk during the first month of lactation (Bertoni et al., 2008). Similarly, cows with lower plasma paraoxonase produced 24% less milk over a 305-d lactation (Bionaz et al., 2007). Use of nonsteroidal anti-inflammatory drug (**NSAID**) sodium salicylate increased whole-lactation milk and fat yield in older cows (3+ parities; Farney et al., 2013b). Carpenter and others (2016) showed similar effects with short term administration (3 days) of sodium salicylate and another NSAID, meloxicam, on whole-lactation milk and protein yields on a commercial dairy farm. As use of NSAIDs during early lactation is considered off-label drug use, natural

18

alternatives such as plant polyphenols provide a more viable approach to achieving similar outcomes.

Several plant polyphenols administered during the transition period have increased milk production. Short term administration of silymarin (flavonolignans) and lycopene to cows for 7 d before expected calving to 14 DIM tended to increase milk yield during the first 21 d of lactation (Garavaglia et al., 2015). Tedesco et al. (2004b) showed that cows given silymarin from d 10 before expected calving to 15 DIM reached peak milk production 1 week sooner and had increased peak yield (41.6 ± 1.05 kg vs. 39.1 ± 1.44 kg/d) relative to control cows. Milk yield did not differ at 7 DIM, but was greater for silymarin cows at 21 and 30 DIM, as well as over 305 d ($9,922.1 \pm 215.7$ vs. $9,597.8 \pm 225.4$ kg). Longer duration grape seed and grape marc meal extract (**GSGME**) supplementation from 3 weeks prepartum to 9 weeks after calving increased milk yield during weeks 4-6, with a tendency for greater yield at weeks 3 and 7 of lactation (Gessner et al., 2015). With the same duration of treatment, Winkler and others (2015) showed an 11% increase in energy-corrected milk production for cows supplemented with a plant product consisting of green tea and curcuma extract (**GTCE**; curcumin) from weeks 2 to 9 of lactation. When only investigated from 3 weeks before to 3 weeks after calving, intraduodenal administration of quercetin did not affect milk production (Stoldt et al., 2015). Given that milk yield responses to polyphenols and NSAID are was typically seen at or after 3 weeks of lactation, it is possible the shorter timeline of the study missed possible milk production responses occurring later in lactation (Carpenter et al., 2016).

Although similar trends in milk yield were observed across studies, polyphenol type had variable effects on early lactation yield of milk components. Milk fat yield increased by 10% (1.39 vs. 1.56 ± 0.05 kg) for cows given GTCE (Winkler et al., 2015) and 13% (1.14 vs. $1.32 \pm$

0.06 kg) for cows given silymarin and lycopene (Garavaglia et al., 2015). In contrast, despite a similar duration of polyphenol administration, Tedesco et al. (2004b) observed a tendency for decreased milk fat yield in cows receiving silymarin, and GSGME had no effect on milk fat (Gessner et al., 2015). Milk protein content was even more variable, likely affected by other dietary factors, especially in studies involving cows further in lactation. Milk protein yield increased by 9% with GTCE supplementation (Winkler et al., 2015). Another study investigating the long term effects of intraduodenal administration of quercetin observed increased milk protein content during the 4 week supplementation period (Leiber et al., 2012). As the rhamnoglucoside of quercetin partly protects dietary protein from ruminal degradation, the authors speculated that intraduodenal application might reduce amino acid oxidation in the small intestine and enhance amino acid availability. Tannins, present in high concentrations in grape marc or grape marc meal, form complexes with protein that decreases ruminal protein degradation and increases protein flux in the small intestine (Patra and Saxena, 2011). Although Gessner et al. (2015) observed no effect of GSGME on early lactation milk protein yield, incorporation of ensiled grape marc in the diet of mid-lactation cows decreased milk protein yield. Silymarin with and without lycopene did not affect milk protein yield (Tedesco et al., 2004b; Garavaglia et al., 2015). Despite potential interactions of polyphenols with dietary proteins, similar increased milk protein yield has also been observed in an NSAID study and interactions with dietary proteins would not be a plausible explanation in that case (Carpenter et al., 2016). Milk lactose content was not affected by polyphenol administration at any stage of lactation (Tedesco et al., 2004b; Gohlke et al., 2013; Moate et al., 2014; Gessner et al., 2015; Stoldt et al., 2015; Stoldt et al., 2016).

Despite increased milk production in periparturient cows receiving flavonoids, dry matter intake was either not affected in these studies (Gessner et al., 2015; Stoldt et al., 20015; Winkler et al., 2015) or not measured (Tedesco et al., 2004; Garavaglia et al., 2015). During wk 2-9 of lactation when ECM was greater for cows receiving GTCE, DMI did not differ between the two groups at any time point (17.1 vs. 17.7 ± 0.59 kg/d; Winkler et al., 2015). Feeding GSGME during the transition period also had no effect on DMI (16.6 vs. 17.2 ± 0.63 kg/d) but increased milk yield and milk protein yield during wk 2-9 of lactation (Gessner et al., 2015). When administered in a TMR to mid-lactation cows and intraduodenally to transition cows, quercetin supplementation had no effect on DMI or energy balance (Stoldt et al., 2015; Stoldt et al; 2016). Authors speculated that the improved milk production, while maintaining energy balance without differences in DMI, could be potentially be attributed to improved utilization of energy and crude protein for milk production or reduced hepatic inflammation and metabolic stress (Winkler et al., 2015).

Insulin Resistance

Insulin resistance in early lactation is stimulated by inflammatory signals and contributes to the reallocation of nutrients to the mammary gland likely to support lactogenesis as well as to immune cells. Although insulin resistance is crucial, exaggerated resistance can encourage excessive lipid mobilization and impair metabolic health (Bradford et al., 2015). Zachut and others (2013) showed that dramatic body weight loss around calving was associated with insulin resistant adipose tissue and lesser milk production. In cows with less body weight loss, adipose tissue was responsive to insulin, plasma NEFA around calving was numerically lower (33%), and they produced more milk. Akt phosphorylation in the liver following a glucose tolerance test was similar for all cows. Increased insulin sensitivity of adipose tissue was also observed in mice

when quercetin, luteolin, or epigallocatechin were fed during an induced inflammatory state (Shoa et al., 2013). Insulin sensitivity of adipose tissue by EGCE supplementation was also reduced in mice fed a high fat diet (Bao et al., 2014). Quercetin has also been observed to differently regulate insulin-mediated glucose transporter 4 translocation, promoting GLUT4 translocation under inflammatory challenge through restoring insulin receptor substrate-1 (IRS-1) response to insulin, whereas under normal conditions quercetin inhibits GLUT4 translocation (Xu et al., 2014). Quercetin supplementation increased sensitivity and insulin release and lowered plasma glucose in late lactation cows during euglycemic, hyperinsulinemic and hyperglycemic clamp studies (Gohlke et al., 2013). Glucose homeostasis is maintained in part through insulin modulation of peripheral glucose uptake and hepatic glucose output (Ohtsuka et al., 2001). As no differences were detected in liver mRNA abundance of enzymes involved in gluconeogenesis or fat metabolism (*G6P*, *PC*, *PEPCK-C*, *PEPCK-M*, and *CPT1A*), authors attributed decreased plasma glucose concentration during quercetin supplementation to increased uptake of glucose by other tissues (Gohlke et al., 2013). Similar glucose responses were reported in cows receiving sodium salicylate during the first week of lactation (Farney et al., 2013a). Even though older cows (3+ parities) experienced hypoglycemia after 7 days of sodium salicylate treatment, 305-d milk yield was increased (Farney et al., 2013b). Improved insulin sensitivity of adipose tissue, and thus potentially lower plasma glucose, is associated with higher milk yield (Zachut et al., 2013; Farney et al., 2013a); however periparturient silymarin supplementation increased milk yield with no effect on plasma glucose concentration (Tedesco et al., 2004b). Despite previously discussed changes in insulin sensitivity by quercetin under inflammatory conditions, intraduodenal supplementation of quercetin during the transition period of dairy cows did not alter plasma glucose concentration. In other studies in which flavonoid supplementation

around calving increased milk yield, plasma glucose concentration was not reported (Gessner et al., 2015; Winkler et al., 2015). Contrary to flavonoid effects during early lactation, 2-wk supplementation of rutin tended to increase plasma glucose concentration (Stoldt et al., 2016).

Metabolic Parameters

The well documented postpartum increase in NEFA concentration was not altered by quercetin supplementation in the transition period (Stoldt et al., 2015), however cows receiving GTCE exhibited decreased NEFA concentrations in the first week of lactation compared to control cows (Winkler et al., 2015). Several other studies supplementing flavonoids during the transition period did not reveal differences in plasma concentrations of NEFA, triglycerides, or cholesterol (Tedesco et al., 2004a; Tedesco et al., 2004b; Gessner et al., 2015; Stoldt et al., 2015). The decreased NEFA concentrations observed in the first week coincided with decreased hepatic triacylglyceride (TAG) and cholesterol concentrations during weeks 1 and 3 in the GTCE group (Winkler et al., 2015), however no differences were observed at the same time points for cows receiving GSGME during the transition period (Gessner et al., 2015). Elevated NEFA and inflammation are two main inducers of endoplasmic reticulum (ER) stress, and ER stress in known to induce fatty liver via elevated de novo lipogenesis (Ringseis et al., 2015). Decreasing NEFA concentrations could attenuate ER stress and potentially explain the differences in lipid metabolism and hepatic lipid accumulation observed across studies.

Plasma β-hydroxybutyric acid (**BHBA**) concentrations were generally not altered by flavonoids supplemented during early or mid-lactation (Tedesco et al., 2004a; Tedesco et al., 2004b; Cui et al., 2015; Stoldt et al., 2015; Winkler et al., 2015). Cows supplemented with GSGME exhibited increased BHBA concentration during weeks 1 to 5 compared to controls (Gessner et al., 2015). Authors attributed the increase in BHBA to low glucose resulting from the

need to support increased milk yield and milk lactose yields in GSGME cows (Gessner et al., 2015).

Endoplasmic reticulum stress

Inflammation-associated factors including proinflammatory cytokines, reactive oxygen species (ROS), microbial components, and NEFA can cause endoplasmic reticulum stress. Three ER stress sensors initiate the unfolded protein response (**UPR**), which functions to restore ER homeostasis through the PERK/eIF2a/ATF4 cascade (Ringseis et al., 2015). Gessner and others (2014) have shown an ER-stress-induced UPR to occur in cows following calving, which can contribute to the pathology of insulin resistance, fatty liver (increased lipid biosynthesis, decreased fatty acid oxidation, decreased secretion of lipids by very low density lipoprotein), ketosis (induction of FGF21 that stimulates the ketone body synthesis), and cell survival (increased inflammation through the NFκB pathway and cytoprotection through activation of Nrf2; Ringseis et al., 2015).

In addition to the positive correlation between plasma NEFA concentration and hepatic mRNA abundance of *FGF21* suggesting a role in fatty liver development (Schlegel et al., 2012b), the direct induction of *FGF21* by ER stress and its elevation during the first week of lactation makes *FGF21* a good marker of ER stress in early lactation dairy cows (Schlegel et al., 2012b; Schaap et al., 2013). Periparturient supplementation of cows with either GSGME or GTCE reduced hepatic mRNA abundance of *FGF21* at weeks 1 and 3 of lactation (Gessner et al., 2015; Winkler et al., 2015), suggesting reduced ER stress. The reduction in *FGF21* aligns with a trend for reduced hepatic mRNA abundance of *AFT4*, the transcription factor regulating *FGF21* (Winkler et al., 2015). Contrary to the role of FGF21 in stimulating ketogenesis,

GSGME cows exhibiting a reduction in *FGF21* actually had greater BHBA concentrations postpartum (Gessner et al., 2015).

The hepatic mRNA abundance of another downstream transcription factor of the PERK/eIF2α/ATF4 cascade, *XBP1*, tended to be reduced during weeks 1 and 3 of lactation in cows supplemented with GSGME (Gessner et al., 2015). Although XBP1 alters transcription to upregulate target genes of NF-kB (*CRP*, *HP*, *TNF*) and Nrf2 pathways (*CAT*, *GPX3*, *MGST3*, *NQO1*, *SOD1*, *UGT1A1*), *UGT1A1* was the only target gene that tended to be reduced in GSGME cows (Gessner et al., 2015). Similarly, no difference in abundance of genes involved in inflammation or the UPR were altered by GTCE supplementation (Winkler et al., 2015). As Nrf2 is a redox-sensitive transcription factor, it works to reduce tissue sensitivity to reactive oxygen species and inflammatory conditions (Ringseis et al., 2015). Overall, mRNA abundance of one gene involved in the UPR tended to be reduced and inflammatory genes were unaffected.

Hepatic Inflammation and Lipid Accumulation

Following parturition, inflammatory cytokines released in response to tissue damage and infection, LPS released into the blood as a result of decreased gut barrier function, and reactive oxygen species (ROS) may all contribute to stimulating inflammation. LPS and fatty acids are sensed by toll-like receptors (**TLR**), resulting in the translocation of NFκB into the nucleus. Excessive infiltration of saturated fatty acids into the endoplasmic reticulum can disrupt the membrane, disrupt function including improper protein folding that causes the UPR and activates JNK, and stimulates the release of Ca^{++} ions that will cause oxidative stress in the mitochondria. Depending on cell type, the JNK activated AP-1 as well as NFκB initiate transcription of inflammation components including acute phase proteins, cytokines, and cell cycle regulators (Bradford et al., 2015).

Rapid lipolysis is an adaptive mechanism to meet the sudden nutrient demand of lactogenesis. However, excessive lipolysis can overwhelm hepatic oxidation capacity, encouraging partial oxidation of fatty acids (ketogenesis), increased peroxisomal oxidation, and reesterification into triglycerides. Interestingly, elevated NEFA concentration itself does not result in development of fatty liver or hepatic steatosis, a condition in which triglyceride storage becomes severe and impairs hepatic function. Restriction of DMI in mid-lactation to induce negative energy balance even beyond that characteristic of early lactation, did not result in hepatic triglyceride accumulation as in early lactation, suggesting other factors such as additional inflammatory challenges exacerbate fatty liver (Gross et al., 2013). The inflammatory condition of liver in postpartum dairy cows and lipid accumulation is similar to hepatic condition in individuals with non-alcoholic fatty liver disease (NAFLD).

Luteolin (flavone) reduced lipid accumulation in HepG2 cells treated to mimic NAFLD through activation of AMP activated protein kinase (**AMPK**), which reduces downstream transcriptional activation of sterol regulatory element binding protein 1, the main transcription factor regulating de novo fatty acid synthesis (Liu et al., 2011). Baicalin also enhanced phosphorylation of AMPK and its downstream target enzyme, acetyl-CoA carboxylase, a key regulator of de novo lipogenesis, in rats on a high fat diet (Guo et al., 2009). Furthermore, baicalin reduced lipid accumulation and reduced systemic inflammation, indicated by lower TNFα. In dairy cows receiving silymarin extract during the transition period, postpartum histological examination of liver biopsies revealed differences in distribution of fat, but overall hepatic fat accumulation was not altered (Tedesco et al., 2004a).

Proinflammatory cytokines trigger the acute phase response in the liver, which increases production of positive acute phase proteins (**+APP**) and reduces synthesis of negative acute

26

phase proteins (**-APP**), such as retinol binding protein (**RBP**) and albumin (Fleck, 1989). In grouping cows by a liver activity index (**LAI**), cows in the lower quartiles were characterized by lower plasma RBP and albumin and greater concentrations of +APP (Bertoni et al., 2008). Although the studies we reviewed using flavonoids during the transition period did not report effects on plasma +APP, cows receiving GSGME or GTCE had greater plasma retinol concentrations than their respective controls (Gessner et al., 2015; Winkler et al., 2015). Given that retinol is released from the liver through binding to RBP, its higher concentration is indicative of reduced liver stress (Bossaert et al., 2012). Although no effect of GSGME on albumin concentration during the transition period was seen (Gessner et al., 2015), mid-lactation cows supplemented with quercetin exhibited increased albumin concentration (Stoldt et al., 2016). Plasma urea concentration was not different between cows on any study we reviewed, regardless of stage of lactation (Tedesco et al., 2004b; Gessner et al., 2015; Winkler et al., 2015).

As fat infiltration can cause hepatocyte death, cytosolic enzymes released upon cell death such as aspartate amino transferase (**AST**), γ-glutamyl transferase (**GGT**), and glutamate dehydrogenase (**GLDH**) can be used as markers of severe hepatic dysfunction. Plasma AST concentrations were decreased in cows supplemented with quercetin during the transition period (Stoldt et al., 2015). Because AST is not liver specific, its concentration in plasma could be influenced by both hepatocyte destruction and muscle catabolism. However, no difference in creatine kinase, a cytosolic muscle enzyme, allowed authors to conclude the observed reduction in plasma AST concentration by quercetin was in response to improved liver function. Glutamate dehydrogenase is specifically expressed in the liver, had the highest degree of positive association with fatty liver, and is indicative of hepatocyte death (Ok et al., 2013). Although plasma GLDH was not significantly decreased by periparturient quercetin supplementation,

authors pointed out that the postpartum increase in GLDH was in the magnitude of 300% for control cows compared to a 50% increase for cows on quercetin ($P < 0.15$, Stoldt et al., 2015). Cows receiving silymarin during the periparturient period did not have different concentrations of GGT relative to control, however GGT levels across cows in the study were not indicative of severe fatty liver (Tedesco et al., 2004b). No difference in plasma AST or alanine aminotransferase was reported in cows supplemented with rutin for 10 wk (Cui et al., 2015). However, because stage of lactation was not specified, these animals may not have been under the same degree of metabolic and hepatic stress as in previous studies.

Oxidative and Antioxidative status

Oxidative stress results from an imbalance of pro-oxidants and anti-oxidants. Abuelo and others (2014) discussed the importance of oxidative status of the transition cow, highlighting that overproduction of ROS in early lactation is a factor contributing to dysfunctional inflammation. Flavonoid supplementation has been reported to improve antioxidative status and reduce lipid peroxidation in sheep (Gladine et al., 2007), beef cattle (Shabtay et al., 2008), and mid-lactation dairy cows (Gobert et al., 2009). When evaluated during the transition period when cows are under more severe oxidative stress, flavonoid supplementation significantly improved antioxidative status in only one of the 4 studies reviewed (Garavaglia et al., 2015). Plasma concentration of TBARS and TEAC were not different between cows supplemented with GSGME or GTCE during the transition period compared to their respective controls (Gessner et al., 2015; Winkler et al., 2015). Hepatic transcript abundance of enzymes involved in free radical defense including superoxide dismutase, catalase, and glutathione peroxidase was not different after 1 or 4 weeks of quercetin supplementation during late lactation (Gohlke et al., 2013). Although cows receiving silymarin and lycopene supplementation for 7 d prior to expected

28

calving date and 14 d following did not have different plasma concentrations of hydroperoxides, TBARS was decreased compared to controls (Garavaglia et al., 2015). Although this provides evidence of improved antioxidative status, the small sample size ($n = 20$) must be considered when evaluating implications. Differences in plant polyphenols' apparent ability to improve antioxidative status could be attributed more to differences in physiologic state (non-lactating, gestating, stage of lactation) than to differences in polyphenol type. Polyphenol structure, specifically the number and position of hydroxyl groups on the phenolic rings, does affect antioxidative activity (Balasundram, 2006). The greater number of phenolic hydroxyl groups allows for increased free radical reducing ability with stabilization of resultant hydroxyl radicals through the aromatic structures (Bors, 1990; Fraga et al., 2010).

Mastitis

Mastitis, the inflammatory response of the mammary gland to infection, represents a major source of economic loss for dairy producers and is typically treated with antibiotics. When bacteria enter the teat canal, resident leukocytes and epithelial cells release cytokines to initiate inflammation in an attempt to eliminate the bacteria, including recruiting of polymorphonuclear neutrophils (PMN). Both bacterial factors and immune reactions contribute to epithelial damage which can be detected as an increase in somatic cell count in milk (Zhoa et al., 2008). Mastitis treatment represents one of the major uses of antibiotics in dairy production. However, the disadvantages to antibiotic use including potential for pathogens to develop resistance, potential for residues in milk if misused, and inability for use in organic dairy production, and consumer fear of antibiotic resistance in the food chain, demonstrates the need for novel therapies to treat mastitis (Gomes and Henriques, 2016).

29

As bacterial infection is the most common cause of mastitis, LPS, a cell wall component of Gram negative bacteria, is used extensively to stimulate inflammatory conditions in vitro. LPS is recognized by TLR4 in mammary epithelial cells and leukocytes, which triggers the NF-κB inflammatory pathway. LPS-induced inflammation in bovine mammary epithelial cells was suppressed by baicalin or morin (flavonol) through suppression of the NF-κB and mitogen-activated protein kinase (MAPK) pathways. Flavonoids decreased phosphorylation of IκB and p65, thus inhibiting NF-κB activation. Reduction in MAPK activation was observed through reductions in p38, ERK, and JNK phosphorylation. Subsequently, mRNA of proinflammtory cytokines including *TNF-α*, *IL-6*, and *IL-1β* were reduced in cells incubated with flavonoids (Wang et al., 2016a; Yang et al., 2016). MAC-T cell incubation with heat-inactivated *Escherichia coli* and *Staphylococcus aureus*, mastitis inducing pathogens, increased *IL-6* and *IL-8* mRNA abundance (Wang et al., 2016b). Pretreatment of cells with Chinese propolis similarly reduced inflammation through the NF-κB pathway while also enhancing transcriptional activity of the Nrf2 pathway (Wang et al., 2016b).

Similar flavonoid suppression of mammary inflammation has been reported in LPS-induced rodent mastitis models. Chen and others (2015) challenged rats 3 d after parturition with intramammary LPS. Rats receiving ECGC prior and during intramammary challenge with LPS exhibited improved mammary morphology with less inflammatory infiltration and less adipose tissue volume than those only challenged with LPS. Inflammatory cytokine (IL-1β, IL-6, and TNF-α) concentrations were reduced in ECGC rats to levels similar to control. Similarly, these cytokines were reduced in LPS-challenged mice fed kaempferol or alpinetin (flavanones; Cao et al., 2014; Haijin et al., 2013). Mammary tissue from mice receiving alpinetin displayed reduced

expression of *TLR4* and both alpinetin and kaemperfol decreased phosphorylation of IκB and NF-κB p65 (Cao et al., 2014; Haijin et al., 2013).

Several different pathogens contribute to mastitis, including the noncontagious species *Streptococcus uberis*, *Streptococcus dysgalactiae*, *Escherichia coli*, and coagulase-negative staphylococcus species, as well as the contagious species *Staphylococcus aureus* and *Streptococcus agalactiae* (Zhoa et al., 2008). The antibacterial activity of flavonoids varies by species. Brazilian propolis extracts exhibited strong antimicrobial activity against *Staphylococcus aureus* strains but were weak against *E. coli* (Fiordalisi et al., 2016). Ethanolic extract of propolis reduced viability of *S. aureus* in brain heart infusion broth and milk; however, bactericidal activity was lesser in milk suggesting possible interactions with milk components (Santanta et al., 2012). In a *S. aureus*-induced mouse mastitis model, baicalin suppressed NF-kB-mediated inflammation and TLR-2 mediated apoptosis (Guo et al., 2013; Guo et al., 2014; Mengyao et al., 2013).

Although many studies have been conducted on in vitro models or LPS-induced rodent mastitis, in vivo research on the impact of polyphenols on mastitis in dairy cows has been limited. Shabtay and others (2012) evaluated concentrated pomegranate extract (**CPE**) between control and CPE cows further classified by the following 3 subgroups: mid-lactation low SCC (<150,000/mL milk) (**L-SCC**), mid-lactation high SCC (>150,000/mL milk) (**H-SCC**), and early lactation cows receiving CPE for 3 weeks before expected calving to 80 DIM. For each of the 3 subgroups receiving CPE, there was a respective control group. L-SCC cows produced 1.9% more milk and H-SCC cows produced 9.4% more milk than their respective controls. Proportion of CPE cows classified as high-SCC (>200,000/mL milk) decreased by 22.8% compared to their control H-SCC counterparts. At both the beginning and end of the experimental period,

coagulate-negative staphylococci (**CNS**) was the main pathogen identified, suggesting CPE did not exhibit antibacterial effects against CNS. When used to challenge MAC-T cells in vitro, *S. aureus* did trigger as large of an inflammatory response as *E. coli* and LPS, with no significant increase in TNF-α concentrations (Wang et al., 2016b). Increased effectiveness of flavonoids to mitigate S. aureus-induced rather than E. coli-induced mastitis suggests greater success of flavonoids for subacute and chronic cases of mastitis compared to acute infection. Cows receiving CPE during the transition period and through 80 DIM produced 6.4% more milk and the percent of cows classified as H-SCC was 36% less than their control counterparts (Shabtay et al., 2012). Early lactation SCC was also reduced in cows receiving silymarin and lycopene during the transition period (Garavaglia et al., 2015). The only other study investigating effects of plant secondary metabolites on udder health and mastitis was conducted by Hashemzadeh-Cigari and others (2014), but the herbal mixture they used mainly contained phenolic acids.

Conclusion

Reviewed literature supports effectiveness of plant polyphenols for improving ruminant health, especially during times of stress. Although polyphenols improved health, no major metabolic parameters were altered and growth was not improved in young growing stock. Some polyphenols were effective at mitigating methane production and reducing acetate to propionate ratio, but those used for their anti-inflammatory properties during the transition period did not alter fermentation. Supplementation during the transition period increased milk yield, reduced inflammatory markers, and improved liver health. Initial research in polyphenol use to combat mastitis is positive; however this use requires future investigation. Overall, it is important to remember that structural differences of these polyphenols make them effective under different situations and to varying degrees.

32

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Chapter 2 - Relative bioavailability of carnitine delivered by ruminal or abomasal infusion or by encapsulation in dairy cattle

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ABSTRACT

These studies evaluated the relative bioavailability of L-carnitine delivered by different methods in dairy cattle. In Experiment 1, 4 Holstein heifers were used in a split-plot design to compare ruminally or abomasally infused L-carnitine. The study included 2 main-plot periods, with infusion routes allocated in a crossover design. Within main-plot periods, each of 3 subplot periods consisted of 4-d infusions separated with 4-d rest periods. Subplot treatments were infusion of 1, 3, and 6 g L-carnitine/d. Doses increased within a period to minimize carryover. Treatments were delivered in two 10-h infusions daily. Blood was collected before the start of infusions and on d 4 of each infusion to obtain baseline and treatment L-carnitine concentrations. There was a dose $\times$ route interaction and route effect for increases in plasma carnitine above baseline, with increases above baseline being greater across all dose levels when infused abomasally compared to ruminally. Results demonstrated superior bioavailability of L-carnitine when ruminal exposure was physically bypassed. In Experiment 2, 56 lactating Holstein cows (143±72 DIM) were used in a randomized complete block design (blocked by parity and milk production) to evaluate 2 rumen-protected products compared to crystalline L-carnitine. Treatments were a) control, b) 3 g/d crystalline L-carnitine (crystalline), c) 6 g/d crystalline, d) 5 g/d 40COAT (40% coating, 60% L-carnitine), e) 10 g/d 40COAT, f) 7.5 g/d 60COAT (60% coating, 40% L-carnitine), and g) 15 g/d 60COAT. Treatments were top-dressed to diets twice daily. The 14-d experiment included a 6-d baseline-measurement period with the final 2 d used for data and sample collection and an 8-d treatment period with the final 2 d used for data and sample collection. Plasma, urine, and milk samples were analyzed for L-carnitine. Crystalline and 40COAT linearly increased plasma L-carnitine, and 60COAT tended to linearly increase plasma L-carnitine. Total daily excretion (milk+urine) of L-carnitine averaged 1.52 ± 0.04 g/d in

controls, increased linearly with crystalline and 40COAT, and increased quadratically with 60COAT. Crystalline increased plasma L-carnitine and milk+urine L-carnitine more than 40COAT and 60COAT. In conclusion, preventing ruminal degradation of L-carnitine increased delivery of bioavailable carnitine to cattle, but effective ruminal protection and post-ruminal bioavailability is challenging.

INTRODUCTION

Fatty liver is a metabolic disease that occurs during the first few weeks of lactation and affects up to 50% of dairy cows (Grummer et al., 1993; Jorritsma et al., 2000). Dry matter intake drops around calving, resulting in rapid lipolysis of adipose tissue to provide energy for insulin independent tissue use and to support gluconeogenesis. As non-esterified fatty acid (**NEFA**) uptake by the liver occurs in proportion to its circulating plasma concentration, it is often supplied in excess of hepatic oxidation capacity. NEFA can then be either partially oxidized to form ketone bodies or re-esterified into triacylglycerides (**TAG**; Vernon et al., 2005). Decreased abundance of very low density lipoproteins to export TAG coupled with large influx of NEFA results in TAG storage in hepatocytes. Liver lipid accumulation damages hepatocytes, decreasing metabolic function of the liver, and fatty liver has been strongly correlated with decreased health status and reproductive performance (Wensing et al., 1997; Bobe et al., 2004). Key control points for hepatic lipid metabolism include delivery of NEFA to the liver and transport into the mitochondria by carnitine palmitoyltransferase 1 (**CPT-1**; Drackley, 1999).

L-carnitine plays an essential role in the transport of long chain fatty acids (**LCFA**) from the cytosol into the mitochondria of hepatocytes (Longo et al., 2006). The conjugation of L-carnitine with LCFA by CPT1 in the outer mitochondrial membrane is considered the rate-limiting step in carnitine-dependent fatty acid oxidation (Louet et al., 2001). From 3 weeks before calving to 1 week postpartum, the concentration of free carnitine decreases while acylcarnitine concentration subsequently increases; however, total postpartum carnitine concentration (free carnitine + carnitine esters) was decreased relative to prepartum values (Schlegel et al., 2012). High fat mobilizing cows, grouped by postpartum liver lipid content, exhibited elevated concentration of acylcarnitines and decreased levels of free carnitine (Humer

et al., 2016). Cows clearly respond to levels of circulating lipid, with hepatic mRNA abundance of CPT1 greatest at day 1 of lactation, coinciding with peak levels of NEFA (Ingvartsen and Anderson, 2000; Loor et al., 2005; Schlegel et al., 2012). Increased CPT1 mRNA expression was also observed in cows with greater BCS at calving or increased postpartum liver fat content (Weber et al., 2013; Akbar et al., 2015). Increased mRNA abundance of CPT-1 and decreased free hepatic carnitine concentration suggest supplemental exogenous carnitine supply could enhance transport and therefore oxidation of LCFAs in the postpartum period.

In vitro incubation of liver slices from early lactation cows with L-carnitine increased β-oxidation of palmitate and decreased palmitate esterification (Drackley et al., 1991). Dietary administration of L-carnitine during the transition period was effective at increasing hepatic carnitine concentration, with a subsequent decrease in total liver lipid content at 10 DIM, the time of peak liver triglyceride (Drackley et al., 2005; Carlson et al., 2007b).

Carnitine is degraded in the rumen, but the extent of degradation is unknown. Abomasal and ruminal infusions of carnitine were equally effective at increasing plasma carnitine concentrations in one study, suggesting some carnitine is able to escape ruminal degradation and be available for intestinal absorption (LaCount et al., 1995). Subsequent research by LaCount and colleagues (1996b) determined degradation rate may be dependent on diet and administration time, as rumen microbes seem to adapt to carnitine supplementation by increasing degradation rate. Previous studies have assumed 80% ruminal degradation for lactating dairy cows fed crystalline L-carnitine and 25% absorption was speculated for growing steers supplemented at 2 and 3 g carnitine per day (Carlson et al., 2007b; Greenwood et al., 2000).

More research is needed to determine the extent of ruminal degradation to evaluate the necessity for a rumen-protected form of L-carnitine. The objective of Experiment 1 was to assess

46

the relative bioavailability of L-carnitine when administered at different sites and different

infusion levels, whereas Experiment 2 assessed the relative bioavailability of two rumen-

protected carnitine products compared to crystalline carnitine. Experiment 2 also determined

production responses to supplemental carnitine, including milk yield, milk components, and feed

intake.

MATERIALS AND METHODS

Experimental procedures were approved by the Kansas State University Institutional

Animal Care and Use Committee.

Experiment 1

Four Holstein heifers previously fitted with ruminal cannulas were used in a split-plot

design to assess the relative bioavailability of ruminally or abomasally administered L-carnitine.

However, one heifer was removed just prior to the end of the first treatment period due to an

intestinal blockage requiring surgery. A second heifer was removed due to an infection during

phase 2 of period 2, and the first heifer removed from the study replaced the second heifer at that

time. The study was therefore an incomplete design. Heifers were housed in a tie-stall facility

and fed a TMR once daily (Table 2.1). The diet met NRC (2001) requirements for all nutrients.

The study was conducted in 2 periods, both preceded by 2 weeks without treatment to

obtain baseline samples and for washout between periods. Each period had 3 phases, each

consisting of 4 d of infusions at a different dose of carnitine, with 4 d between phases. The

treatments were 1) ruminal infusion of L-carnitine at 1, 3, and 6 g L-carnitine/d and 2) abomasal

infusion of 1, 3, and 6 g L-carnitine/d. Each carnitine treatment also included 6 g/d of larch

arabinogalactan as a carrier, and total volume infused was 4 L/d across treatments. The dosage

used in each phase escalated, with phase 1 at 1 g/d, phase 2 at 3 g/d, and phase 3 at 6 g/d. The

site of infusion was randomized; 2 heifers received ruminal infusions in period 1, followed by abomasal infusions in period 2, and the other heifer was treated in the opposite sequence. Daily infusions (throughout each 4-d infusion) were split into 2 equal aliquots, each infused during 10-h infusion periods, allowing 2 h between infusions.

Throughout the study, feed and water intake were recorded daily with the final 3 d of each infusion phase used for analysis. TMR samples were collected every 2 wk and composited for nutrient analysis by Dairy One Forage Laboratory (Ithaca, NY; Table 1). Health was monitored daily.

Prior to the start of infusions and 1.5 h following the first daily infusion on d 4 of each phase, blood samples (coccygeal vein) were collected to obtain baseline and treatment carnitine concentrations. Plasma samples were frozen until carnitine analysis.

Experiment 2

Cows and treatments

Fifty-six mid-lactation Holstein cows (143 ± 72 DIM) were used in a randomized complete block design to determine the relative bioavailability of 2 rumen-protected carnitine products compared to crystalline carnitine. Cows were blocked by parity and level of milk production and then randomly assigned to 1 of 7 treatments within the block ($n = 8$). Cows were housed in a tie-stall facility and adapted to the facility for 4 d prior to 2 d of sample collection for baseline values. Following the 6-d baseline period, treatments were applied for a total of 8 d, with the final 2 d used for data and sample collection. The study was performed in 2 cohorts of cows.

Cows were milked 3 times daily at 0400, 1000, and 1800 h. The basal diet met NRC (2001) requirements for all nutrients and was fed as a total mixed ration twice daily (0600 and

48

1800 h; Table 2.2). Animals had ad libitum access to feed in individual mangers and feed offered was adjusted daily to achieve 12-20% refusals. During the treatment period, the basal diet was top-dressed twice daily with the following treatments: a) control (no supplement), b) 3 g/d crystalline L-carnitine (crystalline; Lonza, Inc.), c) 6 g/d crystalline, d) 5 g/d 40COAT (40% coating, 60% L-carnitine; Lonza, Inc., Allendale, NJ), e) 10 g/d 40COAT, f) 7.5 g/d 60COAT (60% coating, 40% L-carnitine coating; Lonza, Inc.), g) 15 g/d 60COAT.

Data collection and sampling procedures

During the 2-d collection periods, feed and water intake as well as milk yield were recorded. Total mixed ration samples were collected on each day of both baseline and treatment collection periods and composited for nutrient analysis by Dairy One Forage Laboratory (Ithaca, NY; Table 2.2). Health was monitored daily and one cow (7.5 g of 60COAT) was removed from the study due to illness detected by a rapid decline in dry matter intake (DMI).

Over the course of the 48-h collection period, urine and blood samples (coccygeal vein) were collected immediately prior to feeding (2000 h), and 6, 12, and 18 h after. Blood samples were collected into K_3EDTA tubes and immediately placed on ice. Plasma was separated by centrifugation (1,500 $\times$ g for 15 min) and stored in microcentrifuge tubes at -20˚C. Throughout the collection period, urine samples (200 µL) were composited within cow by equal volumes into microcentrifuge tubes and frozen at -20˚C until analysis of total carnitine and creatinine. Sample creatinine concentration and expected excretion of creatinine at 29 mg/kg of BW per day was used to estimate daily urine volume (Valadares et al., 1999). Two milk samples were collected at all 6 milkings during the 2-d collection periods, one used for milk component analysis by Heart of America DHIA (Manhattan, Kansas) and the other frozen until carnitine analysis. Prior to analyses, the milk samples were composited in equal volumes by collection period.

49

Sample Analyses

Milk samples were analyzed for concentrations of fat, true protein, lactose (B-2000 Infrared Analyzer; Bentley Instruments, Chaska, MN), MUN (MUN spectrophotometer, Bentley Instruments), and somatic cells (SCC 500, Bentley Instruments) by Heart of America DHIA (Manhattan, KS).

Plasma was analyzed using enzymatic colorimetric assays to determine concentrations of NEFA (NEFA-HR; Wako Chemicals, Richmond, VA) and BHBA (kit no. H7587-58; Pointe Scientific, Canton, MI).

Concentrations of total carnitine in plasma, milk, and urine were determined by an enzymatic radioisotope method (Carson et al., 2007a). Plasma (0.5 mL), milk (1.0 mL) and urine (1.0 mL) samples were alkalinized with 25 µL, 50 µL, and 60 µL 3 N KOH. To prevent gelling in subsequent steps, 250 µL water was added to plasma samples. Samples were mixed and incubated in a water bath at 60°C for 1 h. Samples were deproteinized by adding of 150 µL 3 M $HClO_4$ to plasma samples and 300 µL to urine and milk samples, mixing, and placing samples on ice for 10 min. After centrifugation at $10,000 \times g$ for 10 min, 500 µL of supernatant was transferred to a separate 1.5 mL micro centrifuge tube and neutralized with 73 µL 3 M KOH for plasma and urine samples and 105 µL for milk samples. After incubation on ice for 10 min, samples were centrifuged for 10 min at $10,000 \times g$. The supernatant was transferred to a clean micro centrifuge tube and frozen until analysis.

To quantify carnitine concentrations, 50 µL of supernatant, standard, or blank was pipetted into 12×75 mm borosilicate tubes in duplicate. 0.75 mL ^{14}C acetyl-CoA reaction mix (0.3 µCi ^{14}C-acetyl CoA, Moravek Biochemicals, Brea, CA), 0.75 mL HEPES-EDTA buffer (pH 7.6), 1

µL acetyl CoA stock (21 mg acetyl CoA in 1 mL deionized water), and 0.25 mg N-ethylmaleimide (Sigma Aldrich, St. Louis, MO) were added to each assay tube. 20 µL of carnitine acyltransferase enzyme (Roche Diagnostics Corp., Indianapolis, IN) was added to all tubes (except blanks) at 5- to 10-second intervals to better equalize incubation times in subsequent steps, and vortexed. After incubation for 30 min in a 37°C water bath, 0.5 mL from each assay was pipetted onto a ion-exchange column (Dowex 1×8 100-200 ION-Exchange Resin; Sigma), draining directly into a scintillation vial. Columns were rinsed 4 times with 0.5 mL deionized water, allowing each aliquot to run through completely before next addition. Scintillation cocktail (10 mL, ScintiSafe 30%, Fisher Scientific, Pittsburgh, PA) was added to each vial to count ^{14}C. Unknowns were quantified using the standard curve, with the sample blank subtracted before applying the regression equation.

Statistical Analyses

In Experiment 1, there were 3 observations for all dose × route combinations, except the 3 g/d abomasally infused carnitine, for which there were 2. Carnitine concentration was expressed as the difference between pre- and post-infusion values. Statistical analysis was performed using JMP (version 12, SAS Inst., Inc., Cary, NC). Dependent variables (feed intake, water intake, change in plasma carnitine concentration) were analyzed to determine the fixed effects of route of administration, dose of carnitine, and their interaction along with the random effects of heifer and phase within period. Contrast statements were used to statistically test linear regression coefficients with increasing doses for ruminal vs. abomasal infusions. To assess relative bioavailability, LS means were regressed against dose for the 2 infusion routes with the intercept forced through 0.

In Experiment 2, statistical analysis was performed using SAS (version 9.3, SAS Inst., Inc., Cary, NC). The mixed procedure was used to model treatment response variables using the

51

covariate for the same variable from the basal period, the fixed effects of treatment and parity, and the random effect of block. Responses were assessed with 9 contrasts that assessed the linear and quadratic responses to raw, 40COAT, and 60COAT carnitine treatments as well as overall contrasts between raw and 40COAT, raw and 60COAT, and 40COAT vs. 60COAT treatments. Significance in Experiments 1 and 2 was declared at $P < 0.05$ and tendencies were declared at $0.05 \leq P < 0.10$.

RESULTS AND DISCUSSION

Experiment 1

Feed and water intake

Water intake was not affected by carnitine infusion across dose or route (all $P > 0.40$; Table 2.3). Although not affected by route ($P = 0.13$), DMI tended to increase quadratically with carnitine dose ($P = 0.07$), being greatest for the 3 g/d carnitine. LaCount and others (1995) conducted a similar study in which water or 6 g/d carnitine was infused abomasally or ruminally in multiparous Holstein cows. Water intake was not reported, but no difference in DMI was observed for treatment or infusion site. Additionally, no effects on DMI were observed when carnitine was abomasally infused at 0, 3, 6, or 12 g/d (LaCount et al., 1996a) or 20 g/d (Carlson et al., 2006).

Plasma Carnitine Response

Plasma carnitine response to ruminal or abomasal carnitine infusion is reported as the difference between baseline and treatment concentrations in Table 2.3. A dose × route interaction was observed ($P = 0.045$), which can largely be attributed to the linear increase in plasma carnitine concentrations with increased dose for abomasal infusion, without a significant effect for ruminal infusions. A route effect was observed ($P = 0.005$) with carnitine being more

bioavailable across all dose levels when infused abomasally compared to ruminally. Increases in plasma carnitine concentrations in response to ruminal infusion appeared to plateau at 3 g/d.

To further characterize the relative bioavailability of carnitine via these 2 routes of administration, a dose-response analysis was conducted (Figure 2.1). This assessment suggests that the relative bioavailability of carnitine is greater when supplied to the abomasum vs. the rumen. It should be noted that this assumes that increases in plasma concentration are directly related to the amount of carnitine absorbed.

Our observed linear increase in plasma carnitine concentration in response to abomasal infusion aligns with the linear increase observed when 3, 6, or 12 g/d carnitine was abomasally infused in mid-lactation cows (LaCount et al., 1996b). Although plasma carnitine concentrations increased linearly, the 12 g/d dose only increased plasma carnitine by approximately 3 nmol/mL; therefore authors identified 6 g/d as the most effective and efficient dose (LaCount et al., 1996b). Contrary to the route difference we observed, LaCount and others (1995) reported similar plasma carnitine increases when carnitine (6 g/d) was infused ruminally or abomasally relative to control. Our least ruminally infused dose (1 g/d) did not change plasma carnitine concentrations, similar to the slight 4 to 5% increase when 0.875 and 1.75 g carnitine was top-dressed on diets of mid-lactation cows (LaCount et al., 1996a). The increase in plasma carnitine concentration was linear, indicating increased effectiveness of greater doses (3.5 and 7 g/d; LaCount et al., 1996a).

The plateau in plasma carnitine concentration at 3 g/d ruminal infusion in our study was unexpected; however, the small sample size ($n = 3$) and large standard error (4.54 µM) relative to the reported difference in baseline and treatment plasma carnitine concentrations at 1, 3, and 6 g/d carnitine ruminally infused/d (-0.57, 12.33, and 9.04 µM, respectively) must be considered. Numerically decreased carnitine concentration in response to 1 g/d ruminally infused carnitine

could be impacted by sequence of treatments, given that adaptation of ruminal microbes may enhance carnitine degradation after a longer period of exposure (LaCount et al., 1996a). Also, percent degradation increases and percent of carnitine absorbed decreases as amount of carnitine ingested increases (Rebouche et al., 1991). Harmeyer and Sporleder (2003) suggested carnitine is not absorbed across the rumen wall, as in vitro incubation of rumen epithelium with high concentrations of carnitine (10 times blood concentrations) did not result in carnitine transport into the bathing media. Therefore, transport of carnitine across the rumen epithelium at 6 g/d is unlikely (Carlson et al., 2007b). The similar increase in plasma carnitine concentration by dietary administration of 50 or 100 g/d carnitine suggests either saturation of intestinal absorption or the high dose provided carnitine above renal reabsorption capacity (Carlson et al., 2007b). Plasma carnitine concentration increased slightly between 6 and 12 g/d abomasally infused carnitine (67.5 vs 64.8 ± 5.4 nmol/mL), but urine carnitine concentration increased by 8-fold at 12 g/d compared to 4-fold at 6 g/d relative to control (LaCount et al., 1996b). Considering our investigated doses appear to be below the threshold suggested by literature, it is unlikely that saturation of ruminal or intestinal absorption are limiting carnitine bioavailability at either route.

Experiment 2

Feed intake and milk production

Production responses to dietary administration of crystalline carnitine, 40COAT, or 60COAT are summarized in Table 2.4. A quadratic effect on water intake was observed with 40COAT, but water intake was not affected by other treatments. The 60COAT product linearly decreased DMI ($P = 0.02$) and crystalline tended to linearly decrease DMI ($P = 0.07$). Dietary carnitine supplementation at doses ranging from 0 to 7 g/d during late lactation did not reduce DMI (LaCount et al., 1996b), however dietary supplementation at a high inclusion rate (100 g/d)

during the periparturient period reduced intake for the first 2 wk of lactation (Carson et al., 2007b). Neither crystalline nor rumen-protected carnitine (**RPC**) fed to feedlot lambs (White et al., 2000; Solhjoo et al., 2014) or RPC administered to mid-lactation dairy cows (Tasdemir et al., 2011) affected DMI.

Dietary carnitine supplementation had no effect on milk yield or yields of milk fat, protein, and lactose. Although crystalline carnitine supplementation by either feeding or infusion did not alter milk yield in most studies (LaCount et al., 1995a, 1996a, b; Carlson et al., 2006), Tasdemir and others (2011) reported a 5% decrease in milk yield in mid-lactation cows fed RPC. However, interpretation of those results in hindered by small sample size ($n = 8$) and low production animals (19.6 kg milk/d). Dietary administration of a high dose (100 g/d) of crystalline carnitine during the periparturient period resulted in a 22% decrease in milk yield compared to control, but the decrease corresponded to the lesser DMI (Carlson et al., 2007b).

Crystalline had a quadratic effect on milk protein percent ($P = 0.04$). The 60COAT product tended to decrease milk protein content ($P = 0.10$) and increase milk lactose content ($P = 0.08$) compared to 40COAT. High dose (100 g/d) carnitine tended to increase milk protein concentration relative medium dose (50 g/d), whereas carnitine supplementation has otherwise not significantly affected milk protein percent or yield (LaCount et al., 1995, 1996a, b; Carlson et al., 2006; Tasdemir et al., 2011). A quadratic effect on milk fat concentration was observed for 60COAT ($P = 0.04$); milk fat concentration was decreased at both doses relative to control. Feeding carnitine at increasing doses (0.875 to 7 g/d) tended to decrease milk fat yield and decreased yield of total solids, resulting in the tendency for decreased FCM (LaCount et al., 1996a). Interestingly, milk fat yield and FCM yield were less in cows abomasally infused compared to ruminally infused with carnitine, and solids-non-fat concentration was increased for

55

carnitine-infused cows compared to control (LaCount et al., 1995). Discrepancies in milk yield and composition response could be attributed to differences in carnitine source, dose, and protection, as well as stage of lactation and production level of the cows.

Metabolic Parameters

No treatment effects were observed for plasma NEFA or BHBA concentrations (all $P >$ 0.15; Table 2.6). Our results are in agreement with previous research in which carnitine administration via the rumen or abomasum to mid- or late-lactation cows did not alter NEFA or BHBA concentrations (LaCount et al., 1995, 1996a, b). Considering lipid mobilization is typically minimal during mid-lactation, it is not surprising that effects on NEFA and BHBA were not observed. However, carnitine supplementation also did not influence plasma NEFA concentrations during feed restriction or early lactation, situations in which fatty acid mobilization is increased (Carlson et al., 2006, 2007b). Intravenous infusion of L-carnitine did not affect plasma BHBA concentration in healthy mid-lactation cows; however, carnitine did decrease plasma BHBA and NEFA concentrations in cows with spontaneous ketosis and feed restriction induced negative energy balance (Erfle et al., 1971). Growing calves supplemented with carnitine linearly increased pre-feeding plasma NEFA concentration, but linearly decreased NEFA 6 h after feeding (Greenwood et al., 2001). The response suggests increased fatty acid oxidation with carnitine during a relatively fasted state but an interaction with dietary components that enhanced lipolysis. In that study, plasma BHBA concentration was not affected by carnitine supplementation, suggesting incomplete oxidation of fatty acids was not influenced by increased fatty acid concentrations (Greenwood et al., 2001).

Plasma Carnitine Response

Plasma, urine, and milk carnitine concentrations were used to evaluate 2 rumen-protected products (40COAT and 60COAT) compared to crystalline (Table 2.5). Although there were no parity effects on baseline-adjusted plasma, milk, or urine carnitine concentrations, and only a tendency for a parity effect on total daily carnitine excretion ($P = 0.10$), baseline plasma carnitine concentrations were greater ($P < 0.001$) in primiparous compared to multiparous cows (161.3 ± 20.0 vs. 137.4 ± 14.1 µM). Plasma samples were collected at 0, 6, 12, and 18 h after feeding to assess diurnal variation; however, there was no effect of time on plasma carnitine concentrations ($P = 0.23$). Supplementation with crystalline carnitine or 40COAT increased plasma carnitine concentrations linearly ($P < 0.001$ and $P = 0.01$, respectively) whereas 60COAT tended to linearly increase plasma carnitine ($P = 0.08$). At 3.5 and 7 g carnitine/d, LaCount and others (1996a) reported 11 and 21% increases in plasma carnitine concentration which is slightly less than the 14 and 42% increases seen in our study feeding 3 and 6 g crystalline/d.

Despite the 40COAT and 60COAT products providing rumen protection in preliminary in vitro incubations (data not shown), crystalline increased plasma carnitine compared to both 40COAT ($P = 0.03$) and the 60COAT ($P < 0.001$). Absence of additional bioavailability with RPC was also observed by White et al. (2002); feedlot lambs supplemented with either RPC (20% carnitine) or unprotected carnitine (50% carnitine) had similar increases in plasma carnitine concentrations. Unfortunately, these results were confounded as doses were not adjusted for actual amount of carnitine present in each product. Rumen-protected carnitine fed to lambs at doses ranging from 0.25 to 10 g/d increased plasma carnitine concentrations relative to control, but there was no dose effect (Walker et al., 2005). Similar effects of crystalline and RPC

observed across studies could potentially be due to hindered release of L-carnitine from

protective coatings, affecting its intestinal absorption.

Excreted carnitine

Milk carnitine concentrations were linearly increased by all sources. There was a

tendency for a quadratic dose effect with 40COAT ($P = 0.08$) with milk carnitine concentration

being similar between control and 5 g L-carnitine/d, but increasing with 10 g L-carnitine/d.

Crystalline increased milk carnitine concentration compared to 60COAT ($P < 0.01$) and tended

to increase it compared to the 40COAT ($P = 0.08$). These effects were mirrored in daily milk

carnitine output, with linear effects for all forms of supplementation (all $P < 0.001$), significantly

greater amounts for crystalline than for 60COAT ($P < 0.01$), and a tendency for greater amounts

for crystalline than for 40COAT ($P = 0.08$).

Total milk carnitine concentrations were increased by ruminal or abomasal infusion and

dietary administration of carnitine (LaCount et al., 1995, 1996b; Carlson et al., 2007a, 2007b).

The quadratic effect observed by LaCount and others (1996b) showed a large increase in milk

carnitine concentration by 3, 6, and 12 g/d infusion (258.8, 370.3, 388.0 μM), similar to the

effect in plasma. Carnitine transport into milk in mid-lactation cows mainly occurs via simple

diffusion and therefore closely relates to plasma concentrations (Shennan et al., 1998). Milk

carnitine is additionally regulated by a high affinity sodium-dependent transport system which

supports its increased concentration in early lactation (Shennan et al., 1998). Dietary carnitine

supplementation (50 and 100 g/d) increased early lactation milk carnitine concentration, but after

supplementation ceased at 21 DIM, milk concentrations were no longer different at week 6 of

lactation (Carlson et al., 2007b). As expected, our observed milk carnitine concentrations were

58

less than those reported in response to abomsal infusion (20 g/d) or early lactation dietary supplementation (50 or 100 g/d; Carlson et al., 2007a, b).

Urine carnitine concentrations increased linearly with the crystalline ($P = 0.03$) and 40COAT ($P = 0.02$). Urinary carnitine excretion increased linearly for all sources ($P < 0.03$). Similarly, urine carnitine concentration and output increased linearly with abomasal infusion (LaCount et al., 1996b). Although carnitine concentration increased linearly in plasma, milk, and urine, the marginal increase between 6 and 12 g/d was lesser in plasma and milk and greater in urine. Thus, at high levels, excess carnitine was excreted in urine.

Total excreted carnitine (milk + urine carnitine) increased linearly for all sources ($P < 0.0001$) and was greater for crystalline carnitine than for 40COAT and 60COAT ($P = 0.02$ and < 0.001, respectively). A quadratic effect was observed for 60COAT ($P = 0.03$), as total amount of excreted carnitine was similar for control and the 7.5 g dose, but increased when 15 g was supplied. Overall, total excreted carnitine increased with increasing supplementation.

CONCLUSION

Carnitine is likely degraded in the rumen, and although the extent of degradation remains unknown, our findings clearly indicate abomasal administration of carnitine results in superior bioavailability compared to ruminal administration. The linear increase in plasma carnitine concentration in response to abomasal infusion does not support a limitation in intestinal absorption across the doses tested. Although crystalline and 40COAT were effective in linearly increasing carnitine concentrations, only subtle responses were observed for 60COAT, which were less than that for crystalline carnitine in plasma, milk, and urine. Ineffectiveness of rumen-protected products in increasing carnitine concentrations more than crystalline may have been due to over-encapsulation that hindered liberation of the carnitine and its absorption in the small

intestine. Effective rumen protection of L-carnitine while maintaining intestinal availability needs further investigation.

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TABLES AND FIGURES

Table 2.1 Ingredient and nutritional composition of the basal diet (Experiment 1).

Item	Value
Ingredient, % of DM	
Alfalfa hay	21.0
Grass hay	1.7
Corn silage	16.1
Wet corn gluten feed[1]	25.7
Cotton seed	4.4
Fine rolled corn	20.4
Micronutrient premix[2]	10.7
Nutrient, % of DM (unless otherwise specified)	
DM, % as-fed	53.5
CP	17.9
Soluble protein, % CP	40.5
ADF	24.75
aNDF	43.8
Lignin	4.55
NFC	26.75
Starch	17.9
Ether extract	4.75

[1]Sweet Bran (Cargill Inc., Blair, NE)

[2]Premix consisted of 58.6% expeller soybean meal (SoyBest, Grain States Soya, West Point, NE), 11.7% limestone, 10.3% sodium bicarbonate, 7.32% Ca salts of long-chain fatty acids (Megalac R, Arm & Hammer Animal Nutrition, Princeton, NJ), 2.34% magnesium oxide, 1.83% XP Yeast (Diamond V, Cedar Rapids, IA), 1.46% stock salt, 1.46% trace mineral salt, 1.46% potassium chloride, 1.46% vitamin E premix (48 kIU/g), 0.91% Biotin 100 (ADM Alliance Nutrition, Quincy, IL), 0.62% NiaShure (65% niacin, Balchem Corp., New Hampton, NY), 0.25% selenium premix (0.06%), 0.23% 4-Plex (Zinpro Corp., Eden Prairie, MN), 0.15% vitamin A premix (30 kIU/g), 0.12% Zinpro 100 (Zinpro Corp.), 0.06% Rumensin 90 (Elanco Animal Health, Greenfield, IN), 0.04% vitamin D premix (30 kIU/g), 0.01% ethylenediamine dihydriodide premix (3.65% I).

Table 2.2 Ingredient and nutritional composition of the basal diet (Experiment 2).

Item	Concentration
Ingredient, % of DM	
Corn silage	35.0
Alfalfa hay	14.2
Wet corn gluten feed[1]	27.3
Cotton seed	2.7
Fine-rolled corn	13.7
Micronutrient Premix[2]	7.0
Nutrient, % of DM (unless otherwise specified)	
DM, % as-fed	49.9
CP	17.5
Soluble protein, % CP	36.3
ADF	23.3
aNDF	36.3
Lignin	4.1
NFC	33.0
Starch	16.2
Ether extract	4.9
NE_L[3], Mcal/kg	1.65

[1]Sweet Bran (Cargill Inc., Blair, NE)

[2]Premix consisted of 54.6% expeller soybean meal (SoyBest, Grain States Soya, West Point, NE), 14.8% limestone, 10.9% sodium bicarbonate, 7.8% Ca salts of long-chain fatty acids (Megalac R, Arm & Hammer Animal Nutrition, Princeton, NJ), 2.49% magnesium oxide, 2.34% stock salt, 1.95% XP Yeast (Diamond V, Cedar Rapids, IA), 1.56% trace mineral salt, 1.56% vitamin E premix (48 kIU/g), 0.97% Biotin 100 (ADM Alliance Nutrition, Quincy, IL), 0.26% selenium premix (0.06%), 0.24% 4-Plex (Zinpro Corp., Eden Prairie, MN), 0.16% potassium chloride, 0.16% vitamin A premix (30 kIU/g), 0.12% Zinpro 100 (Zinpro Corp.), 0.07% Rumensin 90 (Elanco Animal Health, Greenfield, IN), 0.05% vitamin D premix (30 kIU/g), 0.01% ethylenediamine dihydriodide premix (3.65% I).

[3]Estimated according to NRC (2001)

Table 2.3 Effect of carnitine infusion on intake and plasma carnitine concentration (Experiment 1).

Item	Ruminal infusion			Abomasal infusion			SEM[1]	P-value		
	1g	3g	6g	1g	3g	6g		Dose	Route	Dose × Route
DMI, kg/d	18.0	18.8	18.9	16.8	19.0	17.4	1.0	0.07	0.13	0.35
Water intake, L/d	7.7	8.0	8.6	8.3	9.2	7.9	0.7	0.66	0.58	0.41
Change in plasma carnitine[2], µM	-0.6	12.3	9.0	4.5	20.5	35.9	4.8	0.099	< 0.01	0.045

[1]Reported SEM is pooled across route and dose levels.

[2]Plasma concentrations reported are the difference between baseline and treatment concentrations.

Table 2.4 Effect of carnitine supplementation on performance and milk production parameters (Experiment 2).

Item	Control	Raw		40 coat		60 coat		SEM[1]
		3 g	6g	5g	10g	7.5g	15g	
DMI,[2] kg/d	27.21	26.93	26.16	26.83	26.33	26.25	25.78	0.89
Water intake,[3] L/d	128.2	131.4	128.4	136.8	120.6	125.8	125.9	5.6
Milk, kg/d	44.8	45.0	44.7	44.7	45.1	43.0	44.2	1.1
Milk fat,[4] %	3.66	3.53	3.47	3.50	3.47	3.31	3.48	0.11
Milk protein,[5] %	2.86	2.91	2.84	2.90	2.88	2.86	2.85	0.04
Milk lactose, %	4.92	4.94	4.92	4.92	4.89	4.95	4.95	0.03
Milk somatic cell linear score	1.61	1.46	1.96	1.22	1.53	2.01	1.21	0.48
Milk urea nitrogen, mg/dL	13.34	13.41	13.33	13.04	13.10	13.10	13.35	0.30
Milk fat, kg/d	1.64	1.55	1.56	1.59	1.52	1.43	1.55	0.07
Milk protein, kg/d	1.28	1.31	1.26	1.30	1.30	1.23	1.26	0.04
Milk lactose, kg/d	2.20	2.22	2.20	2.20	2.20	2.12	2.19	0.06

[1]Reported SEM is pooled across treatment groups
[2-5]Significance for treatment contrasts declared at $P < 0.05$.
[2]Linear effect of 60% coating product
[3]Quadratic effect of 40% coating product
[4]Quadratic effect of 60% coating product
[5]Quadratic effect of raw carnitine

Table 2.5 Least squares means for concentrations of L-carnitine in plasma, milk, and urine from mid-lactation Holstein cows fed different amounts and sources of L-carnitine (Experiment 2).

Item	Control	Raw		40 coat		60 coat		SEM[1]
		3 g	6g	5g	10g	7.5g	15g	
Plasma, µM[2,3,6,7]	8.59	9.80	12.17	9.36	10.46	8.62	9.77	0.47
Milk								
µM[2,3,4,7]	137.5	166.4	174.3	145.6	176.1	143.2	161.8	5.31
g/d[2,3,4,7]	0.97	1.17	1.22	1.05	1.22	0.99	1.15	0.03
Urine								
µM[2,3]	9.63	10.37	11.47	10.02	11.63	9.93	10.74	0.62
g/d[2,3,4,7]	0.557	0.617	0.701	0.587	0.644	0.557	0.629	0.03
Total excreted carnitine,[8] g/d[2,3,4,5,6,7]	1.52	1.78	1.92	1.62	1.87	1.54	1.79	0.04

[1] Reported SEM is pooled across treatment groups
[2-7]Significance for treatment contrasts declared at $P < 0.05$.
[2]Linear effect of raw carnitine
[3]Linear effect of 40% coating product
[4]Linear effect of 60% coating product
[5]Quadratic effect of 60% coating product
[6]40% coating product vs. raw carnitine
[7]60% coating product vs. raw carnitine
[8]Milk plus urine carnitine

Table 2.6 Blood metabolite concentrations from mid-lactation Holstein cows fed difference amounts and sources of L-carnitine. Least square means were not different for control, crystalline, 40COAT, and 60COAT (Experiment 2).

Item	Control	Crystalline		40COAT		60COAT		SEM[1]
		3g	6g	5g	10g	7.5g	15g	
NEFA, μM	95	105	98	103	98	101	100	6.2
BHBA, μM	802	793	772	816	779	811	813	41.5

[1]Reported SEM is pooled across treatment groups

Figure 2.1 Marginal plasma carnitine responses to carnitine infusion differ by infusion route (Experiment 1). Differences in plasma carnitine concentrations (post minus pre-infusion concentrations) are plotted against infusion amount. The slopes differ between infusion routes (*P* = 0.02), reflecting greater apparent bioavailability for abomasally-delivered carnitine compared to ruminal infusion.
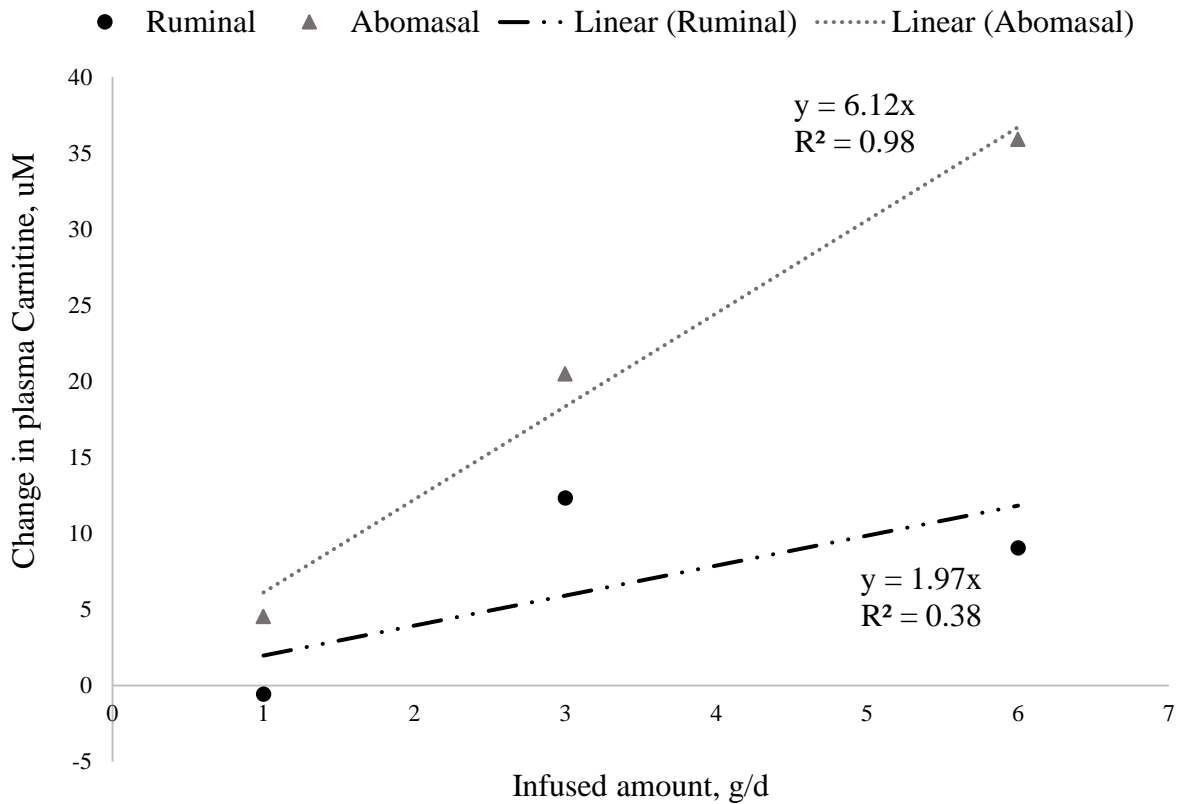
Chapter 3 - Effect of dietary supplementation of *Scutellaria baicalensis* extract during early lactation on milk production of dairy cattle

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ABSTRACT

Multiparous Holstein cows ($n = 122$) were used in a randomized block design to determine the effect of short term and long term postpartum administration of *Scutellaria baicalensis* extract (**SBE**) on 305-d milk yield, 120-d milk component yield, and early lactation milk markers of inflammation and metabolic function. Treatments were 1) control, 2) short term (5-d) administration of the SBE (**SBE5**), and 3) long term (60-d) administration of the SBE (**SBE60**). Treatments were included in a treatment pellet that was identical in ingredient source and composition except for the extract, and control and treatment pellets were provided via an automated milking system. Milk samples were collected on d 1, 3, and once during d 5-12 of lactation, followed by weekly sampling for the remainder of the 120-d collection period. Milk samples collected in the first 2 wk were used for biomarker analysis (haptoglobin and β-hyroxybutyrate [**BHBA**]), and all samples were used for composition analysis. Cows were body condition scored every 2 wk prepartum and postpartum. Milk production, programmed pellet allocation, and actual provision of both pelleted feeds were recorded daily. Results were analyzed with mixed models that accounted for repeated measures over time, and significance was declared at $P < 0.05$. There was no difference in daily treatment pellet feeding between SBE5 and SBE60 for the first 5 d of lactation. Total pellet intake was greater for SBE60 than SBE5 and control cows during the treatment period (wk 1-9), but was not different during the carryover period (wk 10-36). No treatment effects were observed for BCS, milk haptoglobin, or milk BHBA. Whole-lactation milk yield was increased for SBE60 compared to control, but SBE5 did not differ from control. Milk yield showed a treatment × week interaction with both SBE5 and SBE60 being increased compared to control during wk 4-6 and 9-11, but SBE60 being greater than both SBE5 and control during wk 15-20, 23, 26-27, and 34-35. Milk lactose and fat

yields were significantly greater and milk protein yield tended to be greater for SBE60 than control. SBE60 decreased somatic cell count (**SCC**) compared to control during wk 3-5 and 8. SBE5 did not affect SCC. In conclusion, despite no detected treatment effects on BCS or milk biomarkers of inflammation and metabolic status, supplementation of postpartum dairy cows with *Scutellaria baicalensis* extract for 60 d was effective at increasing milk yield.

INTRODUCTION

Inflammation during the transition period of dairy cows has been well established and was recently reviewed (Bradford et al., 2015). Many conditions during the transition to lactation may contribute to inflammation, including metabolic disease, infectious disease, and environmental stressors (Drackley, 1999). Early lactation inflammation, indicated by elevated postpartum serum haptoglobin, has been associated with reduced milk yield and reproductive performance (Huzzey et al., 2012). When allocated into quartiles based on week 1 plasma haptoglobin concentrations, cows in the upper two quartiles had negative shifts in energy metabolism, although cows in the middle quartiles suffered the greatest decline in DMI and BW loss continued through week 9 of lactation (McCarthy et al., 2016). Cows in the highest quartile for liver activity index (LAI; based largely on plasma acute phase proteins) produced 20% more milk in the first month of lactation compared to those in the lowest quartile (Bertoni et al., 2008).

Previous research has shown administration of the nonsteroidal anti-inflammatory drug (**NSAID**) sodium salicylate during the first week of lactation increases whole-lactation milk and fat yield in older cows (3+ parities; Farney et al., 2013). Carpenter and others (2016) showed similar effects of a short term administration (3-d) of sodium salicylate and another NSAID, meloxicam, on whole-lactation milk and protein yields on a commercial dairy farm. As use of NSAIDs during early lactation is considered off-label drug use, natural alternatives such as plant polyphenols are being investigated. Several flavonoids fed during the transition period have increased whole-lactation milk yield (Tedesco et al., 2004), increased whole-lactation ECM yield (Winkler et al., 2015), and reduced liver damage (Stoldt et al., 2015).

Baicalin is a major flavonoid derived from the *Scutellaria baicalensis* plant and has long been associated with anti-inflammatory and antioxidant properties in Chinese herbal medicine. In

vitro studies have shown pretreatment of RAW cells (a macrophage-derived cell line) with baicalin reduced lipopolysaccharide- (**LPS**) induced nitric oxide production in a dose-dependent manner, increased intracellular superoxide dismutase, and attenuated the increased production of proinflammatory cytokines (Liu et al., 2008). Similarly, when used in the pretreatment of bovine mammary epithelial cells, baicalin decreased LPS-induced cellular inflammatory responses and apoptosis (Yang et al., 2016). The objective of this study was to determine the effect of short term (5-d) and long term (60-d) administration of *Scutellaria baicalensis* extract (**SBE**) after calving on milk yield and milk markers of inflammation and metabolic function. Secondary outcomes examined were effects of SBE on milk components and somatic cell count.

MATERIALS AND METHODS

Cows and treatments

Multiparous Holstein cows ($n = 122$) on a commercial farm were used in a randomized block design to determine the effect of short term (5-d) and long term (60-d) postpartum administration of SBE on 301-day milk yield and early lactation milk markers of inflammation and metabolic function. Cows were blocked by parity (2 and 3+), calving date, and risk factors (high risk block: calving difficulty score ≥ 3 or twins), then randomly assigned within block to one of three treatments. Prepartum cows were housed in pens of approximately 10 cows on a bedded straw pack. Upon calving, cows were moved into a fresh pen where they had free access to an automatic milking system (**AMS**; Austronaut A3, Lely Ltd., Maassluis, the Netherlands), but were encouraged through the AMS if their voluntary attendance was less than 3 visits that day. After 3 to 5 days, unless experiencing health issues, cows were moved to one of three free-stall barns with voluntary access to three AMS units within their pen. Cows were managed per site SOP.

Assigned milking frequency, defined as the number of AMS visits a cow is allowed to make daily, was adjusted throughout lactation on an individual cow basis accounting for production level and stage of lactation. Targeted milking frequency was determined by an algorithm accounting for both time interval and optimum expected yield per milking. Time interval between allowed visits was based on the maximum number of daily milkings which ranged from 3.5 to 6. Milking attendance was monitored twice daily and cows identified for inadequate milking frequency (last AMS visit > 8 h before) were moved to the robot for milking.

Cows were fed a partial mixed ration (**PMR**) twice daily and were provided with pelleted concentrate feed in the AMS. *S. baicalensis* extract (Groupe CCPA, Janze, France) was combined with the dairy's standard robot feed formulation and pelleted. The control and treatment pelleted feeds were stored in two feed bins that independently supplied the milking robots. Treatments were 1) control ($n = 39$), 2) short term (5-d) administration after calving of the Scutellaria pellet ($n = 42$; **SBE5**), and 3) long term (60-d) administration after calving of the Scutellaria pellets ($n = 40$; **SBE60**). Treatments began within 24 h after calving. All cows received the control pellet, with the amount based on stage of lactation and milk production. Treatment cows were allocated 1.8 kg of the treatment pellet (delivering 100 g test material/d) in place of an equal amount of control pellet across all milkings for either 5 or 60 d. During the first 50 d of lactation total pellet allocation is based solely on DIM, with cows allocated 3.6 kg from 0-20 DIM and 5.4 kg from 21-50 DIM. From d 51 until 2 weeks prior to dry off, total pellet allocation was based on a feed table which incorporated milk production as a factor. Cows producing < 27.2 kg milk were allocated 4.5 kg pellet, whereas pellet allowance for cows producing between 27.2 and 65.8 kg milk ranged from a minimum of 4.5 to a maximum of 7.3 kg, with the exact amount determined according to the following equation (pellet allocation (kg/d) = 0.033 × milk yield (kg/d) + 2.524). The feeding

program distributed the target amount of treatment feed across the average number of daily milkings per cow. Due to the nature of AMS, voluntary deviations from a cow's average number of milkings resulted in slight excesses or shortfall in actual provision of pellet compared to the targeted allocation, and instances in which not all the feed allocated for that particular milking was dispensed were recorded as rest feed. Reported pellet intake is the difference between total pellet allowance and rest feed.

Data collection and sampling procedures

The PMR, control pellets, and treatment pellets were sampled biweekly and; composited by month for nutrient analysis by Dairy One Forage Laboratory (Ithaca, NY). Nutrient analyses are reported as averages across the study for the PMR in Table 3.1 and the pelleted feeds in Table 3.2.

Milk samples were collected on days 1, 3, and once during days 5-12 of lactation, followed by weekly sampling for the remainder of the 120-d collection period, into vials containing a preservative tablet (2-bromo-2-nitropropane-1,3diol). Milk samples collected in the first 2 wk of lactation were allocated for biomarker analysis (haptoglobin and β-hydroxybutyrate [**BHBA**]) and component analysis; subsequent samples were used only for composition analysis. Subsampled milk was centrifuged at $2,600 \times g$ for 10 min. Skim milk was transferred to microcentrifuge tubes and frozen 20˚C until biomarker analysis.

Cows were scored every 2 wk for body condition on a 5-point scale [1 = extremely thin to 5 = extremely obese (Edmonson et al., 1989)] from wk -3 to wk 17 relative to calving. Daily milk production, DIM, number of milkings per day, programmed feed daily allocated and feed provided for both pelleted feeds, and rumination data were recorded on an individual cow basis and collected using the management software, Time for Cows (**T4C**; Lely). Cows all wore neck

collars containing rumination monitors (Qwes-HR, Lely) with a microphone that uses the distinctive sounds of rumination and regurgitation to generate data surrounding rumination. A reliability score (1-100%) is assigned to daily data and indicated the proportion of the day in which sound bites were recorded. Only rumination data with ≥ 80% reliability was used for analysis. Culling data were reported in PC Dart (Dairy Records Management Services, Raleigh, NC) by the farm staff.

Sample Analyses

Milk samples were analyzed for concentrations of fat, true protein, lactose (B-2000 Infrared Analyzer; Bentley Instruments, Chaska, MN), MUN (MUN spectrophotometer, Bentley Instruments), and log10-transformed somatic cell count (SCC 500, Bentley Instruments) by MQT labs (Kansas City, MO).

Milk samples were analyzed for haptoglobin (ELISA kit #2410-7; Life Diagnostics, West Chester, PA). The preservative tablet was validated to have no effect on the results of the assay (data not shown). Prior to BHBA analysis, milk samples were deproteinized. Milk samples were first alkalinized (pH > 9) with 50 µL 3 M KOH; this step was inadvertent but did not impact the analyte of interest, so we subsequently proceeded with the protocol as intended. A greater amount of sample (1000 µL vs. 500 µL) was used for d 1 samples than d 3 and 9 samples as total solids in colostrum samples interfered with the amount of supernatant in subsequent steps. Samples were incubated in a water bath at 60˚C for 1 h, then deproteinized by adding either 600 µL (1 DIM samples) or 300 µL (3 and 9 DIM samples) 3 M $HClO_4$, mixing, and placing on ice for 10 min. After centrifugation at $10,000 \times g$ for 10 min, 500 µL of supernatant was transferred to a separate 1.5 mL micro centrifuge tube and neutralized with 105 µL 3 M KOH. After incubation on ice for 10 min, samples were centrifuged for 10 min at $10,000 \times g$. The supernatant

was transferred to a clean micro centrifuge tube and frozen until analysis by enzymatic kit (kit #H7587-58; Pointe Scientific Inc., Canton, MI).

Statistical Analysis

Milk yield, milk composition, milking frequency, pellet offered, rumination time, and body condition score (BCS) data were summarized by week relative to calving for statistical analysis. Milk yield, milk composition, milking frequency, and total pellet intake were analyzed separately for the 60-d treatment period (wk 1-9) and the carryover period. Statistical analysis was performed using SAS (version 9.4, SAS Institute., Cary, NC) to model the fixed effects of treatment, week, parity and two-way interactions of these variables, as well as the random effects of barn and cow. Additional variables (including their treatment interactions), were also tested and removed when they did not contribute significantly to the model ($P > 0.10$). Additional variables tested included risk block (high risk vs. low risk), BCS category (< 4 vs. ≥ 4 at calving), and month of calving. Body condition score and month of calving were not retained in any model. Values with Studentized residuals > 4 or < -4 were removed as outliers. Because there was a lesser proportion of high-risk cows ($n = 3$ per treatment) than low-risk cows, variables for which risk or its interactions remained in the model were analyzed again with risk removed from the model to generate unbiased LS means and SEM; *P*-values from the full model were used for assessing treatment effects and interactions. Repeated measures within cow were modeled with autoregressive and heterogeneous autoregressive covariance structures, and the one with the least Bayesian information criterion was selected for each dependent variable. Repeated measures within cow for BCS and milk haptoglobin were modeled with spatial power covariance structures because of unequal spacing of time points.

Values for milk haptoglobin were log-transformed prior to statistical analysis; reported data were back-transformed. The statistical analysis of BHBA and haptoglobin in milk samples taken during d 5-12 of lactation were analyzed with day as a median value of d 9. Analysis using the exact day of the third sample (d 5-12) did not reveal any treatment effect or interaction (all $P > 0.20$), so there was no impact on interpretation (results not shown).

Treatment effects were evaluated by contrasts between control and SBE60 and control and SBE5 for both the treatment (d $1 – 60$) and carryover periods. If an interaction with time was observed, contrasts within week were evaluated using the SLICE option of PROC MIXED.

RESULTS

Treatment provision and total pellet offered

Test material delivered for the first 5 DIM is shown in Figure 3.1, with no difference between SBE5 and SBE60 observed ($P = 0.41$; 80.78 and 83.08 $\pm$ 0.34 g/d, respectively). There was an effect of DIM ($P < 0.001$) as cows adapted to the AMS; however, there was no treatment $\times$ DIM interaction ($P = 0.94$). Mean test material provision for SBE60 ranged between 92.15 and 97.82 g/d during wk 1-9 of lactation. Pellet feeding records (T4C) confirmed that no treatment feed was allocated to control cows nor to SBE5 cows after d 5 of lactation. Total pellet offered over the first 63 DIM (Table 3.3) differed by treatment and week, and had a treatment $\times$ week interaction (all $P < 0.001$; Figure 3.2). Pellet offered was greater for SBE60 cows compared to control cows during wk 1-9 ($P > 0.001$) and tended to be increased across wk 1-36 ($P < 0.10$). No overall treatment effect was seen from $64 – 252$ DIM ($P = 0.25$); however, there was a treatment $\times$ week interaction ($P < 0.001$) with greater amounts of pellet offered to SBE60 than control in wk 10-13, 15, 16, and a tendency for difference in wk 17. Daily rumination time through 120 DIM was not different for control cows compared to either SBE5 or SBE60 from

both wk 1-9 and wk 10-17 (all $P > 0.55$) and no treatment × week interaction observed ($P = 0.39$; Table 3.3).

Milk production and composition

Milk yield did not differ between SBE5 and control either during wk 1-9 ($P = 0.35$) or wk 10-43 ($P = 0.73$). Milk yield tended to increase for SBE60 compared to control during wk 1-9 ($P = 0.07$) and was significantly increased during wk 10-43 ($P = 0.04$). An overall treatment × week interaction was observed with tendencies for differences during wk 4-6, 9-11, 15-16, 22, 24, and 28 and significant differences in wk 17-21, 23, and 26-27 (Figure 3.3). Whole-lactation milk yield (305-d) was 11,245, 11,608, and 12,664 ± 465.3 kg for control, SBE5, and SBE60, with significant differences between SBE60 and control ($P = 0.03$), but not between SBE5 and control ($P = 0.60$). Milking frequency was not affected by either SBE5 ($P = 0.60$) or SBE60 ($P = 0.19$) during the first 63 DIM, but milking frequency was increased for SBE60 during the carryover period compared to control ($P = 0.04$) whereas no difference was detected between SBE5 and control ($P = 0.48$). As expected, milking frequency differed by week ($P < 0.001$), but no overall treatment × week interaction was observed ($P = 0.11$). Despite the difference in milking frequency, milk yield per milking did not differ by treatment during the treatment or carryover periods (all $P > 0.65$).

Milk composition data during the first 17 wk of lactation are summarized in Table 3.4. There were no treatment effects on milk fat or protein content during the treatment or carryover periods (all $P \geq 0.15$). Milk lactose concentration tended to be increased for SBE60 compared to control during the treatment period ($P = 0.06$), but not the carryover period ($P = 0.25$), and was not different for SBE5 compared to control during either wk 1-9 or wk 10-17 ($P = 0.54$ and 0.46, respectively). Milk fat yield was increased in SBE60 during both the treatment and carryover

period compared to control (both $P = 0.04$), whereas SBE5 was not different from control in either period (both $P \geq 0.50$). Milk protein yield tended to be increased for SBE60 compared to control in the treatment period ($P = 0.09$) and was statistically greater during the carryover period ($P = 0.01$), but again did not differ between SBE5 and control ($P \geq 0.13$). Milk lactose yield was increased for SBE60 but not SBE5 compared to control during the treatment period ($P = 0.03$ and 0.26, respectively). During the carryover period, milk lactose yield continued to be greater for SBE60 compared to control ($P = 0.02$), and SBE5 tended to increase milk lactose yield compared to control ($P = 0.07$). There was a tendency for an overall treatment × week interaction for milk lactose yield ($P = 0.08$) with significantly greater values for SBE60 compared to control during wk 5-6 and 8-11, and tendencies for increases during wk 4, 14, and 15. Milk lactose yield was also greater for second lactation cows compared to cows in lactation 3+ (2.31 vs. 2.15 ± 0.06 kg/d; $P = 0.03$).

Somatic cell count was decreased by SBE60 compared to control during the treatment period ($P = 0.02$) with a tendency for a difference in wk 3 and significant effects in wk 4-6 and 8 (Figure 3.4). SBE5 did not affect SCC ($P = 0.37$) during wk 1-9, and neither SBE5 or SBE60 affected SCC during the carryover period ($P = 0.29$ and 0.13, respectively).

Overall there was no treatment effect on BCS ($P = 0.44$) with means being 3.40, 3.30, and 3.31 ± 0.06 for control, SBE5, and SBE60. As anticipated, body condition score differed by week ($P < 0.001$), but there was no treatment effect on prepartum or postpartum BCS (treatment × week: $P = 0.57$). Treatment means for BCS from 3 wk prior to calving and through 29 wk of lactation are shown in Figure 3.5.

Milk markers of inflammation and metabolism

Neither milk haptoglobin nor milk BHBA showed significant treatment effects ($P = 0.97$ and 0.89, respectively) or treatment $\times$ DIM effects ($P = 0.45$ and 0.47). Milk haptoglobin concentrations were greatest the day after calving (when inflammation is greatest) and subsequently declined for d 3 and d 5-12 milk samples ($P < 0.001$). BHBA concentration also had a DIM effect ($P < 0.0001$), increasing from d 1 to d 5-12 samples.

DISCUSSION

The objective of the study was to determine the effects of *Scutellaria baicalensis* extract supplementation during early lactation on whole lactation milk production. The main flavonoids of SBE, baicalin, baicalein, wogonin, and wogonoside are known to have anti-inflammatory properties (Wang et al., 2013). The effect of time for test material delivered during the first 5 DIM is attributed to AMS adaptation during the first 2 DIM. From d 3 of lactation onward, the target mean dose of test material was successfully delivered by the AMS.

Scutellaria administration for 60 d effectively increased whole-lactation milk yield compared to control cows, whereas milk yield was not significantly altered in response to 5 d of supplementation. Treatment differences for SBE60 began at wk 4 of lactation, encompassed peak milk (wk 5), and progressed beyond the end of the treatment period, with nearly 6 kg/d increase in peak milk compared to control (51.8, 50.0, 45.9 $\pm$ 1.96 kg/d). Similarly, cows administered the flavonoid silymarin from d 10 before expected calving to 15 DIM peaked 1 wk earlier and had increased peak yield (41.6 $\pm$ 1.05 vs. 39.1 $\pm$ 1.44 kg/d). Milk yield did not differ at 7 DIM, but was greater for silymarin cows at 21 and 30 DIM, as well as overall 305 d (9,922.1 $\pm$ 215.7 vs. 9,597.8 $\pm$ 225.4 kg; Tedesco et al., 2004). Administration of silymarin and lycopene to cows 7 d

before expected calving to 14 DIM tended to increase milk yield during the first 21 d of lactation (Garavaglia et al., 2015).

Longer duration of grape seed and grape marc meal extract (**GSGME**) supplementation from 3 wk prepartum to 9 wk after calving also increased milk yield during wk 4-6, with a tendency for greater yields at wk 3 and 7 of lactation (Gessner et al., 2015). Treatment for the same duration caused an 11% increase in ECM for cows supplemented with green tea and curcuma extract (**GTCE**) during wk 2-9 of lactation. When only investigated from 3 wk before to 3 wk after calving, intraduodenal administration of quercetin did not affect milk production (Stoldt et al., 2015); however, given that milk yield responses to polyphenols are typically seen at or after wk 3 of lactation, the shorter timeline of that study may have missed milk production responses occurring later in lactation (Carpenter et al., 2016).

Although previous polyphenol studies differed in duration of treatment administration, supplementation ranging from as little as 1 wk prior to expected calving to 14 d postpartum tended to increase milk yield. Even shorter postpartum treatment administration of NSAID (3 d) was effective at increasing whole lactation milk yield (Carpenter et al., 2016). Despite the long-term impact of polyphenols or NSAIDs with treatment durations less than 60 d, milk yield response of SBE5 did not differ from either control. Therefore, it is possible 5 d administration of SBE increases milk production, but our observed response was insufficient to reach significance.

Considering total pellet offered increased for SBE60 2 wk before milk yield differences, greater consumption of high energy concentrate feed could have contributed to increased milk yield. Although total pellet allocation during the first 50 d of lactation was determined solely based on stage of lactation, there are scenarios in which daily total pellet allowance does not

match daily pellet intake, and thus can result in the treatment effect observed for pellet intake. Daily pellet allowance is divided into equal amounts across the average number of milkings for individual cows. Decreased pellet intake may result from failed or shortened milkings, or cases in which the cow visits the AMS fewer times than her average milkings per day. As a result, steadily increasing milking frequency can result in pellet intake repeatedly exceeding daily allowance, albeit by a small amount. Although milking frequency was not statistically different between SBE60 and control when analyzed from wk 1-9, it is possible that slight increases in milking frequency for SBE60 cows could have contributed to the increased total pellet intake. After 50 DIM, feed allowance was based on milk yield, so after that point, increased milk yield of SBE60 cows triggered increased pellet allocation during the last 2 wk of the treatment period and throughout the carryover period. Although pellet provision was greater in SBE60 compared to SBE5 or control cows, the additional energy provided could not account for the magnitude of difference detected in milk yield. During d 1-63, SBE60 cows produced 4.46 kg more daily milk than control cows which equates to 2.58 Mcal per day (0.69 Mcal/kg milk; NRC, 2001). The additional 0.42 kg pellet intake for SBE60 cows only supplies an additional 0.82 Mcal/d. Although not measured in this study, it is likely SBE60 cows consumed more PMR. In previous polyphenol studies the observed increases in milk yield were not matched by increases in DMI. Overall DMI during wk 2-9 was not influenced by periparturient feeding of GSGME (16.6 vs. 17.2 ± 0.63; Gessner et al., 2015) or GTCE (17.1 vs. 17.7 ± 0.59; Winkler et al., 2015). Additionally, DMI did not differ between treatments at any specific time point during wk 2-9 in either study.

Similar to the treatment differences observed for milk yield, milk lactose yield was greater in SBE60 compared to control, but not different between SBE5 and control. Timing of

the observed differences in milk lactose yield also coincides with increases in milk yield. Increased milk lactose synthesis could possibly indicate some interaction of Scutellaria extract with glucose metabolism, thereby increasing the amount of glucose available to the mammary gland for lactose synthesis. Investigation of intraduodenal quercetin supplementation on glucose metabolism revealed no difference in hepatic mRNA abundance of enzymes involved in gluconeogenesis; however, no difference in lactose content was detected in that study (Gohlke et al., 2013). Although those findings were in late lactation cows, previous studies supplementing polyphenols during the transition period also did not observe difference in milk lactose yield (Tedesco et al., 2004; Stoldt et al., 2015). Similar to our observations, Garavaglia and others (2015) observed a tendency for increased milk lactose yield (1.61 vs. 1.81 ± 0.07 kg/d) during the first 21 DIM for cows receiving silymarin and lycopene during the transition period.

Long term administration of Scutellaria also increased milk fat and protein yield. In previous transition cow studies, polyphenols either had no effect on milk protein yield (Tedesco et al., 204, Garavaglia et al., 2015; Gessner et al., 2015) or increased milk protein by 9% (Winkler et al., 2015). Effects on milk fat yield tended to vary more in those studies, including decreased milk fat in cows receiving silymarin (Tedesco et al., 2004), no effect with GSGME supplementation (Gessner et al., 2015), and 10 to 13% milk fat yield increases by GTCE (Winkler et al., 2015) and silymarin and lycopene (Garavaglia et al., 2015), respectively.

Increased mammary gland function indicated by increased lactogenesis and milk fat synthesis may be in response to improved mammary gland health. Somatic cell count was decreased in SBE60 with differences occurring during wk 4-6, coinciding with increases in milk and milk lactose yields. Somatic cell count is indicative of mammary gland inflammation, which reduces synthetic capacity of the gland. Thus, SCC is negatively associated with milk yield (Fox

et al., 1985) and yields of milk fat and lactose (Harmon, 1994). Similar to our results, Garavaglia and others (2015) observed reduced early lactation SCC coinciding with increased milk fat yield and tendencies for increased milk yield and milk lactose yield. Although feeding a concentrated pomegranate extract (**CPE**) to early lactation cows did not significantly reduce SCC, milk yield was increased by 6.4% (Shabtay et al., 2012). Furthermore, Shabtay et al. (2012) grouped mid-lactation cows into low SCC (**L-SCC**; <150,000 cells/mL milk) and high SCC cows (**H-SCC**; >150,000 cells/mL milk). L-SCC cows receiving CPE produced 1.9% more milk compared to their control and the proportion of CPE cows classified as H-SCC (>200,000 cells/mL milk) decreased by 22.8% compared to their control H-SSC counterparts. The reduction in milk and component yield during mammary gland inflammation can be at least partially attributed to the following factors: increased permeability of the blood milk barrier resulting in leakage of milk components into circulation (Shuster et al., 1991), partitioning of glucose to support the increased presence of leukocytes, decreased uptake of nutrients by the mammary epithelium, and overall reduced synthetic capacity of the mammary epithelium (Ballou et al., 2012). Therefore, improved mammary gland health may provide a mechanism by which polyphenol supplementation increases milk production.

Milk haptoglobin concentration can be used to detect subclinical and clinical mastitis (Thomas et al., 2015); however, despite the observed decrease in SCC for SBE60, milk haptoglobin concentration did not differ by treatment. Given that milk samples analyzed for haptoglobin concentration did not extend beyond wk 2 of lactation and effects of SBE60 were not observed until wk 3 of lactation, it is likely SBE effects on mammary gland inflammation take longer to appear.

Haptoglobin concentration in plasma is commonly used as a marker of systemic inflammation; however due to the ease of milk sampling with an AMS, milk haptoglobin concentration was used in this study to investigate mammary inflammation. Hiss and others (2009) measured haptoglobin concentrations in both milk and serum samples. Haptoglobin concentrations were greatest in milk during wk 1 of lactation (54 ± 14 µg/mL) but decreased to basal levels by wk 6 (3.9 ± 0.8 µg/mL). Similarly, plasma concentrations were greatest during wk 1 (1600 ± 270 µg/mL) and decreased to basal levels by wk 3 (340 ± 90 µg/mL). Clearly, haptoglobin concentrations are greater in serum than milk, but the measures were correlated ($r = 0.6$). Mammary inflammation induced by LPS increased milk haptoglobin concentration relative to control quarters (152.2 ± 226 vs. 2.4 ± 1.1 µg/mL, respectively), but haptoglobin concentration in response to mammary LPS was greater in blood (371.7 ± 37.5 µg/mL; Hiss et al., 2004). Although our d 1 milk haptoglobin concentrations are much less than those reported by Hiss et al. (2009), values did decrease with DIM and reached a concentration lower than previously reported at wk 2.

The lack of treatment effects or treatment $\times$ DIM interactions for milk haptoglobin provides no evidence to support that *Scutellaria baicalensis* decreases early lactation inflammation. Similarly, GSGME supplementation during the periparturient period did not alter hepatic mRNA abundance of haptoglobin during week 1 or 3 relative to control, although abundance did tend to decrease from week 1 to 3 (Gessner et al., 2015). Winkler et al. (2015) did, however, observe a tendency for GTCE to decrease hepatic mRNA abundance of haptoglobin by 81%, with a tendency to decrease between wk 1 and 3.

Klein et al. (2010) showed milk BHBA concentration to be effective for identifying cows in metabolic stress and likely to develop ketosis. Previous studies have shown strong correlations

between blood BHBA and milk BHBA (r = 0.66 and 0.73; Enjalbert et al., 2001; Bjerre-Harpoth et al., 2016). Thus, even though a portion of BHBA is utilized for de novo fatty acid synthesis in the mammary gland (Bauman and Griinari, 2003), milk BHBA concentration remains reflective of plasma BHBA concentrations. Milk BHBA concentrations are typically less than plasma concentrations (69 ± 7 vs 584 ± 27 µM, respectively), are increased in cases of clinical ketosis, and typically begin to increase by the second wk of lactation (Enjalbert et al., 2001; Bjerre-Harpoth et al., 2016).

Milk BHBA concentrations in our study are greater than previously reported; however, BHBA concentration did increase with DIM similar to time effects seen in plasma BHBA concentrations (Tedesco et al., 2004). *S. baicalensis* supplementation had no effect on milk BHBA concentrations, which aligns with previous studies supplementing flavonoids and indicating no difference in measures of hepatic ketogenesis (Tedesco et al., 2004; Stoldt et al., 2015; Winkler et al., 2015). Cows supplemented with GSGME did exhibit increased plasma BHBA concentration during wk 1 to 5 of lactation compared to control (Gessner et al., 2015). Authors attributed the increase in BHBA concentration to low glucose resulting from the need to support milk and milk lactose yields in GSGME cows.

Despite the greater energy demand that accompanies increased milk production, prepartum and postpartum body condition score was not affected by treatment. Similarly, Tedesco et al. (2004) observed no difference in prepartum BCS, but BCS losses from 15 d before calving to 30 DIM tended to be less for cows receiving silymarin. Other studies investigating polyphenol supplementation during the periparturient period did not report BCS (Garavaglia et al., 2015; Gessner et al., 2015; Winkler et al., 2015).

CONCLUSION

Supplementation of dairy cows with *Scutellaria baicalensis* for 60 d increased whole-lactation milk yield compared to control cows. Milk fat, protein, and lactose yields increased through 120 DIM and SCC was decreased during the treatment period for the 60-d treatment compared to control cows. Milk production parameters were not different for short term administration (5-d) compared to control cows. Other than milk SCC, there were no suggestions of impacts on health outcomes. Overall, long term administration of *S. baicalensis* effectively increased milk production, however the mechanism by which this was achieved is unknown. Possible mechanisms that warrant further investigation include effects on glucose metabolism and improved mammary gland health, amongst others.

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TABLES AND FIGURES

Table 3.1: Nutritional composition of the partial mixed ration (PMR).

Nutrient	% of DM	SD
DM, % as-fed	57.06	0.27
CP	18.71	0.37
ADF	20.89	1.54
aNDF	31.96	2.31
NE_L[1], Mcal/kg	1.65	0.04

[1]Estimated according to NRC (2001)

Table 3.2: Ingredient and nutritional composition of the control and treatment pellet.

Item	Control pellet	Treatment pellet	SD
Ingredient, % of DM			
Ground corn	42.47	42.44	
Wheat middlings	27.76	27.23	
Wheat flour	15.16	10.10	
Soybean meal (47.5%)	10.92	10.92	
Molasses	3.16	3.16	
Super bind[1]	0.53	0.53	
Test feed premix[2]	-	5.62	
Nutrient, analyzed, % of DM (unless otherwise specified)			
DM, % as-fed	87.60	87.44	0.81
CP	17.33	17.30	0.38
ADF	6.77	5.30	0.70
aNDF	15.33	14.52	0.94
NE_L[3], Mcal/kg.	1.94	1.94	0.02

[1]Modified lignin sulfonate pellet binder (Bonaventure Chemicals, Inc., Weston, FL)

[2]Test feed premix included wheat flour, calcium carbonate, natural flavoring and *Scutellaria baicalensis* extract

[3]Estimated according to NRC (2001)

Table 3.3: LS means for weekly total pellet offered, milk yield, and milking frequency for cows fed control or *S. baicalensis* extract for either 5 d (SBE5) or 60 d (SBE60) following calving. Data were analyzed by treatment period (1 – 63 DIM) and carryover period (64-301 DIM).

	Control	SBE5	SBE60	SEM[1]	*P*-values	
					Con v. SBE5	Con v. SBE60
Total pellet offered, kg/d						
d 1 - 63	5.25	5.30	5.66	0.14	0.77	< 0.01
d 64 - 301	4.97	5.18	5.28	0.15	0.13	0.02
Milk yield, kg/d						
d 1 - 63	42.46	44.95	47.19	2.01	0.35	0.07
d 64 - 301	35.39	36.23	40.02	1.91	0.73	0.04
Milking frequency, d^{-1}						
d 1 - 63	3.24	3.34	3.48	0.21	0.60	0.19
d 64 - 301	2.56	2.67	2.84	0.18	0.48	0.04
Milk per visit, kg						
d 1 - 63	13.88	14.13	14.12	0.75	0.70	0.75
S 64 - 301	14.05	13.89	14.07	0.63	0.92	0.99

[1]Reported SEM is pooled across treatment groups.

Table 3.4: Rumination time through 120 DIM and milk composition for the first 17 weeks of lactation of control cows and cows supplemented with *S. baicalensis* extract (SBE) for either 5 d (SBE5) or 60d (SBE60) following calving.

	Control	SBE5	SBE60	SEM[1]	P-values	
					Con v. SBE5	Con v. SBE60
Rumination, min/d						
d 1-63	429.9	427.3	429.0	8.20	0.76	0.92
d 64-120	410.3	405.8	409.9	7.30	0.58	0.95
Milk fat, %						
d 1-63	3.84	3.84	3.84	0.17	0.95	0.99
d 64-120	3.24	3.08	3.29	0.18	0.28	0.77
Milk protein, %						
d 1-63	3.16	3.10	3.12	0.06	0.40	0.54
d 64-120	2.97	2.89	2.97	0.05	0.15	0.99
Milk lactose, %						
d 1-63	4.87	4.89	4.95	0.04	0.54	0.06
d 64-120	4.92	4.95	4.97	0.04	0.46	0.25
Milk fat, kg/d						
d 1-63	1.61	1.67	1.77	0.08	0.50	0.04
d 64-120	1.35	1.38	1.51	0.08	0.73	0.04
Milk protein, kg/d						
d 1-63	1.34	1.40	1.46	0.06	0.42	0.09
d 64-120	1.23	1.34	1.41	0.05	0.13	0.01
Milk lactose, kg/d						
d 1-63	2.10	2.23	2.36	0.10	0.26	0.03
d 64-120	2.07	2.28	2.35	0.09	0.07	0.02
SCC, $\log_{10}$ cells/mL						
d 1-63	2.19	2.07	1.86	0.13	0.37	0.02
d 64-120	2.13	1.98	1.91	0.14	0.29	0.13

[1]Reported SEM is pooled across treatment groups

Table 3.5: Milk haptoglobin and BHBA on days 1, 3, and 5-12 of lactation for control cows and cows receiving *S. baicalensis* extract (SBE) for either 5 d (SBE5) or 60 d (SBE60) after calving.

	Control	SBE5	SBE60	SEM[1]	Trt	DIM	Trt×DIM
Haptoglobin, µg/mL					0.97	< 0.001	0.45
Day 1	4.98	3.54	5.47	1.04			
Day 3	1.53	1.70	1.44	0.35			
Day 5-12	0.59	0.69	0.50	0.13			
BHBA, µM					0.89	< 0.001	0.47
Day 1	264.0	265.3	249.4	23.6			
Day 3	639.7	609.7	632.2	22.6			
Day 5-12	729.1	746.7	717.8	18.6			

[1]Reported SEM is pooled across treatment groups. Trt: *P*-value for treatment effect; DIM: *P*-value for time effect.

Figure 3.1: Test material offered to cows during the first 5 d of lactation for cows receiving *S. baicalensis* extract (SBE) for either 5 d (SBE5) or 60 d (SBE60) after calving. Target test material intake was 100 g/d. There was no difference between the treatments shown ($P = 0.41$).
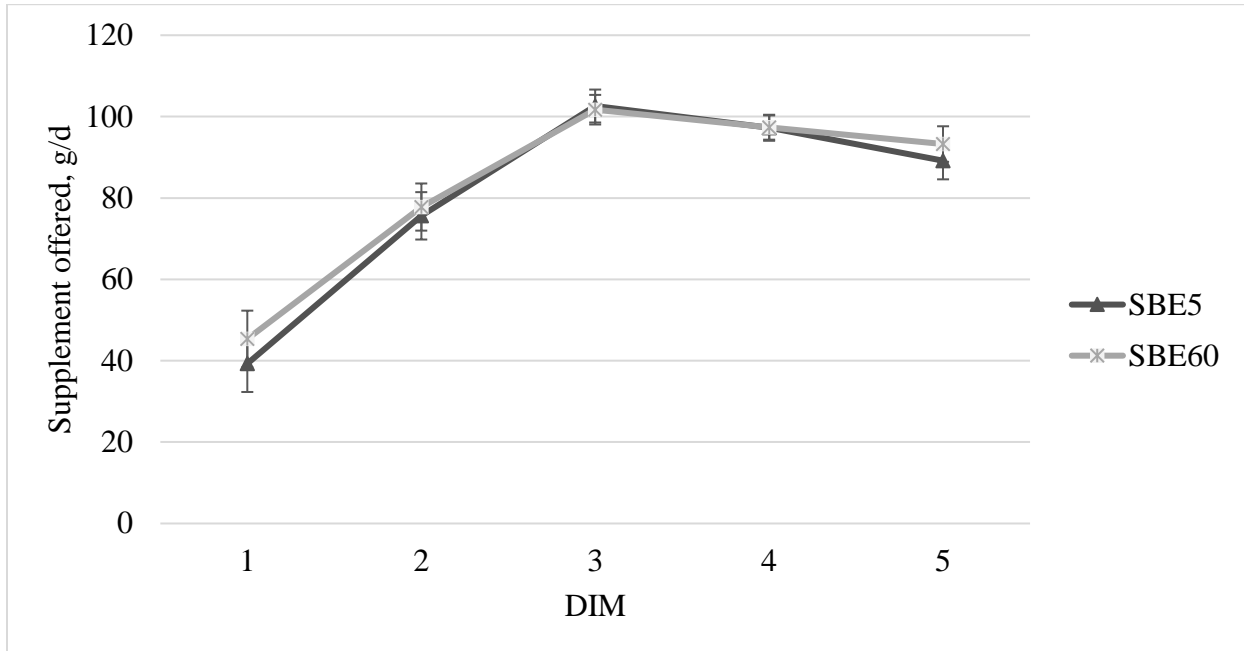
Figure 3.2: Weekly total pellet offered (control + treatment) of control cows and cows supplemented with *S. baicalensis* during the first 5 d (SBE5) or 60 d (SBE60) of lactation. Data were analyzed by treatment period (wk 1-9) and carryover period (wk 10-36). Total pellet offered was increased for SBE60 compared to control during the wk 1-9 ($P < 0.01$) and from wk 10-43 ($P = 0.02$). Total pellet offered was not different between SBE5 and control during either wk 1-9 ($P = 0.77$) or wk 10-43 ($P = 0.13$). A treatment × week interaction was detected ($P < 0.001$), and differences between SBE60 and control are indicated by *($P < 0.05$) and † ($P < 0.10$).
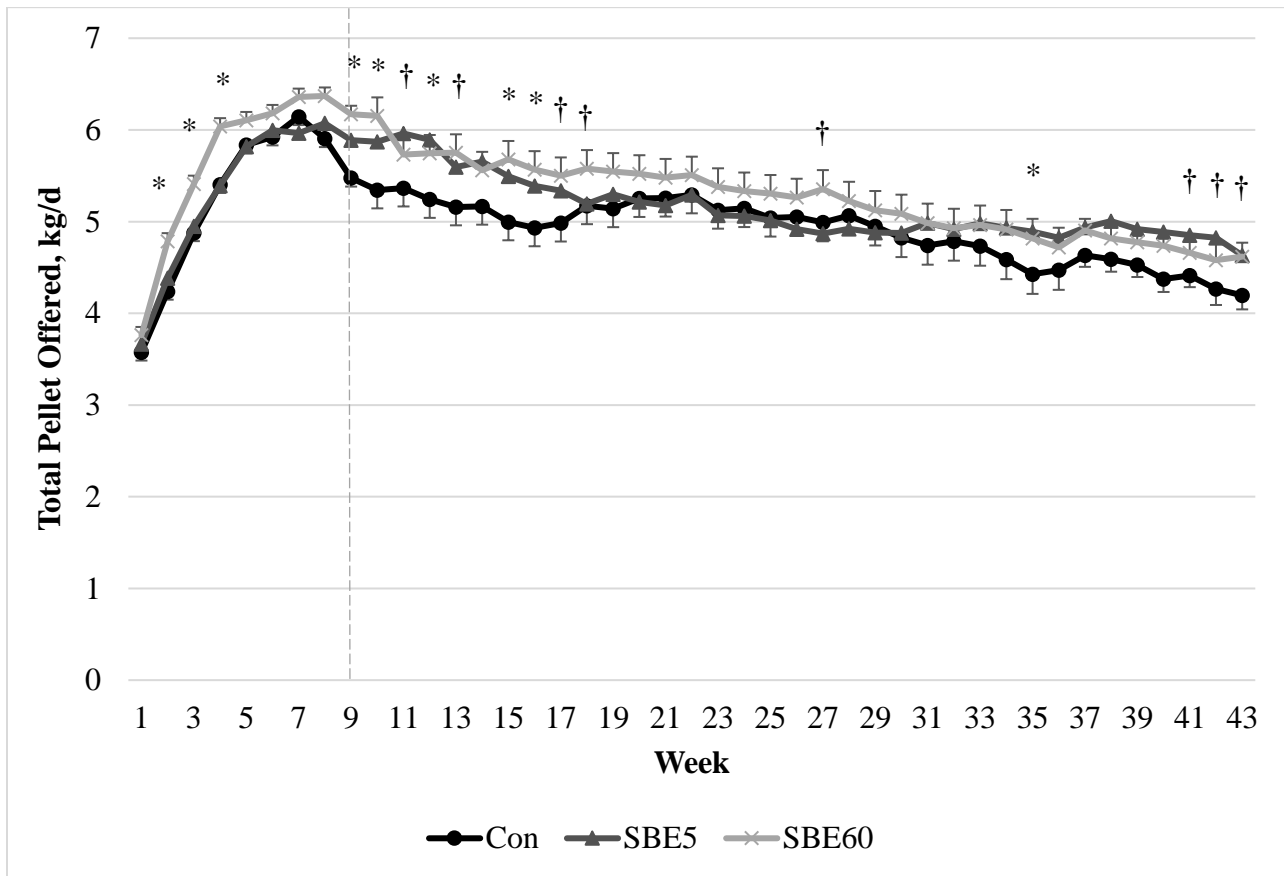
Figure 3.3: Milk yield of control cows and cows supplemented with *S. baicalensis* extract (SBE) during the first 5 d (SBE5) or 60 d (SBE60) of lactation. Data were analyzed by treatment period (wk 1 – 9) and carryover period (wk 10 – 43). Milk yield tended to be increased for SBE60 compared to control from wk 1-9 ($P = 0.07$) and was significantly increased from wk 10-43 ($P = 0.04$). Milk yield was not different between SBE5 and control during wk 1-9 ($P = 0.35$) or wk 10-43 ($P = 0.73$). A treatment × week interaction was detected ($P < 0.03$), and differences between SBE60 and control are indicated by *($P < 0.05$) and † ($P < 0.10$).
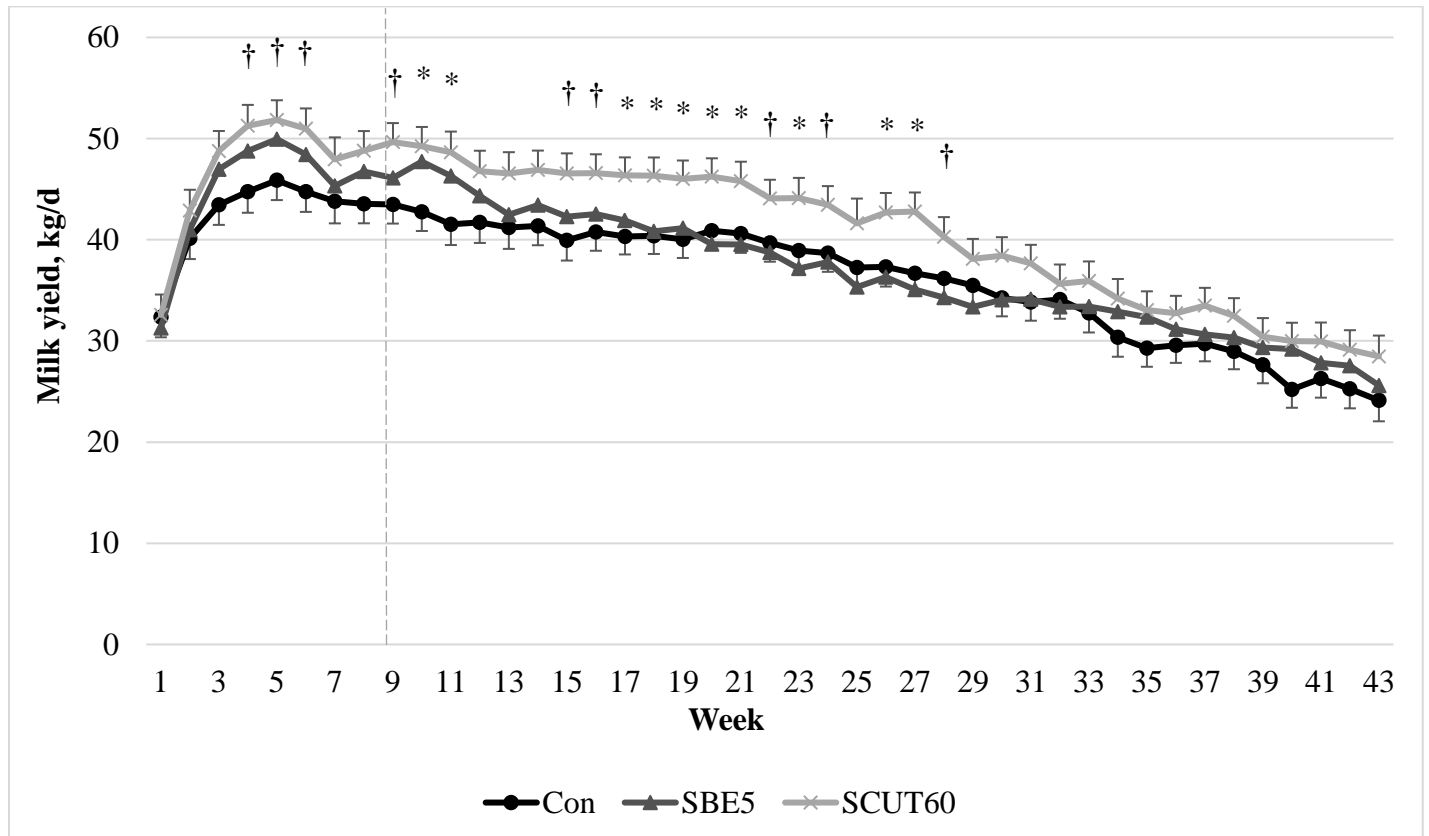
Figure 3.4: Somatic cell count (SCC) of control cows and cows supplemented with *S. baicalensis* extract (SBE) during the first 5 d (SBE5) or 60 d (SBE60) of lactation. Data were analyzed for the treatment period (wk 1-9) and carryover period (wk 10-17). Somatic cell count was not different between SBE5 and control during wk 1-9 ($P = 0.37$) or wk 10-17 ($P = 0.29$). Somatic cell count was decreased for SBE60 compared to control during wk 1-9 ($P = 0.02$), but not during wk 10-17 ($P = 0.13$). No treatment $\times$ week interaction was detected ($P = 0.16$).
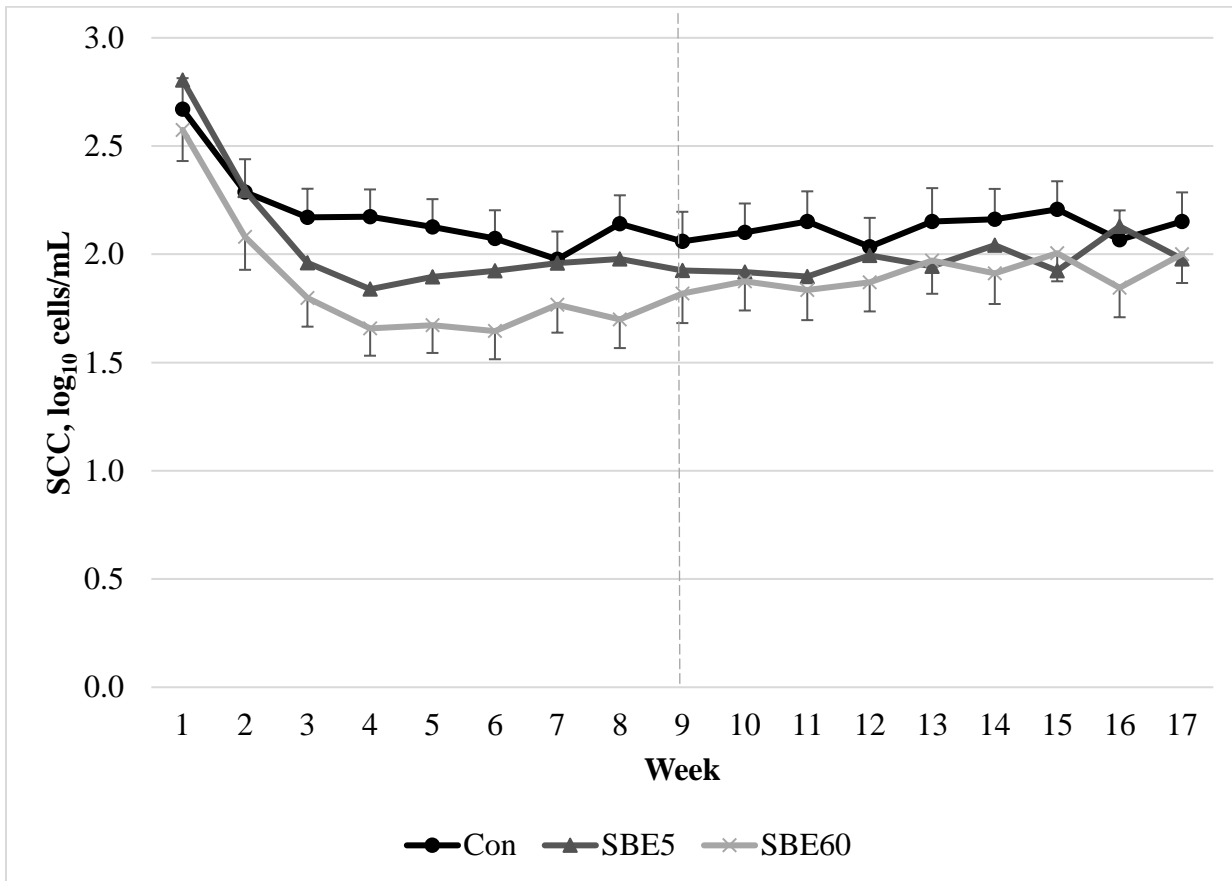
Figure 3.5: Body condition score (5 point scale) from 3 weeks prepartum to 29 weeks postpartum for control cows and cows supplemented with *S. baicalensis* extract (SBE) for the first 5 d (SBE5) or 60 d (SBE60) of lactation. Least square means across time were 3.40, 3.30, 3.31 ± 0.06 for CON, SBE5, and SBE60, respectively. BCS differed by week ($P < 0.001$), but there were no differences by treatment ($P = 0.44$) or treatment × week ($P = 0.57$).