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EFFECTS OF *CAPSICUM* OLEORESIN ON FEED INTAKE, MILK PRODUCTION, IMMUNE RESPONSES, BLOOD METABOLITES, AND HORMONES IN LACTATING DAIRY COWS

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Abstract

In both dairy and beef production systems, there has been interest in using plant extracts to enhance productivity and feed efficiency. *Capsicum* oleoresin (**CO**), an ethanolic extract from the fruit of *Capsicum* plants, has been reported to modify rumen fermentation in ruminants. This is attributed to an antibacterial effect of capsaicinoids, the active compounds of *Capsicum*. In addition, capsaicinoids reportedly alter host responses in rats and humans. Studies have also shown that capsaicinoids had immunoregulatory effects, stimulate digestive enzymes, regulated appetite-related hormones, and alter blood metabolites. In a series of experiments, the effects of CO were investigated on the host responses as well as rumen fermentation in lactating dairy cows.

The objective of the first experiment was to investigate the effect of CO supplied postruminally on nutrient utilization, gut microbial ecology, immune response, and productivity of lactating dairy cows. Treatments were control (no CO) and daily doses of 2 g/cow of either CO for 9d. Milk yield was decreased by CO treatment compared with the control. The treatments did not affect nutrient utilization and gut microbial ecology. The relative proportion of lymphocytes was increased by the CO treatment compared with the control. The CO treatment increased the proportion of total $CD4^+$ cells and total $CD4^+$ cells that co-expressed the activation status signal CD25 in blood. Collectively, the CO used in the first trial appeared to have an immune-stimulatory effect by activating and inducing the expansion of CD4 cells in dairy cows. Although CO treatment

decreased milk yield, this should be interpreted with caution because of the short duration of treatment. The objective of the second experiment was to investigate the effect of *Capsicum* oleoresin in granular form (CAP) on feed intake, immune responses, oxidative stress markers, rumen fermentation, and productivity of lactating dairy cows. Treatments included control (no CAP) and daily supplementation of 250, 500, or 1,000 mg CAP/cow. Dry matter intake was not affected by CAP, but milk yield tended to quadratically increase with CAP supplementation. The CAP treatments quadratically increased energy-corrected milk yield. The CAP treatment did not affect oxidative stress markers, rumen fermentation. Blood serum β-hydroxybutyrate was quadratically increased by CAP, whereas the concentration of nonesterified fatty acids was similar among treatments. Mean fluorescence intensity for phagocytic activity of neutrophils tended to be quadratically increased by CAP. Numbers of neutrophils and eosinophils and the ratio of neutrophils to lymphocytes in peripheral blood linearly increased with increasing CAP. Overall, in the conditions of the trial, energy-corrected milk yield was quadratically increased by CAP, possibly as a result of enhanced mobilization of body fat reserves with CAP supplementation. In addition, CAP increased neutrophil activity and immune cells related to acute phase immune response. The objective of the third experiment was to investigate the effects of rumen-protected *Capsicum* oleoresin (RPC) supplementation on feed intake, digestibility, milk production, and responses to an intravenous glucose challenge and an immune challenge in lactating dairy cows. Treatments were 0 (control), 100, and 200 mg RPC/cow/d. Dry matter intake and milk yield tended to quadratically increase with RPC. Feed efficiency linearly increased with RPC supplementation. Apparent total tract digestibility of DM, OM, and CP and fecal N

proportions of N intake were linearly increased by RPC supplementation. During a glucose tolerace test, serum insulin was decreased by RPC while glucose concentration in serum was not affected. After an immune challenge, cortisol and haptoglobin concentration in serum were lower in RPC treatment than the control. In conclusion, *Capsicum* may stimulate productivity and insulin activity and alleviate acute phase responses in lactating dairy cows.

Key words: *Capsicum* oleoresin, milk production, feed intake, immune response, dairy cow

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Abbreviations

ADF	Acid detergent fiber
BUN	Blood urea nitrogen
BW	Body weight
CAP	Capsicum oleoresin in granular form
CCK	Cholecystokinin
CD	Cluster of differentiation
CGRP	Calcium gene-related peptide
CO	Capsicum oleoresin
CP	Crude fiber
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
ECM	Energy-corrected milk
FA	Fatty acid
FCM	Fat-corrected milk
GLP-1	Glucagon-like peptide-1
HAP	Hyper-ammonia producing
IFNG	Interferon gamma
IL10	Interleukin 10
IL-1B	Interleukin 1 beta
iNDF	Indigestible NDF
LPS	Lipopolysaccharide
MP	Metabolizable protein
MPN	Milk protein N
MUN	Milk urea nitrogen
N	Nitrogen
NDF	Neutral detergent fiber
NE_L	Net energy for lactation
NFC	Non-fiber carbohydrate
OM	Organic matter
ORAC	Oxygen radical absorbance capacity
PBMC	Peripheral blood mononuclear cells
PD	Purine derivatives
PN	Phytonutrients

PP	Peyer's Patch
RDP	Rumen degradable protein
ROS	Reactive oxygen species
RPC	Rumen-protected Capsicum
RUP	Rumen undegradable protein
SCC	Somatic cell counts
SEM	Standard error of mean
TBARS	Thiobarbituric acid reactive substances
TMR	Total mixed ration
TNF	Tumor necrosis factor alpha
TRPV1	Transient receptor potential vanilloid receptor 1
UUN	Urine urea nitrogen
VFA	Volatile fatty acid

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Chapter 1

Introduction

Ruminants have a symbiotic relationship with microbes living in the rumen. As host animals, ruminants provide an ideal environment for microbes to live while microbes supply energy and protein back to the host animals by degrading feeds, digesting nutrients and producing end-products. However, the efficiency of utilizing feeds is not ideal in ruminants. Microbial degradation and utilization result in a loss of energy and nitrogen mainly through methane and ammonia, respectively (Shirley, 1986).

Ionophoric antibiotics have been used to minimize the losses of energy and nitrogen in ruminants. The antibiotics selectively inhibit gram-positive rather than gram-negative bacteria, thereby modifying rumen fermentation (Duffield et al., 2008). From numerous studies, antibiotic ionophores have been proven to increase propionate and decrease acetate, ammonia, and methane (Guan et al., 2006; Kobayashi, 2010). Decreases in methane and ammonia production enhance the efficiency of energy and nitrogen utilization in ruminants. However, due to emerging concerns of customers about residues in animal products, antibiotics used as growth promoters have been taken out of many diets (European Union, 2003).

Capsicum, a genus of flowering plants, has been reported to act as a rumen modifier due to an antibacterial effect of its active compounds, capsaicinoids

(Calsamiglia et al., 2007). Dietary *Capsicum* supplementation reportedly decreased ruminal acetate concentration in beef cattle (Cardozo et al., 2006; Fandiño et al., 2008) although it did not affect ruminal VFA concentration in dairy cows (Tager and Krause, 2011). In addition to the effect on rumen fermentation, studies have reported that the effects of capsaicinoids on feed (or food in human studies) intake, digestive enzymes in the gastrointestinal tract, immune responses, insulin sensitivity, and lipid metabolism in rats and humans (Luo, 2011; Srinivasan, 2015). Although data have been conflicting among studies, capsaicinoid treatment stimulated gastric emptying and decreased blood leptin level, which increased feed intake (Hsu and Yen, 2007). Activity of digestive enzymes such as lipase and trypsin was increased by capsaicinoids in the gastrointestinal tract (Platel and Srinivasan, 2000). Capsaicinoids were also reported to have immunoregulatory effects on macrophages, neutrophils, T cells, and B cells depending on dose amount (Nilsson et al., 1991; Takano et al., 1997; Franco-Penteado et al., 2006; Nevius et al., 2012). Interestingly, capsaicinoids treatment has increased insulin sensitivity in rats (van de Wall et al., 2006). In addition, capsaicinoids reportedly enhanced fat mobilization and reduced adipose tissue (Yoshioka et al., 2000).

Based on this evidence, the overall hypothesis in this dissertation is that capsaicinoids could not only modify rumen fermentation, but also exhibit an array of activities in the lower part of the ruminant digestive tract if they bypass the rumen. Improvements in feed intake, digestibility, rumen fermentation parameters, and immune responses could lead to improved milk production and feed efficiency in dairy cattle. Capsaicinoids were used in an oleoresin form of *Capsicum* in animal trials (Tager and

Kraused, 2011; Rodríguez-Prado et al., 2012). The strong pungency of *Capsicum* oleoresin and its possible degradation in the rumen can be decreased by fat protection when it is used as a feed additive in ruminants (Meunier et al., 2007). Thus, the main objective of this dissertation was to investigate the effects of *Capsicum* oleoresin as a feed additive on feed intake, milk production, digestibility, rumen microbial population and fermentation parameters, oxidative stress, immune responses, and glucose and lipid metabolism in dairy cows.

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Chapter 2

Literature Review

Capsicum and its active compounds, capsaicinoids

Capsicum is a genus of flowering plant. There are many cultivars in *Capsicum* plants. They have different shapes, colors, flavors, and pungency. Major species are *Capsicum annuum, Capsicum baccatum, Capsicum chinense, Capsicum pubescens*, and *Capsicum frutescens* (Pickersgill, 1971).

Capsaicinoids are a group of active compounds in *Capsicum* fruits, which include capsaicin, dihydrocapsaicin, norhydrocapsaicin, homodihydrocapsaicin, homocapsaicin, and norcapsaicin. Capsaicinoids are categorized as terpenoids (Calsamiglia et al., 2007), and they are also classified as phenolic acids (Patra, 2012). Capsaicin and dihydrocapsaicin are the most common capsaicinoids and capsaicin has one double bond in a fatty acid side chain, which is different from dihydrocapsaicin (Figure 2-1). Other capsaicinoids differ in length of the side chain and degree of unsaturation. The amount of capsaicinoids among *Capsicum* species differs. A study using gas chromatography has shown 0.22 to 20.0 mg total capsaicinoids/g dry *Capsicum* (Thomas et al., 1998).

The capsaicinoid profile is different by *Capsicum* species. Bennett and Kirby (1968) have shown the composition of capsaicinoids in *Capsicum annuum* as: capsaicin (69%), dihydrocapsaicin (22%), nordihydrocapsaicin (7%), homocapsaicin (1%), and

homodihydrocapsaicin (1%). Recently, Nwokem et al. (2014) reported capsaicinoid profiles of varieties in *Capsicum annuum* where capsaicin accounted for 32 to 49%, dihydrocapsaicin for 11 to 34%, and nordihydrocapsaicin for 6 to 27%. Wesolowska et al. (2011) also demonstrated 37% capsaicin, 29% dihydrocapsaicin, and 2% homodihydrocapsaicin in *Capsicum annuum* using gas chromatography-mass spectrometry. Although capsaicin and dihydrocapsaicin were the major capsaicinoids in *Capsicum annuum*, other *Capsicum* species such as *Capsicum pubescens* has a different capsaicinoid profile. According to Zewdie et al. (1998), an isomer of dihydrocapsaicin (39%) was the largest proportion in *Capsicum pubescens* and was followed by homodihydrocapsaicin (17%), and capsaicin (13%).

Capsaicin and dihydrocapsaicin account for about 90% of pungency in capsaicinoids and it is known that pungency of capsaicin is similar to that of dihydrocapsaicin (Kosuge and Furuta, 1970). Pungency of capsaicinoids can be characterized by Scoville heat units (SHU) based on human perception, and capsaicin and dihydrocapsaicin have 16.1×10^6 SHU; homocapsaicin, 6.9×10^6 SHU; homodihydrocapsaicin, 8.1×10^6 SHU; and nordihydrocapsaicin 9.3×10^6 SHU. (Scoville, 1912; Legin, 1996). It is generally thought that moderate pungency is 3,000-25,000 SHU, high pungency is 25,000-70,000 SHU, and very high pungency is >80,000 SHU (Othman et al., 2011).

Biosynthesis of capsaicinoids

Capsaicinoids are synthesized by two pathways in *Capsicum* plants, the phenylpropanoid pathway and the leucine/valine-fatty acid pathway (Gururaj et al., 2012). The phenylpropanoid pathway begins with phenylalanine and ends up with vanillylamine. Whereas, the leucine/valine-fatty acid pathway produces a pool of C9–C11 fatty acids from leucine or valine. Capsaicinoids are made by a condensation reaction between vanillylamine and C9–C11 fatty acids by capsaicin synthase (Bennet and Kirby, 1968; Gururaj et al., 2012). The placental tissue, the region of attachment of seeds on inner fruit wall, serves as major storage of capsaicinoids with less stored in the flesh of the fruits. This was confirmed by Stewart et al. (2007) who found capsaicinoids only in the epidermal cells of the interlocular septa which are locules in the fruit connected to the placenta.

Absorption and Metabolism

Although absorption of capsaicinoids into the rumen epithelial cells has not been reported, the intestinal absorption of capsaicinoids was well documented in rats. Kawada et al. (1984) demonstrated rapid absorption of capsaicinoids from the stomach and the small intestine in rats. A capsaicin mixture suspended in basal diet was dosed at 3 mg to fasted rats (220 g) by a stomach tube. At various time interval, stomach, duodenum, jejunum, ileum, cecum, and large intestine were removed from the rats and analyzed for capsaicin and dihydrocapsaicin. In the whole gastrointestinal tract, 50, 30, 20, and 15 % of the dose were found at 5 min, 30 min, 1 h, and 3 h post administration, respectively,

which means 85% of the dose was absorbed within 3h in the gastrointestinal tract in rats. Capsaicin and dihydrocapsaicin were absorbed at the same rate. Interestingly, a significant amount of the dose was found in the stomach tissue within 5 min, although the small intestine served as the major absorption site at 3h post administration. Less than 10% of the dose was found in feces after 48h. These findings are somewhat different from those of a study by Suresh and Srinivasan (2010), who orally dosed 30 mg capsaicin to rats (150 – 160g). They analyzed capsaicin concentration or amount in blood, liver, kidney, intestine, feces, and urine in time courses after capsaicin administration. About 24% of the dose was detected in blood and the tissues within 1h post dose, and 1.2% at 24h. Capsaicin showed the highest concentration at 1h (1.9 μg/ mL) in blood serum and at 3 h (44.7 μg/whole tissue) in the liver. Capsaicin remaining in feces was 6.3% over a period of 4 days, which means approximately 94% of dosed capsaicin was absorbed in the tissues and blood. Only a small amount of capsaicin (0.095%) was detected in urine, and this could be because capsaicin was metabolized by the liver (Chanda et al., 2008) These results are comparable with results of Kawada and Iwai (1985), who found 8.7 % intact dihydrocapsaicin and eight of its metabolites such as vanillylamine, vanillin, vanillyl alcohol, and vanillic acid in urine within 48h post dihydrocapsaicin dose (20 mg/kg) in rats.

It has been known that capsaicinoids are metabolized in the liver to a large extent and in other tissues to a lesser extent (Reilly and Yost, 2006; Chanda et al., 2008). Donnerer et al. (1990) reported that an *in vitro* incubation of dihydrocapsaicin with liver or brain tissue for 30 min showed a significant decrease of dihydrocapsaicin

concentration only in liver tissue. Another *in vitro* study compared dihydrocapsaicin-hydrolyzing enzyme activity in various organs of rats (Kawada and Iwai, 1985). The study found the liver had the highest activity and is followed by kidney, lung, small intestine, stomach, and brain. Kadawa et al. (1984) demonstrated hydrolyzing enzyme activity for dihydrocapsaicin in the jejunum tissue. They found that 15% of dihydrocapsaicin absorbed into the portal vein was transformed into its metabolites. Donnerer et al. (1990) also indicated degradation process of capsaicin and dihydrocapsaicin in the gastrointestinal tract by intragastric application. The percentage of unchanged capsaicin and dihydrocapsaicin decreased from the gastrointestinal lumen to the portal vein blood.

A variety of metabolites are produced in the metabolism of capsaicin by P450 enzymes in the liver (Reilly and Yost, 2006). Processes metabolizing capsaicin include aromatic and alkyl hydroxylation, O-demethylation and alkyl dehydrogenation, and an additional ring oxygenation (Reilly et al., 2003). Hydroxy-capsaicin, 17-hydroxy-capsaicin, 16,17-dehydro-capsaicin, 16-hydroxy-capsaicin, vanillylamine, and vanillin arise from the detoxification of capsaicin in the liver (Chanda et al., 2008). Among the metabolites, vanillin has been known to have antimicrobial and antioxidant properties (Tai et al., 2011; Patra and Yu, 2014). However, the extent of metabolism is different by animal species (Chanda et al., 2008). For example, vanillin was found in the liver of rats at a high level, but not in dogs. And it is unknown how capsaicin is metabolized in the liver of ruminants.

Transient receptor potential cation channel subfamily V member 1

Capsaicinoids exert their effects by binding to and activating the transient receptor potential cation channel subfamily V member 1 (TRPV1) (Reyes-Escogido et al., 2011). This receptor is a cation channel primarily expressed in the sensory neurons. It has been also reported that TRPV1 was located in the brain, bladder, kidneys, intestines, keratinocytes of epidermis, glial cells, and liver as well as polymorphonuclear granulocytes, mast cells, and macrophages (Cortright and Szallasi, 2004). Although 6 TRPV channels were found in mammals, only TRPV1 is activated by capsaicinoids (Vriens et al., 2009). TRPV1 is also stimulated by acidic pH and high temperature (>42 ℃), resulting in the perception of burning, pain, and discomfort. Binding of capsaicinoids to TRPV1 in the plasma membrane of neurons increases intracellular calcium and release of neuropeptides such as substance P, calcium gene-related peptide (CGRP), and somatostatin (Reyes-Escogido et al., 2011). The simulation of sensory neurons through TRPV1 produced vasodilation, plasma extravasation, and hypersensitivity (Richardoson and Vasko, 2002). The neuropeptides released from neurons by the TRPV1 activation can also affect immune cells by regulating cytokine production (Tsuji et al., 2010; Tsuji et al., 2011). The interaction between activated neurons and immune cells will be described in detail later in this review. TRPV1 has been known to play a critical role in body temperature regulation (Romanovsky, 2009), which is related to thermogenesis and energy expenditure. On the other hand, prolonged stimulation of capsaicinoids functionally inactivates TRPV1, which is the underlying

mechanism of topical application with capsaicinoids for pain relief and provides approaches to neurological studies (Bley, 2004).

Capsicum oleoresin

Capsicum can be used as a form of oleoresin. *Capsicum* oleoresin is made by extraction with solvent such as hexane, acetone, and ethanol. Constituents in *Capsicum* oleoresin are different by cultivars and extraction methods. It generally consists of fatty oils, dyes, sterols, waxes with 4 to 6% capsaicinoids (Legin, 1996; Wesolowska et al., 2011). Vitamin E has been found in *Capsicum* oleoresin in hexane and acetone extracts of *Capsicum annuum* L., however its amount ranged from 0.1 to 0.6% (Wesolowska et al., 2011). *Capsicum* oleoresin has been commercially marketed for personal self-defense and experimentally used in food animals such as pigs, chicken, and cattle (Liu et al., 2014; Lee et al., 2011; Cardozo et al., 2008). Extremely strong pungency of *Capsicum* oleoresin can be alleviated by fat protection when it is used as feed additive in animal trials (Meunier et al., 2007). In ruminants, the fat protection also increases rumen bypass rate, which enhances availability of capsaicinoids in the lower gut of ruminants.

Capsicum as a rumen modifer

Mode of action

Studies indicated that capsaicinoids have an antimicrobial effect *in vitro* and *in vivo* (Patra, 2012). Capsaicinoids have a phenolic ring as a functional group, which is

known to have higher antimicrobial activity than other essential oil (EO) components such as cinnamic aldehyde, alcohols, aldehydes, ketones, ethers, and hydrocarbons (Kalemba and Kunicka, 2003). Calsamiglia et al. (2007) described mechanisms of action of EO for antimicrobial properties in a review. Since capsaicinoids are categorized as terpenoids in EO, the mechanisms in the review could be considered as modes of action for capsaicinoids (Calsamiglia et al., 2007). To summarize, one of the well-known modes of action of terpenoids is its interaction with the cell membrane of bacteria. The hydrophobic property of the cyclic hydrocarbons is able to change the conformation of the membrane structure by accumulating in the lipid bilayer of bacteria. This results in the loss of membrane stability allowing the leakage of ions across the membrane, which causes cell death or suppression of growth due to a large energy loss. However, the hydrophobicity of terpenoids does not work on gram-negative bacteria because of the cell wall structure. It has been reported that only small molecules in terpenoids could diffuse into the layer of lipopolysaccharides by forming hydrogen bridges with water. Capsaicinoids have been reported to have antibacterial effects on both gram positive and gram negative bacteria (Deans and Ritchie, 1987). In addition to the interaction with the cell membrane, phenolic compounds of terpenoids may coagulate cell constituents by protein denaturation.

Due to antibacterial properties of capsaicinoids, it is speculated that use of capsaicinoids as a feed additive in ruminants could modify the ruminal microbial population as well as affect rumen fermentation parameters such as VFA and ammonia production.

Ruminal fermentation parameters

The effects of *Capsicum* oleoresin on ruminal VFA concentration has been investigated *in vitro* and *in vivo* (Calsamiglia et al., 2007). Cardozo et al. (2005) tested the effects of *Capsicum* oleoresin *in vitro* on rumen fluid from beef cattle fed a high concentrate diet at two different pH. *Capsicum* oleoresin decreased total VFA and propionate concentration, but increased acetate and butyrate at pH 7.0. In contrast, at pH 5.5, *Capsicum* oleoresin increased VFA and propionate concentration, but decreased acetate. Thus, the acetate:propionate ratio decreased with *Capsicum* oleoresin at pH 5.5. This is possibly because low pH might make capsaicinoids more hydrophobic, which is more active on certain types of bacterial cell membrane (Calsamiglia et al., 2007). Cardozo et al. (2006) supplemented 1 g/d *Capsicum* oleoresin in beef cattle fed a 90:10 concentrate:forage diet. They found that the supplementation did not affect pH, total VFA concentration, and butyrate proportion, but decreased acetate proportion in the rumen. This is consistent with Fandiño et al. (2008) who observed a decrease of acetate proportion and no effect on total VFA concentration by 500 mg/d *Capsicum* oleoresin in beef cattle fed high concentrate diet. Rodríguez-Prado et al. (2012) tested ruminal fermentation with 3 doses of *Capsicum* oleoresin (125, 250, and 500 mg/d) in a solid form using beef cattle. The solid form contained 20% liquid *Capsicum* oleoresin. *Capsicum* oleoresin tended to linearly decrease pH and acetate proportion while increasing total VFA concentration in the rumen. In dairy cows, Tager and Krause (2011) found no effect of *Capsicum* oleoresin (250 mg/d) in a solid form on VFA

concentration in the rumen. The different results from those of beef cattle studies might be attributed to the relatively low dose amount (Tager and Krause, 2011).

It has been known that hyper-ammonia producing (HAP) bacteria are sensitive to EO (Patra, 2011). *Capsicum* oleoresin is expected to reduce ammonia production in the rumen because it also belongs to EO. However, the effects of *Capsicum* oleoresin on ruminal ammonia concentration are not consistent among studies. Rodríguez-Prado et al. (2012) found a tendency of increase in ruminal ammonia concentration with *Capsicum* oleoresin supplementation in beef cattle. Whereas, Cardozo et al. (2006) and Fandiño et al. (2008) observe no effect of *Capsicum* oleoresin on ruminal ammonia concentration.

Collectively, *Capsicum* oleoresin has shown subtle or no effects on rumen fermentation parameters in beef cattle or dairy cows. Calsamiglia et al. (2007) indicated that a negligible effect of *Capsicum* on rumen fermentation is due to a low number of oxygen molecules attached to the phenolic ring in capsaicin compared with other terpenoids.

Rumen microbes

Although no data have been published about the effects of capsaicinoids on microbes in the rumen, antimicrobial effects of other EO, terpenoids, phenolic compounds, or mixture of several compounds were observed *in vitro* and *in vivo* (Patra, 2012).

Benchaar et al. (2007) supplemented an EO mixture (750 mg/d) containing thymol, eugenol, vanillin and limonene to diets in dairy cows, but they found no effect on total viable cellulolytic bacteria in the rumen. In an earlier study, the same mixture was used in sheep, but no change in total viable rumen bacteria counts was observed (Wallace et al., 2002). Studies have shown that EO could inhibit HAP bacteria in the rumen, which decreases deamination of amino acids (Patra, 2011). McIntosh et al. (2003) demonstrated *in vitro* that an EO mixture reduced the activity of HAP bacterial species such as *Clostridium sticklandii* and *Peptostreptococcus anaerobius* while other bacteria were adapted to EO. Supplementation with EO at 100 mg/d in an *in vivo* trial decreased number of HAP bacteria by 77% in sheep fed a low protein diet (Wallace, 2004). However, EO did not work for a high protein diet in the same experiment, which indicates the inhibitory effect of EO on HAP bacteria depends on diets. It has been reported that the rumen bacteria were affected differently by the dose amount of EO. The growth of *Selenomonas ruminantium* was selectively inhibited by thymol at a low dose (90 mg/L) *in vitro*, whereas thymol could inhibit all other ruminal bacteria at a high dose (400 mg/L) in the trial (Evans and Martin, 2000). Recently, in dairy cows, supplementation of oregano leaves containing carvacrol decreased *Ruminococcus flavefaciens* which is one of the major rumen fibrolytic species (Hristov et al., 2013).

The effect of EO on protozoa has not been consistent in studies. Extracts of clove containing eugenol decreased the numbers of total protozoa, small entodiniomorphs and holotrichs *in vitro* (Patra et al., 2010). Cardozo et al. (2006) also observed decreases of the counts of holotrichs and entodiniomorphs in beef cattle fed 2 g/d anise extract (100

g/kg anethol). However, Yang et al. (2010) found no effect on ruminal protozoa including *Isotricha, Dasytricha* and *Entodinium* sp. in steer fed 0.4 to 1.6 g/d cinnamaldehyde. Benchaar et al. (2007) also observed in dairy cows that 750 mg/d of EO mixture did not affect ruminal protozoa counts. It should be noted that rumen microbes can adapt to EO supplementation (Busquet et al., 2005). Although the antimicrobial effects of EO may be observed in short term experiments, their effects have to be proven in a long term experiments.

Host responses to *Capsicum*

In ruminant studies, *Capsicum* was originally considered as modifier of rumen fermentation and microbial population to optimize rumen function (Calsamiglia et al., 2007). However, recent studies suggest that the improvement in animal performance may be due to a host response (physiological and immunological) rather than antimicrobial effects of capsaicinoids in the rumen (Karadas et al., 2014; Liu et al., 2013; Liu et al., 2014a).

Immune response

Binding of capsaicin, a main active compound in *Capsicum*, to TRPV1 could modify immune cells including macrophages, neutrophils, T lymphocytes, and B lymphocytes by stimulating or inhibiting cytokines and antibodies (Nilsson et al., 1991;

Takano et al., 1997; Franco-Penteado et al., 2006; Nevius et al., 2012). Capsaicin could directly act on immune cells through TRPV1 expressed on the membrane in mammalian (Cortright and Szallasi, 2004), and also indirectly affect immune cells by binding to TRPV1 on afferent neurons that stimulate release of neuropeptides such as CGRP, substance P, and tachykinin (Tsuji and Aono, 2012). It has been known that CGRP plays an anti-inflammatory role, and substance P and tachykinin are associated with vasodilation, plasma extravasation, and pro-inflammatory actions (Zimmerman et al., 1992; Pennefather et al., 2004). Demirbilek et al. (2004) found decreases of pro-inflammatory cytokines (TNF, IL6) and an increase of an anti-inflammatory cytokine (IL10) with a subcutaneous dose of capsaicin in the blood of septic rats. An increase of CGRP in septic rats by the capsaicin treatment in the same experiment could exhibit anti-inflammatory effects. Nilsson et al. (1991) have reported the anti-inflammatory effects of capsaicin on immunoglobulins. Concentrations of IgG and IgA were decreased by capsaicin treatment in lymphoid tissue from immunologically challenged rats. It was not certain in this experiment whether the anti-inflammatory effect resulted from the direct effect of capsaicin on the tissue or the indirect effect of capsaicin through stimulation on neurons. Joe and Lokesh (1997) found that capsaicin exhibited anti-inflammatory effects *in vitro* by directly inhibiting the incorporation of arachidonic acid and prostaglandin E2 into membrane lipids of peritoneal macrophages in the rat. Meanwhile, some studies found capsaicin exhibited pro-inflammatory effects on immune cells. Capsaicin reportedly stimulated production and activity of neutrophils (Franco-Penteado et al., 2006). Neutrophils were increased in rats neonatally treated with capsaicin. Neonatal treatment with capsaicin has been known to desensitize sensory

afferent neurons in rats, and this might affect the neutrophil production (Camara et al., 2008). Functional activity of neutrophils was also facilitated by capsaicin treatment in rats (Zhukova and Makarova, 2002).

The immunoregulatory effects of capsaicin may depend on dose amount of capsaicin. Capsaicin exhibited both anti-inflammatory and pro-inflammatory effects at different doses in a rat study (Demirbilek et al., 2004). A low dose of capsaicin (1 mg/kg BW) increased CGRP, a potent anti-inflammatory mediator released from sensory neurons, whereas high dose capsaicin (150 mg/kg BW) decreased CGRP in blood compared with non-treated septic rats. The changes in CGRP resulted in increases of pro-inflammatory cytokines in the high dose group. The decrease of CGRP with the high dose of capsaicin might be caused by ablation of the sensory fibers (Buck and Bulks, 1986). Nevius et al. (2012) demonstrated the immunoregulatory effects of capsaicin on T cells in type I diabetes model mice. Type I diabetes is an autoimmune disease in which β-cells in the pancreatic islets of Langerhans are attacked by immune cells (Cnop et al., 2005). Orally dosed capsaicin (10 μg) decreased T cell proliferation in pancreatic lymph nodes and prevented the mice from clinical diabetes for 20 weeks, while the control (0 μg), lower (0.1 or 1 μg), and higher (25 or 50 μg) doses did not protect the mice from diabetes. The decrease of T cells was consistent with an increase of the anti-inflammatory cytokine, IL-10. The medium dose (10 μg) of capsaicin exhibited an anti-inflammatory effect on T cells attacking β-cells in the pancreatic islets in this experiment. Notably, the mechanism of action was investigated separately in this trial whether capsaicin had the immunoregulatory effects directly on the immune cells or the effects

were exhibited by neuropeptides of activated neurons by dosed capsaicin in the gut. The results indicated that capsaicin could suppress T cell activation independent of neurons (Nevius et al., 2012).

Some studies suggested capsaicin might also act on immune cells via a different way as well as a TRPV1 dependent mechanism. In an ex vivo trial, $CD3^+$ T lymphocyte population was decreased in Peyer's Patch (PP) collected from mice fed capsaicin for 4 days (Takano et al., 2007). Capsaicin-induced reduction in $CD3^+$ T cells was not seen in rats injected with capsaicin plus capsazepine, a TRPV1 antagonist. This result shows that the reduction in T lymphocytes in PP was mediated by the TRPV1 activation. However, capsaicin increased IL-2 and IFNG production of PP in the same trial and these increases were not inhibited by simultaneous administration of capsazepine to the mice. The authors concluded that the effects of capsaicin on the pro-inflammatory cytokines of PP were exerted independent of TRPV1. Kim et al. (2003) also found that the anti-inflammatory effect of capsaicin on prostaglandin E2 production was not abolished by capsazepine, which means the anti-inflammatory action of capsaicin in this study might occur through a different mechanism, not by TRPV1. Kim et al. (2014) in a recent study indicated that capsaicin inhibited cytotoxicity of natural killer cells and cytokine production (IFNG and TNF) through a TRPV1 independent pathway.

There are limited data about the immunoregulatory effects of capsaicin or *Capsicum* oleoresin in food animals. Liu et al. (2012) reported the immunoregulatory effects of *Capsicum* oleoresin in porcine macrophages *in vitro*. *Capsicum* oleoresin treatment (0, 25, 50, 100, and 200 μg/ml) linearly decreased TNF, IL-1β, and TGF-β

produced by porcine macrophages challenged with LPS in a dose-dependent manner. However, IL-1β concentration was quadratically increased in non-LPS treated macrophages by *Capsicum* oleoresin, which means the immunoregulatory effects of *Capsicum* oleoresin on IL-1β might depend on activation status in this trial. These data are consistent with results of Liu et al. (2013), who found *in vivo* that serum TNF and IL-1β concentration in virus-challenged pigs were attenuated by inclusion of *Capsicum* oleoresin (10 mg/kg) in the diet. In recent studies, Liu et al. (2014a, 2014b) reported that *Capsicum* oleoresin supplementation in pigs could alter gene expression profiles of ileal mucosa, which were associated with immune responses including innate immunity, T cells, and antigen presentation. There is a study in the chicken investigating the effects of capsaicin on blood cell types (Prieto and Campo, 2010). Capsaicin treatment reduced serum heterophil (neutrophil) number and heterophil : lymphocyte ratio elevated by heat stress in chickens, suggesting that capsaicin could alleviate oxidative injuries induced by high temperature.

Oxidative stress

Reactive oxygen species (ROS) are produced by normal metabolic processes. High producing dairy cows are exposed to oxidative stress because high energy demand and increase in oxygen requirements result in an accumulation of ROS, in particular, for cows in the transition period (Castillo et al., 2005). Cows that produce more ROS than available antioxidants are likely to be susceptible to a variety of health disorders (Sordillo and Aitken, 2009).

Capsaicin was reported to serve as an antioxidant against lipid peroxidation in animal tissues in studies (De and Ghosh, 1992; Asai et al., 1999). Dietary capsaicin (0.015%) reduced serum concentration of thiobarbituric acid reactive substances (TBARS) in rats having experimentally induced oxidative stress (Manjunatha and Srinivasan, 2006). The results indicated that capsaicin inhibited the lipid oxidation in the rats because TBARS are formed as a byproduct of lipid peroxidation. In the same trial, capsaicin supplementation lowered liver lipid peroxide level and liver enzymes such as alanine aminotransferase, aspartate aminotransferase, and lactate dehydrogenase, which indicate that capsaicin inhibited the hepatotoxicity and ameliorated the damage of the liver caused by induced oxidative stress (Senior, 2012). Demirbilek et al. (2004) reported that subcutaneous administration of capsaicin (1 mg/kg) attenuated the increases of plasma nitrite/nitrate concentration and tissue malondialdehyde in rats with sepsis. Nitrite/nitrate and malondialdehyde are byproducts of nitric oxide metabolism and lipid peroxidation, respectively (Nielsen et al., 1997; Shiva, 2013). According to Okada et al. (2010), the antioxidant property of capsaicin is due to its capability of scavenging ROS. Phenolic hydroxyl groups of capsaicin plays a role of the site for radical scavenging.

It is known that plant secondary metabolites are subject to detoxification *in vivo* by so-called phase I, II, and III metabolisms, which include hydrolytic biotransformation, conjugation reactions, and transporter-mediated efflux into the gut lumen or bile, respectively (Gurley, 2011). Due to the detoxifications, concentration of exogenous phenolic compounds in plasma or tissue is generally lower than that of other endogenous antioxidants *in vivo* (Masella et al., 2005). Instead, researchers have found that

phytochemicals could induce upregulations in genes and transcription factors relating to endogenous antioxidants, which include xenobiotic response elements and antioxidant response elements for the genes, and aryl hydrocarbon receptor and NF-E2 related factor (Seymour et al., 2013). Although data about capsaicin in the gene regulation have not been reported, effects of capsaicin on concentration of endogenous antioxidants in rat tissues have been demonstrated in literatures. Hassan et al. (2012) experimentally induced liver injury in rats and measured activities of endogenous antioxidants including, superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) in liver tissue of the rats after oral treatment of capsaicin. The results showed that capsaicin treatment significantly increased activities of SOD, and CAT, and content of GSH in injured liver tissue compared with non-treated liver tissue. Figure 2-2 shows roles of the endogenous antioxidants. SOD serves as the first line of defense against ROS and catalyzes the reduction of superoxide ($O^{2\bullet-}$) to hydrogen peroxide (H_2O_2) and O_2. Hydrogen peroxide is converted to water and oxygen by CAT. In addition, GSH also reduces H_2O_2 to water through GSH peroxidase (Sen and Chakraborty, 2010). Abdel-Salam et al. (2012) also found that intragastric and intraperitoneal administration of capsaicin increased GSH in brain, liver, and lung of rats challenged by LPS. Mechanisms of the effects of capsaicin on the endogenous antioxidants still need to be elucidated.

Glucose and insulin

Islet hormones, insulin and glucagon, are critical in maintaining glucose homeostasis in blood. Hyperglycemia stimulates secretion of insulin from the islets of

langerhans, in turn, glucose is stored in the liver as a form of glycogen. In contrast, glucagon increases glucose level in blood. It is known that the secretion and regulation of insulin and glucagon are controlled by the nervous system (Osundiji and Evans, 2013). Innervation of the pancreas independantly enables the islet to be capable of altering the hormone release. Early studies have shown that stimulations on vagus nerves regulated insulin secretion *in vitro* (Kaneto et al., 1967; Frohman et al., 1967). In addition, the brain is able to systemically control the hormone regulation of the pancreas (Osundiji and Evans, 2013). For example, Pocai et al. (2005) found that the mediobasal hypothalamus could lower blood glucose levels and control the effects of systemic insulin on hepatic glucose production via efferent vagal nerves.

In this sense, capsaicin, causing activation or ablation of sensory afferent nerves, has been used to investigate the hormone regulation in the pancreas in studies. Van de Wall et al. (2005) investigated the role of sensory afferent nerves in glucose tolerance using rats neonatally treated with capsaicin, and found that plasma insulin concentration was reduced in capsaicin-treated rats after intravenous glucose infusion while glucose and glucagon levels were not different. The authors suggested that capsaicin treatment increased insulin sensitivity. This is consistent with Koopmans et al. (1998), who found a enhancement of insulin sensitivity in rats neonatally treated by capsaicin. Increased insulin sensitivity in capsaicin-treated rats might have resulted from the inhibition of CGRP which was known to decrease insulin sensitivity at high level (Rasmussen et al., 1998).

Nevertheless, data on the effects of capsaicin on glucose and insulin levels have been conflicting. A single dose of 5 g *Capsicum frutescens* (26.6 mg capsaicin) decreased plasma glucose concentration at 30 and 45 min post oral glucose challenge, and increased plasma insulin concentration at 60, 75, 105, and 120 min post oral glucose challenge in a human study (Chaiyasit et al., 2009). Akiba et al. (2004) demonstrated that capsaicin treatment increased insulin concentration released from pancreatic islet β cells, and showed capsaicin exhibited the effect by increasing Ca^{2+} influx through TRPV1 expressed on β cells. However, Dömötör et al. (2006) observed increases of glucose and glucagon concentration in blood serum after oral glucose challenge when 400 μg capsaicin was administrated to human subjects. Serum insulin level was not affected by capsaicin. The authors suggested that these results were mediated by a direct release of somatostatin by the stimulation of capsaicin-sensitive afferent nerves in rats (Szolcsányi et al., 2004).

Fat mobilization

Studies have shown the effects of capsaicin on fat metabolism including deposition, distribution, and mobilization of fat in rats and humans (Yoshioka et al., 2000; Leung, 2008). In an early study, capsaicin supplementation (0.014%) to a high fat diet reduced serum triglyceride level and perirenal adipose tissue in rats (Kawada et al., 1986). Yoshioka et al. (2000) found that capsaicin inclusion (0.014%) in the diet reduced epididymal adipose tissue weight and increased serum free fatty acids in exercise-trained rats. Capsaicin intake increased the negative energy balance in the rats

in this trial. Shin and Moritani (2007) reported ingestion of 150 mg capsaicin before physical exercise exhibited higher fat oxidation in humans. In a recent study, topically administrated capsaicin was reported to reduce visceral adipose tissue as well as body weight in rats (Lee et al., 2013). They also found increases in gene expression of lipoprotein lipase and adiponectin in mesenteric adipose tissues of capsaicin-treated rats; these substances are related to fat accumulation reduction and fatty acid combustion (Yamauchi et al., 2001; Koike et al., 2004).

Underlying mechanisms of the capsaicin's effects on fat metabolism have been suggested *in vitro* and *in vivo*. Capsaicin was reported to directly act on adipose tissue in studies. Kang et al. (2007) incubated mesenteric adipose tissues with capsaicin, and found increases of adiponectin in capsaicin-treated tissues. They also demostrated that the DNA binding activity of NF-κB, a protein complex controling DNA transcription, was decreased by capsaicin treatment. It was suggested that capsaicin could have the inhibiting effects on fat accumulation by altering the NF-κB activity. Hsu and Yen (2007) reported comparable results that capsaicin treatment on adipocytes reduced protein expression of adiponectin *in vitro*. Adipose tissues are known to have TRPV1 in mice and humans and capsaicin is likely to act on adipose tissues through TRPV1 (Zhang et al., 2007). In addition to the direct effects of capsaicin on adipose tissues, capsaicin may indrectly affect fat metabolism as well. Watanabe et al. (1987) demonstrated that intravenous infusion of capsaicin in rats increased catecholamine released from the adrenal medulla. Since catacholamine has been known to increase

energy expenditure, capsaicin treatment could affect the energy homeostatsis, followed by increased fat utilization (Pritzlaff et al., 2000).

On the other hand, there is a report that capsaicin could affect fat distribution by altering blood flow to different tissue (Leung et al., 2007). Intragastric capsaicin treatment in rats decreased visceral fat, but increased subcutaneous while total fat did not change. Repeated dose of capsaicin induced a higher blood flow in the visceral site and a lower flow in the subcutaenous site. Because fat accumulation depends on blood flow (Crandall et al., 1997), it was suggested that capsaicin treatment could shift the fat distribution from visceral to subcutaneous tissues.

Feed (food) intake

Due to its property to stimulate or desensitize vagal afferent nerves, capsaicin has been studied on regulating feed (food) intake (Whiting et al., 2014). The studies suggested that capsaicin could affect energy intake or appetite through altering production and secretion of hormones and neuropeptide such as catecholamine.

A human study has shown the effects of capsaicin on intake-regulating factors, ghrelin and GLP-1 (Smeets and Westerterp-Plantenga, 2009). Consuming 1.03 g red peper tended to decrease ghrelin, a hunger-stimulating hormone, in plasma, while plasma GLP-1 was increased by the treatment, which is a peptide secreted from the ileum and reported to reduce food intake (Naslund et al., 2004). GLP-1 is known to inhibit gastric emptying and reduce the flow rate of nutrients for intestinal absorption (Imeryuz et al.,

1997). Furthermore, capsaicin may have an inhibitory effect on food intake through neuropeptide hormones. Capsaicin treatment has increased catecholamine release from the adrenal medulla in studies (Watanabe et al., 1987; Hursel and Westerterp-Plantenga, 2010) and catecholamine is reported to act as a food suppressor in conjunction with the fight-or-flight response (Adan et al., 2008).

However, studies have shown conflicting data about the effect of capsaicin on food intake. McCann et al. (1988) reported that the inhibitory effect of cholecystokinin (CCK) on feed intake was reduced in rats having desensitized afferent nerves by capsaicin. This effect was observed in another study in which a reduction in food intake by CCK injection was attenuated in rats neonatally treated with capsaicin (van de Wall et al., 2006). These results indicate that CCK acts through the vagal nerves on food intake and capsaicin treatment could inhibit the effect of CCK by desensitization of the vagal nerves. Hsu and Yen (2007) found a decrease in protein expression of leptin in adipocytes treated with capsaicin. Because leptin is known to inhibit hunger, it is suggested capsaicin could stimulate food intake (Klok et al., 2007). In addition, Debreceni et al. (1999) reported that ingestion of 400 μg capsaicin increased gastric emptying in heathy human subjects, which is inconsistent with the results of Smeets and Westerterp (2009), who found the inhibitory effect of capsaicin on food intake.

On the other hand, *Capsicum* treatment increased feed intake in beef cattle, which might be due to pungency of capsaicin (Cardozo et al., 2006; Fandiño et al., 2008; Rodríguez-Prado et al., 2012). *Capsicum* supplementation also increased water consumption in these studies, perhaps leading to increased feed intake. According to

Rodríguez-Prado et al. (2012), a linear increase in DMI was observed with increasing *Capsicum* supplementation, and they found a strong relationship between water intake and DMI (R^2=0.98). In dairy cows, *Capsicum* treatment did not affect DMI although length of the first meal time was shorter for cows fed *Capsicum* than the control cows (Tager and Krause, 2011). The authors indicated that relatively low dose of *Capsicum* in their experiment was the reason for the different response in DMI compared with the beef cattle studies (Cardozo et al., 2006; Rodríguez-Prado et al., 2012).

Gut health and digestive enzymes

The gastrointestinal tract is also known to be innervated and react with capsaicin. Studies have shown that capsaicin treatment had protective effects on the gastrointestinal tract (Luo, 2011). Abdel-Salam et al. (1995) reported that intragastric capsaicin treatment (0.1 μg/kg) decreased lesion severity induced by aspirin, ethanol, and HCl in rats. However, a high concentration of capsaicin (10 mg/kg) aggravated the lesion severity. A review including observations in 98 healthy humans and 178 patients with gastrointestinal diseases has indicated that capsaicin treatment increased gastric secretory responses and gastric emptying, and prevented gastric mucosal injury (Mózsik et al., 2007). With a large dose of capsaicin, however, the sensory nerves might be desensitized and lose the protective effects of capsaicin in the rats. Maggi et al. (1987) demonstrated that capsaicin desensitization could increase intestinal ulcers in rats.

A mode of action for the protective effects of capsaicin has been suggested by Gray et al. (1994). When capsaicin was administered into the stomach in rats, the number of gastric ulcers was decreased by the treatment. However, capsaicin treatment failed to reduce the ulcers in CGRP-depleted rats. Because CGRP secretion is stimulated by TRPV1 on afferent neurons with capsaicin and CGRP is known to have anti-inflammatory effects (Zimmerman et al., 1992; Tsuji and Aono, 2012), it is thought that capsaicin treatment could reduce gastric ulcers through CGRP activity.

According to studies of Platel and Srinivasan (1996, 2000) in rats, dietary capsaicin (0.015 %) for 8 weeks could increase secretions of digestive enzymes such as lipase, amylase, trypsin, and chymotrypsin from the pancreas. Meanwhile, a single dose of capsaicin (100 mg) did not affect the enzymes except for an increase of amylase in the pancreas and intestinal mucosa. Rao et al. (2003) measured the enzyme activities in two concentrations of capsaicin (0.5 and 5.0 mg/ml), and found amylase activity was enhanced in the pancreas homogenate with only 5.0 mg/ml capsaicin. These results, collectively, suggest that the stimulatory effects of capsaicin on digestive enzymes depend on the dose amount. This could be because of the desensitizing ability of capsaicin on nerves.

From the data shown in rats that capsaicin stimulates digestive enzymes in the gastrointestinal tract, similar effects could be exerted by capsaicin treatment in ruminants. Enhanced secretion of digestive enzymes may increase digestibility of nutrients in ruminants because the contribution of the lower gut to total tract digestibility has been reported to be 35.3, 21.2, and 19.5 % for OM, cell wall, and starch (Archimède et al.,

1996). However, Tager and Krause (2011) found no effect on total tract digestibility of OM, DM, NDF, ADF, CP, or starch when dairy cows were fed *Capsicum* oleoresin. It might be because *Capsicum* oleoresin in the experiment was administrated in a non-protected form and degraded by microbes in the rumen before reaching the lower gut.

Effects of *Capsicum* on productivity in food animals

The antimicrobial and the modulatory effects of capsaicionids on the host responses as shown in rats and humans studies could be beneficial to productivity in food animals such as pig, chicken, beef cattle, and dairy cows. Nevertheless, studies that investigated the effects of capsaicinoids are limiting in food animals, although some studies used EO mixtures containing *Capsicum* oleoresin (Matysiak et al., 2012; Bravo et al., 2014). Liu et al. (2013a) found that dietary inclusion of *Capsicum* oleoresin (10 mg/kg) increased average daily gain (ADG), accompanied with reduced diarrhea in weaned pigs. However, the effects on ADG and diarrhea were diminished after 9 days of the dose. Tager and Krause (2011) reported no effect of *Capsicum* oleoresin on productivity in dairy cows. Milk yield and composition in cows fed *Capsicum* oleoresin (0.25 g/d) were also not different from those of non-treated cows. Studies are necessary to investigate the effects of *Capsicum* on rumen microbial ecosystem, ruminal fermentation, feed intake, productivity, immunity, hormones, and blood metabolites in dairy cows as shown in monogastric animals. Rumen-protected *Capsicum* can be utilized to supply capsaicinoids to the lower guts thus exerting physiological effects in dairy cows.

Summary

Capsicum is a genus of flowering plants. It has various species with a variety of names depending on locations and types. Capsaicinoids are active compounds in the fruit of *Capsicum* plants, which are responsible for its pungency. Capsaicin and dihydrocapsaicin are major compounds in capsaicinoids and they account for about 90% of pungency. Capsaicinoids are synthesized via a phenylpropanoid pathway and a leucine/valine-fatty acid pathway, and stored mainly in the placenta of the *Capsicum* fruit. Capsaicinoids are readily absorbed in the gastrointestinal tract and metabolized in the liver to a large extent. TRPV1 is a receptor for capsaicinoids to exhibit their effects on nerves and tissues. *Capsicum* has been used for food preservation and medicine due to its antimicrobial property and is considered a potential rumen modifier in ruminant research. Studies have shown that *Capsicum* had little or no effect on rumen fermentation parameters such as VFA and ammonia concentration in dairy or beef cattle. There are limited data of the effect of *Capsicum* on rumen microbial population. Capsaicin showed immunoregulatory effects in rats by altering the production of cytokines and antibodies and stimulating activity and proliferation of immune cells. Capsaicin directly acts on immune cells through TRPV1, but also indirectly affects immune cells by stimulating neuropeptides release from neurons. Capsaicin is reported to serve as an antioxidant against lipid peroxidation. In addition, endogenous antioxidants can be enhanced by capsaicin. The innervation in the pancreas allows capsaicin treatment to alter insulin and glucagon secretion. Capsaicin treatment is reported to increase insulin sensitivity or glucose tolerance, although data are conflicting.

The inhibitory effects of capsaicin on fat are attributed to activation of TRPV1 on adipocytes and/or increased energy expenditure. Feed (food) intake data are not consistent among studies. Capsaicin has been reported to stimulate or inhibit appetite-related hormones. On the other hand, *Capsicum* supplementation in beef cattle increased feed intake, which was due to pungency of *Capsicum*. In addition, protective effects on the gastrointestinal tract and stimulating effect on digestive enzymes are observed with capsaicin treatment in rats and humans. The effects of capsaicin on rumen or gut microflora and the host animals may lead to improved productivity, health, and immunity in dairy cows.

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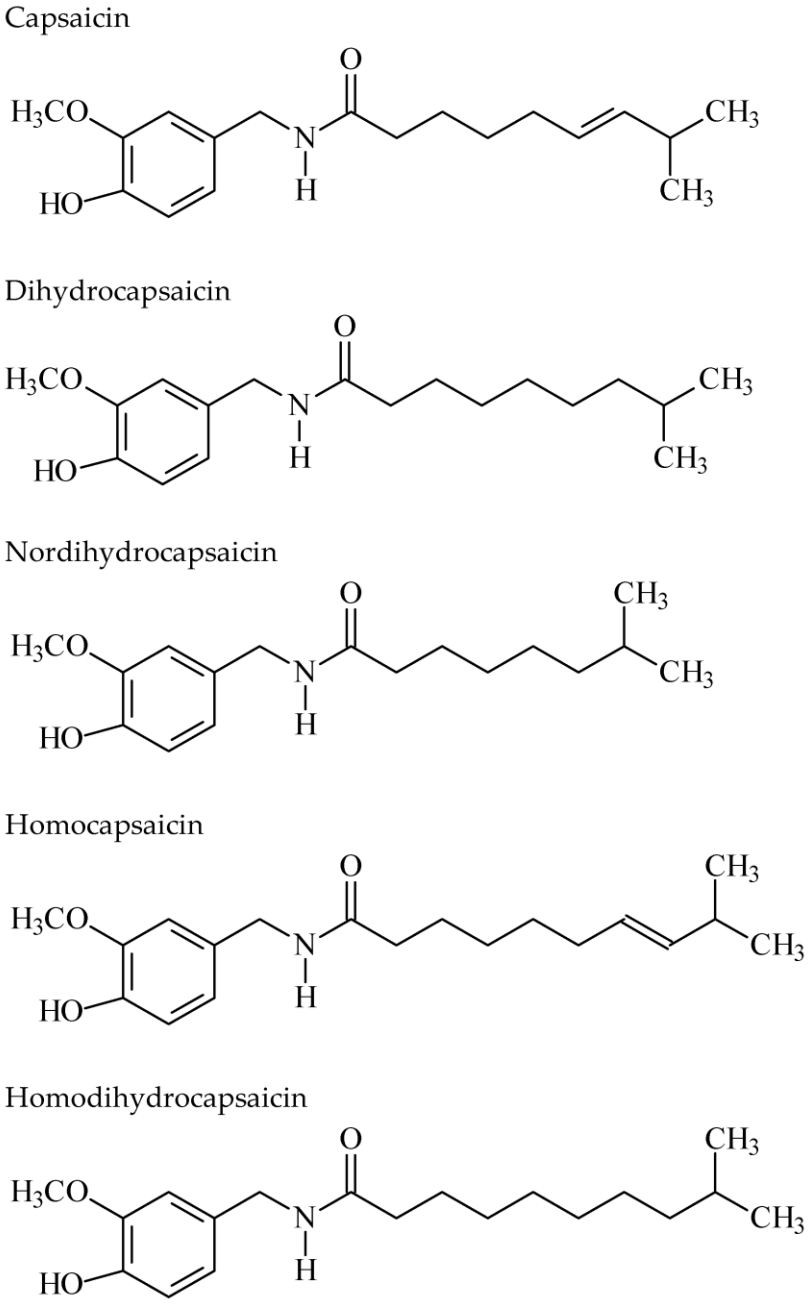

Figure 2-1. Chemical structures of capsaicinoids adapted from Mózsik et al. (2014)

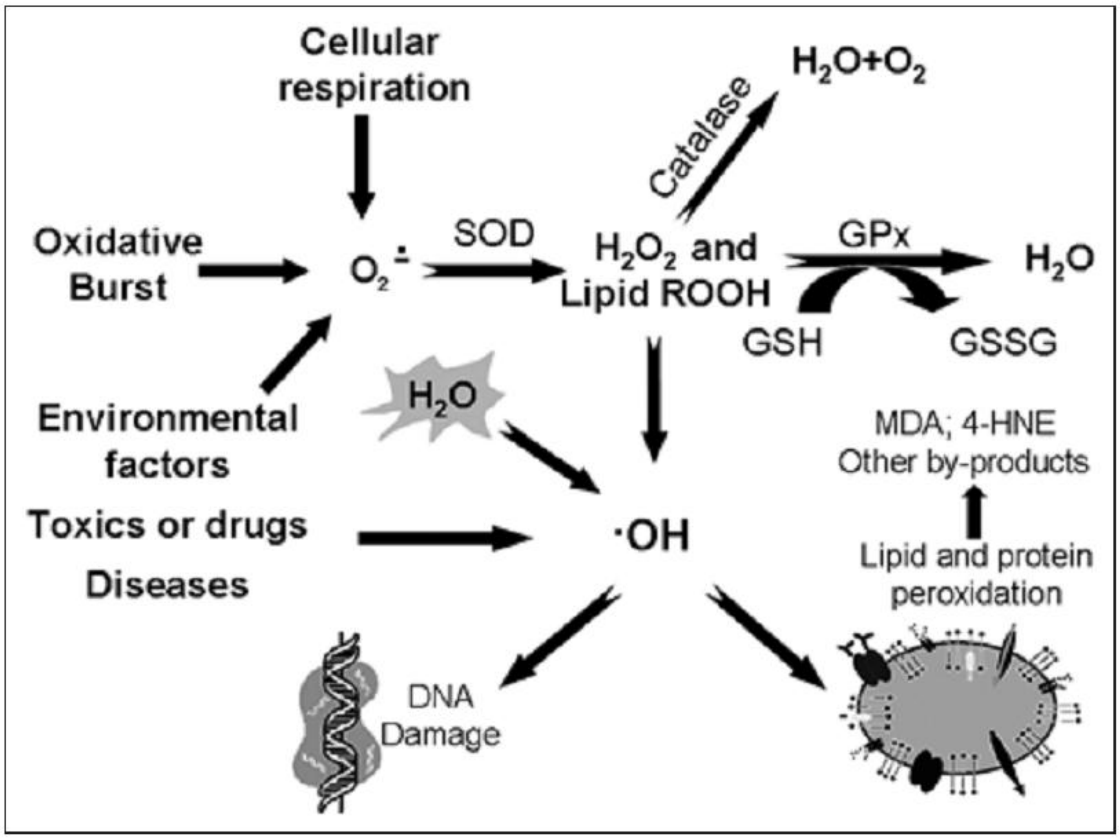

Figure 2-2. Endogenous antioxidants adapted from Rizzo et al. (2010). SOD, superoxide dismutase; GSH, glutathione; GPx, glutathione peroxidase; GSSG, Glutathione disulfide; MDA, malondialdehyde.

Chapter 3

Immune and production responses of dairy cows to postruminal supplementation with phytonutrients

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Abstract

This study investigated the effect of phytonutrients (PN) supplied postruminally on nutrient utilization, gut microbial ecology, immune response, and productivity of lactating dairy cows. Eight ruminally cannulated Holstein cows were used in a replicated 4 × 4 Latin square. Experimental periods lasted 23 d, including 14-d washout and 9-d treatment periods. Treatments were control (no PN) and daily doses of 2 g/cow of either *Curcuma* oleoresin (curcumin), garlic extract (garlic), or *Capsicum* oleoresin (capsicum). Phytonutrients were pulse-dosed into the abomasum of the cows, through the rumen cannula, 2 h after feeding during the last 9 d of each experimental period. Dry matter intake was not affected by PN, although it tended to be lower for the garlic treatment compared with the control. Milk yield was decreased (2.2 kg/d) by capsicum treatment compared with the control. Feed efficiency, milk composition, milk fat and protein

yields, milk N efficiency, and 4.0% fat-corrected milk yield were not affected by treatment. Rumen fermentation variables, apparent total-tract digestibility of nutrients, N excretion with feces and urine, and diversity of fecal bacteria were also not affected by treatment. Phytonutrients had no effect on blood chemistry, but the relative proportion of lymphocytes was increased by the capsicum treatment compared with the control. All PN increased the proportion of total $CD4^+$ cells and total $CD4^+$ cells that co-expressed the activation status signal and CD25 in blood. The percentage of peripheral blood mononuclear cells (PBMC) that proliferated in response to concanavalin A and viability of PBMC were not affected by treatment. Cytokine production by PBMC was not different between control and PN. Expression of mRNA in liver for key enzymes in gluconeogenesis, fatty acid oxidation, and response to reactive oxygen species were not affected by treatment. No difference was observed due to treatment in the oxygen radical absorbance capacity of blood plasma but, compared with the control, garlic treatment increased 8-isoprostane levels. Overall, the PN used in this study had subtle or no effects on blood cells and blood chemistry, nutrient digestibility, and fecal bacterial diversity, but appeared to have an immune-stimulatory effect by activating and inducing the expansion of CD4 cells in dairy cows. Capsicum treatment decreased milk yield, but this and other effects observed in this study should be interpreted with caution because of the short duration of treatment.

Key words: Capsicum, curcumin, garlic, dairy cow

Introduction

Many plants produce secondary metabolites that may be useful as feed additives because of their biologically active constituents (Wallace, 2004). Plant-derived bioactive compounds, also referred to as phytonutrients (PN) or phytobiotics, such as phenolic compounds, essential oils, and saponins have been shown to express antimicrobial activities (Cowan, 1999; Bakkali et al., 2008) and have been investigated as alternatives to rumen modifiers, such as ionophoric antibiotics, in animal nutrition (Greathead, 2003; Rochfort et al., 2008). Phytonutrients have also been studied as inhibitors of pathogens having an effect on animal health and productivity (Panda et al., 2006; Jouany and Morgavi, 2007; Giannenas and Kyriazakis, 2009).

Natural products originating from plants have been used in traditional medicine and as feed supplements to livestock diets for centuries. Antimicrobial activities, immune enhancement, and stress reduction are among the beneficial characteristics of these preparations (Wang et al., 1998). Garlic (*Allium sativum*) and garlic oil, for example, have a wide spectrum of activities from antimicrobial, antioxidant, and anticarcinogenic properties to beneficial effects on the cardiovascular and immune systems (Lang et al., 2004; Mirunalini et al., 2010). The antimicrobial properties of PN, demonstrated in monogastric farm species (Youn and Noh, 2001; Ando et al., 2003; Youn et al., 2004; Michiels et al., 2005, 2007), can also be advantageous in suppressing intestinal protozoa and pathogenic bacteria in cattle. Other effects such as immune stimulation, again primarily through inhibition of intestinal parasites (Applegate, 2009),

and prebiotic effects have been reported for PN in pigs and poultry (Guo et al., 2004a, b; Maass et al., 2005; Applegate, 2009).

Some PN (particularly phenols) are known to be resistant to microbial degradation in the rumen and could reach the small intestine in a biologically active form. Franz et al. (2010), for example, reported resistance (up to 60% recovery) of phenolic compounds, such as carvacrol, to microbial degradation in a continuous culture fermentor study. It is possible that these escape rates of phenolic compounds occur in vivo because the rumen is a dynamic system and the passage rate of the liquid phase of ruminal contents can be as high as 20%/h (Hristov et al., 2003). Thus, ruminal fluid and any compound associated with it may have a short residence time in the rumen (around 5 h) and a high probability of reaching the intestine of the host animal. Once PN reach the absorptive sites in the small intestine, they can be rapidly absorbed (as shown recently for essential oil terpenes from caraway, *Carum carvi*, and oregano, *Origanum vulgare*; Lejonklev et al., 2013) and have various physiological effects. Thus, if PN bypass the rumen, they could exhibit the same activities in the lower part of the ruminant digestive tract as previously described in monogastric animals. Improvements in digestibility, of the mucosal and antioxidant status, and immune response could lead to improved animal health, milk production, and feed efficiency in dairy cattle.

Based on evidence in monogastric species, we hypothesized that PN could have immune-stimulating and other beneficial effects in dairy cows. The specific objectives of the study were to investigate postruminal physiological effects of PN in relation to

nutrient utilization, gut microbial ecology, immune response, and productivity of lactating dairy cows.

Materials and Methods

Animals and treatments

This experiment and all procedures were reviewed and approved by The Pennsylvania State University Animal Care and Use Committee (IACUC protocol no. 35632). Animals were cared for according to the guidelines of the committee.

The design of the experiment was a replicated 4 × 4 Latin square with 1 multiparous and 7 primiparous Holstein cows averaging 533 ± 75.3 kg of BW, 175 ± 19.8 DIM, and 30.3 ± 8.01 kg/d of milk yield at the beginning of the trial. All cows were fitted with soft plastic rumen cannula (10-cm internal diameter; Bar Diamond Inc., Parma, ID). Experimental periods were 23 d, including 14-d washout and 9-d treatment periods. Treatments were control (99.9% ethanol dissolved in distilled water, see below, without PN) and 3 PN (Pancosma S.A., Geneva, Switzerland): a daily pulse dose of 2 g/cow of *Curcuma* oleoresin (curcumin; from *Curcuma* longs L., containing 95% curcumin), a daily pulse dose of 2 g/cow of garlic extract (garlic; containing by weight, 60% polysorbate 80 and 40% organosulfur compounds), or a daily pulse dose of 2 g/cow of *Capsicum* oleoresin (capsicum; from *Capsicum* frutescens L. and *Capsicum* anum L. var.concoides, containing 6% capsaicin). Phytonutrients were dissolved in 99.9% ethanol (2 g of PN in 40 mL of ethanol) and further diluted with 250 mL of distilled water.

Solutions were pulse-dosed into the abomasum of the cows through the rumen cannula, 2 h after feeding during the last 9 d of each experimental period (i.e., the treatment period) using a 500-mL plastic bottle attached to a 155-cm-long polyvinyl tube (10-mm internal diameter). The infusion tube was flushed with an additional 500 mL of distilled water following the delivery of the treatment solutions. The relatively short treatment period was necessary to reduce, as much as possible, stress to the cows caused by the invasive abomasal infusion procedure. Cows were housed in a tiestall barn, were fed once daily at approximately 0800 h, and had free access to fresh water. The basal diet (Table 3-1) was fed ad libitum as a TMR to achieve approximately 5 to 10% refusals. Feed was pushed up 3 to 5 times daily. Cows were milked twice daily at 0500 and 1700 h and treated with recombinant bST (Posilac; Elanco Co., Greenfield, IN; 500 mg, i.m.) at 14-d intervals. Although not analyzed, we assumed that, because of the experimental design of the trial (i.e., Latin square), the bST treatment did not affect the experimental results in this study.

Sampling and analysis

Feed intake was measured daily, TMR samples were collected twice weekly, and individual feed ingredients were sampled once weekly. Composite samples of the TMR, forages, and concentrates were stored frozen, oven-dried for 48 h at 65℃ (forced-air oven), and ground in a Wiley mill (A. H. Thomas Co., Philadelphia, PA) through a 1-mm screen before being analyzed for CP (AOAC International, 2000), NDF (Van Soest et al., 1991), ADF (AOAC International, 2000), ether extract (AOAC International, 2006), Ca (AOAC International, 2000), P (AOAC International, 2000), NFC (NRC,

2001), and NE_L (NRC, 2001) by Cumberland Valley Analytical Services (Maugansville, MD). Total mixed ration and fecal samples were ashed for 4 h at 600°C for analysis of OM. Samples of the TMR were also analyzed for indigestible NDF (iNDF; see below).

Milk production of the cows was recorded daily, and milk yield, DMI, and estimated feed efficiency data for the last 6 d of each treatment period (i.e., after 3 d of PN administration) were used in the statistical analysis. Dry matter intake was calculated by adjusting daily as-fed feed intake to DM content (measured for 48 h at 65°C) of the weekly TMR and refusals composited samples. Samples for milk composition were collected on the last 2 d of each treatment period, from a.m. and p.m. milkings, preserved with 2-bromo-2-nitropropane-1,3 diol, and submitted to Dairy One laboratory (Pennsylvania DHIA, University Park) for analysis of milk fat, true protein, lactose, and MUN using infrared spectroscopy (MilkoScan 4000; Foss Electric, Hillerød, Denmark). Milk composition data of the 2 sampling days were averaged and the average values used in the statistical analysis. Averaged milk yield and DMI for the 6 data collection days and averaged milk composition data were used to calculate milk fat, protein, lactose, 4.0% FCM, and NE_L yield and efficiency.

Rumen samples were collected once during each experimental period (on d 7 of the treatment period), 3 h after feeding. Whole ruminal contents were sampled, processed, and analyzed for pH, VFA, and ammonia concentration as described elsewhere (Hristov et al., 2013).

Spot fecal and urine samples were collected by stimulating defecation or from the rectum and by massaging the vulva, respectively, at 0900, 1500, and 2100 h on d 6 and

at 0300, 0600, 1200, 1800, and 0000 h on d 7 of each treatment period. Approximately 300-g wet fecal and 500-mL urine samples were collected per sampling. Aliquots of the fecal samples (approximately 50 g per sampling, wet basis) were composited, per cow and period, and stored frozen in −80°C for bacterial population analysis using tag-encoded FLX amplicon pyrosequencing and Gray28F 5′GAGTTTGATCNTGGCTCAG and Gray519R 5′GTNTTACNGCGGCKGCTG primers (Dowd et al., 2008a,b). For details on these analyses, see Hristov et al. (2013)). The remaining fecal sample was oven-dried at 65°C in a forced-air oven for 48 h. After drying, samples were ground through a 1-mm sieve (Wiley mill), composited per cow and period, and analyzed for OM and amylase-treated NDF (aNDF) and ADF (Ankom A200 fiber analyzer; Ankom Technology, Macedon, NY, and Van Soest et al., 1991). Heat-stable amylase (Ankom Technology) and sodium sulfite (Fisher Scientific, Waltham, MA) were used in the aNDF procedure. Fecal samples were pulverized at 30 Hz/s for 2 min in a Mixer Mill MM 200 (Retsch GmbH, Haan, Germany) and analyzed for CP (N × 6.25) on a Costech ECS 4010 C/N/S elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA). Apparent total-tract digestibility of nutrients was estimated using iNDF as an intrinsic digestibility marker (Schneider and Flatt, 1975). Fecal and TMR samples were analyzed for iNDF according to Huhtanen et al. (1994)), with the exception that 25-µm pore size Ankom filter bags (Ankom Technology) were used for the rumen incubation. Urine samples were acidified (pH <3) using 2M H_2SO_4 (pH was verified using litmus paper), diluted 1:10 with distilled water, composited per cow and period, and stored frozen at −20°C. Urine samples were analyzed for N (as for feces), urea-N (Stanbio Urea Nitrogen Kit 580; Stanbio Laboratory Inc., San Antonio, TX), and creatinine (Stanbio

Creatinine Kit 0400; Stanbio Laboratory Inc.). Daily volume of excreted urine was estimated based on urinary creatinine concentration, assuming a creatinine excretion rate of 29 mg/kg of BW (determined based on total urine collection samples from Hristov et al., 2011). Estimated urine output was used to calculate daily total N and urinary urea-N excretions.

Blood samples were collected from the coccygeal tail vein or artery at 2 and 4 h after PN administration on d 6 and 7 of each treatment period. Samples (approximately 10 mL) were collected into Vacutainer tubes containing EDTA (BD Biosciences, Franklin Lakes, NJ), kept refrigerated (4°C), and analyzed the same day for hematology analyses. The analyses included red blood cell count, hemoglobin, hematocrit, red blood cell parameters (mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, platelet count, and mean platelet volume), and total white blood cell count, including total count for neutrophils, eosinophils, lymphocytes, monocytes, and basophils using an automated hematology analyzer (HemaVet, Drew Scientific, Oxford, CT). A separate set of blood samples was collected into Vacutainer tubes containing silica clot activator (SST tube, BD Biosciences); blood serum was separated (after clotting) through centrifugation at 3,000 × g and room temperature for 15 min, and analyzed for albumin, alkaline phosphatase, alanine aminotransferase, amylase, BUN, Ca, cholesterol, creatinine, globulin, glucose, P, K, Na, total bilirubin, total protein (Idexx VetTest and VetLyte Chemistry and Electrolyte Analyzers, Idexx Laboratories Inc., Westbrook, ME). A third set of blood samples was collected into Vacutainer tubes containing EDTA (BD

Biosciences). Plasma was obtained as described elsewhere (Tekippe et al., 2011), composited per cow, period, and sampling time point, and stored frozen at −80°C. Plasma was analyzed for the oxidative stress markers 8-isoprostane and oxygen radical absorbance capacity (ORAC), as described elsewhere (Ju et al., 2009 and Cao and Prior, 1999, respectively).

Whole blood (approximately 250 mL) was collected via jugular venipuncture before feeding from 4 cows on d 8 and from the other 4 cows on d 9 of each treatment period for immune response analyses. Peripheral blood mononuclear cells (PBMC) were isolated following procedures described by Ndiaye et al. (2008)) for analysis of proliferation, T-cell phenotypes, and cytokine production. For the proliferation assay, 25 $\times 10^6$ PBMC were incubated with carboxyfluorescein succinimidyl ester (1.75 μM; Sigma Chemical Co., St. Louis, MO) in serum-free AIM-V medium (Invitrogen, Grand Island, NY) at 37℃ for 15 min and then at room temperature for another 10 min. The cells were diluted 5-fold with medium containing 10% fetal calf serum (Invitrogen) and washed 3 times at 300 × g for 10 min. Labeled PBMC were cultured and treated with either concanavalin A (Sigma Chemical Co.) or PBS (as a control) in 96-well plates for 72 h. Proliferation was determined by flow cytometery (GuavaEasyCyte Plus, Millipore, Billerica, MA).

To determine the effect of PN on T-lymphocyte phenotypes, 1×10^6 PBMC were directly labeled with Fluor-conjugated primary antibodies against T-lymphocyte surface antigens following a protocol described by Poole and Pate (2012)). The antibodies used were against cluster of differentiation (CD)4 (MCA1653F; AbD Serotec, Raleigh, NC)

and CD25α (MCA2430PE; AbD Serotec), CD8α (MCA837F; AbD Serotec) and CD8β (MCA1654PE; AbD Serotec), CD8α, ACT2 [an activation marker, ACT2 (CACT26A; VMRD, Pullman, WA], δ T-cell receptor (δTCR; GB21A, VMRD) and CD8α, δTCR, and WC1 (MCA838F; AbD Serotec). The δTCR antibody was conjugated to phycoerythrin using the Zenon R-phycoerythrin mouse IgG2b labeling kit (Z25255; Invitrogen). Each sample was labeled in duplicate with the following combinations of antibodies: CD4 and CD25α, CD8α and CD8β, CD8α and ACT2, δTCR and CD8α, and δTCR and WC1. Control antibodies used were IgG2b negative control (MCA691PE; AbD Serotec), IgG2a negative control (MCA929F; AbD Serotec), and IgG1 negative control (MCA928PE and MCA928F; AbD Serotec). The proportion of stained cells was determined by flow cytometry (GuavaEasyCyte Plus, Millipore).

Production of the proinflammatory cytokines tumor necrosis factor (TNF), IFNγ, and IL6 was measured in the medium of cultured PBMC by ELISA (ELISA plates for all cytokines were purchased from Thermo Fisher Scientific, Waltham, MA). The PBMC were cultured for 48 h in the presence or absence of LPS at 1µg/ml.

Liver tissue samples were collected by blind percutaneous needle biopsy as described previously (Greenfield et al., 2000) from 4 cows on d 8 and from the other 4 cows on d 9 of each treatment period, 4 h after PN administration. Samples were rinsed once with ice-cold saline and an aliquot (500 to 1,000 mg) was transferred to a tube containing Trizol solution (Invitrogen) for RNA isolation and immediately frozen at −80°C. Liver tissue was analyzed for key transcripts that represent control points for gluconeogenesis (phosphoenolpyruvate carboxykinase-C, glucose 6-phosphatase, and

pyruvate carboxylase), fatty acid oxidation (carnitine palmitoyltransferase 1A), response to reactive oxygen species (catalase and glutathione peroxidase), and protease inhibitors (α-2-macroglobulin). Abundance of mRNA transcripts was quantified using real-time PCR, brilliant SYBR Green reagent and QPCR Master Mix (Stratagene, Cedar Creek, TX), and the following primers: PC, CCACGAGTTCTCCAACACCT (forward), TTCTCCTCCAGCTCCTCGTA (reverse); phosphoenolpyruvate carboxykinase-C, AACCTGGCCATGATGAACCCTACT (forward), ACTCCTTGCCCTTCCAGGAAATGA (reverse); bovine 18S, ACCCATTCGAACGTCTGCCCTATT (forward), TCCTTGGATTGTGGTAGCCGTTTCT (reverse), carnitine palmitoyltransferase 1A TCCAGCTGGCTCATTACAAGGACA (forward), TCTCAGACGCGATCCTGAACAACT (reverse), catalase ATCTCACTCAGGTGCGGACTTTCT (forward), AGGTGTGAACACCATGCTCTGCTT (reverse), and glutathione peroxidase AAAGTGCGAGGTGAATGGCGAGAA (forward), ACAGCAGGGTTTCAATGTCAGGCT (reverse). The no-reverse-transcriptase control was formed by combining an equivalent quantity of purified RNA for each sample, and water served as the no-template control. A cDNA pool was formed from an equivalent quantity of cDNA from each sample diluted with nuclease-free water in a 1:4 dilution series to generate a standard curve. The relative abundance of each mRNA was determined using dilutions of the cDNA pool as a reference. The PCR reactions were as follows: 1 cycle at 95°C for 10 min; 40 cycles of 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s; and 1 cycle of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. Reaction

efficiencies were between 90 and 110%, and all samples, standards, and controls were analyzed in triplicate and values normalized to 18S mRNA abundance within each sample.

Statistical analysis

All data were analyzed using the MIXED procedure of SAS (2003; SAS Institute Inc., Cary, NC) by ANOVA Latin square. The model included square, cow within square, period, and treatment, with the error term assumed to be normally distributed with mean = 0 and constant variance. Square and cow within square were random effects and all others were fixed. Feed intake and milk yield data were analyzed as repeated measures assuming an autoregressive first-order covariance structure. The model was similar to that described above but contained time of sampling effect and treatment × time interaction. When the main effect of treatment was significant, means were separated by pairwise t-test (pdiff option of PROC MIXED). Statistical differences were declared at $P \leq 0.05$. Differences between treatments at $0.05 < P \leq 0.10$ were considered as a trend toward significance.

Results

The basal diet fed in this trial (Table 3-1) was identical to a diet fed in a concurrent trial (the adequate-MP diet in Lee et al., 2012) and was formulated to meet the NE_L and MP requirements of the cows. Due to lower-than-expected feed intake,

however, the diet supplied inadequate amounts of NE_L and MP for all treatments; NE_L deficiency was from 1 (capsicum treatment) to 8% (garlic treatment) and MP deficiency was from 7 (capsicum treatment) to 13% (garlic treatment; according to NRC, 2001). Body weight of the cows was not affected by treatment (average of 564 kg, SEM = 26.1; P = 0.66), but as a result of nutrient supply deficiencies, all cows lost BW during the trial; BW loss was on average 22 ± 3.13 kg (approximately 240 g/d).

Dry matter intake was similar between the control, curcumin, and capsicum treatments, but tended to be lower (P = 0.09; by about 1.3 kg/d compared with the control) for the garlic treatment compared with all other treatments (Table 3-2). We detected a trend for effect of time (i.e., day of PN treatment; P = 0.09) on DMI but no time × treatment interaction (P = 0.93). Intake of DM varied from day to day (20.0 to 21.5 kg/d) with no trend for a steady increase or decrease with the initiation of PN administration (data not shown in tables). Milk yield was decreased (by 2.2 kg/d; P = 0.05) by the capsicum treatment compared with the control. We observed no time or time × treatment effect for milk yield (P = 0.34 and 0.98, respectively). Because of the lower DMI, feed efficiency was numerically greater (P = 0.15) by about 4.5% for the garlic treatment compared with the control but was about 6% lower for capsicum (again, compared with the control). Phytonutrients had no effects on milk composition, milk fat and protein yields, milk N efficiency, 4.0% FCM yield, or the efficiency of feed NE_L use.

Rumen fermentation variables were not affected (P > 0.29; data not shown in tables) by treatment. Rumen pH (overall mean followed by SEM; 6.10, 0.043), ammonia concentration (9.0 mM, 0.76), total VFA (107 mM, 3.4), individual VFA concentrations,

and acetate:propionate ratio (2.6, 0.14) were within normal ranges and similar between control and PN treatments. Apparent total-tract digestibility of DM (%, overall mean followed by SEM; 64.3, 0.67), OM (65.6, 0.64), CP (61.7, 1.24), aNDF (37.2, 0.85), and ADF (34.5, 0.88) were not affected by treatment ($P > 0.52$; data not shown in tables). Fecal bacteria were also not affected by treatment (Table 3-3). The 5 most predominant bacterial genera in fecal mass were *Clostridium* (on average 10.5% of the total copies of 16S rDNA), *Eubacterium* (10.2%), *Ruminococcus* (9.8%), *Bacteroides* (8.4%), and *Oscillospira* (8.4%). Nitrogen intake, nitrogen excretion with feces or urine, urinary urea N excretion, and total excreta N did not differ among treatments (Table 3-4).

Chemical analysis of blood serum showed no differences ($P > 0.15$; data not shown in tables) due to treatment. Blood glucose (overall mean followed by SEM; 64.5 mg/dL, 3.63), BUN (9.6 mg/dL, 0.68), creatinine (0.76 mg/dL, 0.059), total protein (7.15 g/dL, 0.258), albumin (2.83 g/dL, 0.175), globulin (4.37 g/dL, 0.168), alkaline phosphatase (69.7 U/L, 7.76), total bilirubin (0.25 mg/dL, 0.065), amylase (25.0 U/L, 3.28), Ca (10.1 mg/dL, 1.84), P (5.77 mg/dL, 0.358), Na, K, and Cl were within the normal ranges for cattle (Bos taurus). Alanine aminotransferase (50.1 U/L, 2.77) and cholesterol (235 mg/dL, 17.1) concentrations were slightly elevated in all cows, except those in the garlic treatment ($P = 0.93$ and 0.21, respectively), relative to normal ranges for dairy cattle. Blood cell counts were generally not affected by treatment (Table 3-5). Compared with the control, the relative proportion of neutrophils tended to be slightly lower ($P = 0.09$) for cows in the curcumin and capsicum groups and slightly higher for garlic; that of lymphocytes was increased ($P = 0.04$) in the capsicum treatment compared

with the control and garlic treatments. Concentration of red blood cells, hematocrit percentage ($P = 0.11$), platelet count, and hemoglobin ($P = 0.11$) concentration did not differ between control and PN treatments.

With one notable exception, PN had no effects on blood T-cell phenotypes, proliferation, or viability (Table 3-6). The proportion of total $CD4^+$ (and $CD4^+CD25^-$) cells was greater ($P \leq 0.03$) in blood samples from cows treated with PN than in the control cows. We also detected numerical trends for increased (2-fold; $P = 0.28$) total $CD4^+$ that co-expressed the activation status signal CD25 (IL2 receptor) in PN compared with the control. The percentage of PBMC that proliferated (without concanavalin A) increased numerically ($P = 0.24$), with more than twice as many cells proliferating in the PN groups compared with the control group. Viability of PBMC was not affected by treatment. Cytokine production also did not differ between control and PN.

Phytonutrients had no effect on expression of mRNA in liver for key enzymes (Table 3-7), although we observed numerical increases in pyruvate carboxylase (on average 55%), glucose 6-phosphatase (52%), and glutathionine peroxidase (45%) and a numerical decrease in α-2-macroglobulin (40%) with PN compared with the control group ($P > 0.57$). Phytonutrients did not affect the ORAC of blood plasma, but garlic increased ($P = 0.02$) 8-isoprostane levels compared with the control (and capsicum; Figure 1).

Discussion

Various forms of plant extracts, based on some of the active compounds used in the current study, have been investigated as rumen modifiers in dairy cows, having limited or no effects on feed intake and milk production or composition (for example, Yang et al., 2007; Tager and Krause, 2011; Boyd et al., 2012). To our knowledge, however, this is the first study to report production effects of PN supplied postruminally. Cows received PN treatments for a relatively short period, 9 d, to avoid stress from the abomasal infusion procedure. Production and feed intake data were collected following a short adaptation period, which might not have been sufficient to trigger stable feed intake and production responses. The lack of time effect (i.e., days of PN treatment) on milk production, however, is an indication that, within the 9 d of treatment, milk production did not change significantly across treatments. As indicated earlier, there also was no clear trend in DMI during the 9-d treatment period, despite the trend for effect of time (no time × treatment interaction). Thus, within the specific conditions of this trial, the curcumin and capsicum treatments did not affect DMI but numerically decreased milk yield, whereas the garlic treatment tended to decrease both DMI and milk yield. Although the effect of garlic on milk production can clearly be attributed to the decreased DMI, the trend for decreased milk yield with the other PN is difficult to explain. Specific plant extracts and PN may have an objectionable odor and decrease DMI through palatability mechanisms when offered mixed with the TMR (a clear example with carvacrol in oregano was reported by Hristov et al., 2013), but apparently this could not have been the case in the current study because PN were infused into the

abomasum. Thus, the mechanism involved in the decreased milk production with all PN remains unclear. In studies with rats, capsaicin increased energy expenditure by enhancing adrenal sympathetic nerve activity and epinephrine secretion (Kawada et al., 1986; Watanabe et al., 1987, 1988). Similarly, an oral dose of red pepper containing capsaicin induced thermogenesis and increased heat loss in humans (Hachiya et al., 2007). These properties of capsaicin could have caused less-efficient use of dietary energy, which might have resulted in the decreased milk production observed in the current study. Variable effects on DMI by essential oil preparations have been reported for dairy cows in the literature (Kung et al., 2008; Tassoul and Shaver, 2009; Tager and Krause, 2011).

Similar to the feed intake and production data, no dairy cow studies are known to us that investigated digestibility and urinary N loss effects of PN supplied postruminally. Several studies have reported a lack of effect of PN, specifically various blends of essential oils or their active ingredients, on ruminal or total-tract nutrient digestibility (Benchaar et al., 2007; Tager and Krause, 2011) or urinary N losses (Benchaar et al., 2006, 2007). In some cases, however, DM or fiber degradability and digestibility were remarkably increased (Benchaar et al., 2006; Yang et al., 2007; about a 12% increase in OM degradability with garlic essential oils in Boyd et al., 2012). In the current study, postruminal supplementation of 2 g/d of PN had no effect on total-tract digestibility or urinary N losses.

Gut digesta or tissue could not be sampled in the current study and, therefore, fecal samples were used to assess the effect of PN on intestinal microflora. Bacterial

composition of fecal matter is closely related to the microfloral composition of the cecum in vertebrate animals (Smith, 1965), and fecal microbial cultures have been routinely used to study intestinal microbial composition (Tannock, 2001). The only genus that was numerically affected by PN in the current study was Oscillospira. These large bacteria are common inhabitants of the rumen and large intestine of cattle (Mackie et al., 2003; Li et al., 2012), and their decreased population in feces is likely an indication of decreased large intestinal populations. The implications of this observation, however, are unclear.

The only difference in blood cells in this study was a slight increase in the proportion of lymphocytes in the total white blood cells with the curcumin and capsicum treatments. This increase was likely due to a slightly lower total white blood cell count in these treatments compared with the control and a slight decrease in neutrophil count, resulting in a relatively greater percentage of lymphocytes in these treatments. However, white blood cell counts were within the normal variable range for adult cattle (Weiss and Wardrop, 2010). The neutrophil:lymphocyte ratio for the curcumin and capsicum treatments (arithmetic means of 1.34 and 1.31, respectively) were similar to that of the control (1.36), whereas the neutrophil:lymphocyte ratio in the garlic treatment was slightly higher (1.56; $P = 0.02$; SEM = 0.288). This increase was suggested as indicative of an acute stress response (Weiss and Wardrop, 2010). Whether or not cattle on the garlic treatment experienced a stronger acute stress response compared with the control or the other PN treatments is unknown but may be of interest to explore in future research. Feng et al. (2012)) indicated that allicin, a major active component of garlic,

enhances proinflammatory responses in acute phase infection by parasite in mice (Mus spp.). In contrast, Kuo et al. (2011)) reported that garlic oil has an antiinflammatory effect on neutrophil infiltration by lowering adhesion molecules in rats (Rattus spp.).

The activity of peripheral blood immune cells measured by proliferation and cytokine production ex vivo was not significantly affected by any of the PN tested in the present study. However, further analysis of PBMC revealed that the proportion of $CD4^+$ cells increased in response to garlic, curcumin, and capsicum treatments compared with the control. This indicates that compared with other immune cells in peripheral blood, $CD4^+$ cells may be more sensitive to the effects of PN. Although not statistically significant, all PN also doubled the proportion of $CD4^+$ cells that co-expressed CD25α (the high affinity receptor for IL2). Indeed, CD25α is considered an indicator of the activation status of bovine T lymphocytes (Waters et al., 2003). Dietary supplementation of garlic in a fermented form enhanced the proportion of $CD4^+$ in T lymphocytes isolated from pigs (Sus scrofa domesticus) challenged with and without LPS (Wang et al., 2011). Garlic has been shown to induce proliferation of human PBMC (Salman et al., 1999), rat T lymphocytes (Colić et al., 2002), and mouse splenocytes (Patya et al., 2004) in vitro. In the present study, the lack of a significant effect of garlic on proliferation may be attributed to both the variation in individual animal responses and the concentration of garlic in peripheral circulation. In vitro studies have shown that garlic increases the expression of CD25 in rat splenocytes stimulated with either phorbol myristate acetate or concanavalin A (Colić and Savić, 2000; Liu et al., 2009). Diets supplemented with both capsicum and curcumin increased splenocyte proliferation and peripheral blood $CD4^+$

cells in chicken (Gallus gallus domesticus) challenged with Eimeria tenella (Lee et al., 2011). Curcumin alone has also been shown to increase mouse $CD4^+$ cells in the spleen (Yasni et al., 1993) and intestinal mucosa (Churchill et al., 2000). In experimental autoimmune encephalomyelitis, curcumin increased mouse $CD4^+CD25^+$ T-regulatory cells in the spleen (Kanakasabai et al., 2012). However, curcumin has also been shown to inhibit the expansion of splenic $CD4^+$ cells and their expression of CD25 in vitro (Forward et al., 2011). In the present study, it was not determined if the $CD4^+CD25^+$ population were regulatory T cells. When CD4 T lymphocytes are activated, they produce cytokines that activate cells of the innate immune system such as macrophages, and stimulate antibody production from B cells. However, the induction of TNF, IFNγ, and IL6 with LPS in vitro was not affected by PN in the current study. Although the nature of the immune response cannot be characterized as pro- or antiinflammatory, garlic, curcumin, and capsicum might modulate the function of the adaptive immune system, as reported in monogastric species.

Liver has been recognized as a potential site of action for curcumin (Maradana et al., 2013), capsicum (Kang et al., 2011), and garlic oils (Iciek et al., 2012), and these PN act to improve lipid metabolism, insulin sensitivity, and enhanced capacity to respond to oxidative stressors in the attenuation of metabolic dysregulation caused by obesity (Kang et al., 2011) or excessive alcohol consumption (Raghu et al., 2012). Several PN have been shown to be safe and effective in enhancing metabolism, but the blood concentrations necessary to elicit a biological response are difficult to attain using oral supplementation due to low bioavailability, causing a combination of poor absorption,

rapid metabolism, and rapid systemic elimination (Anand et al., 2007; Ferruzzi, 2010). Methods are being actively sought to enhance PN bioavailability through the use of nanoparticles, chemically enhanced derivatives, and other technologies (Anand et al., 2007). Although the effect of rumen metabolism on PN was circumvented in the present study by abomasal infusion, we did not attempt to enhance postruminal bioavailability; consequently, the PN used are subject to the same limitations observed in monogastric species. The lack of observed response for liver transcripts and other postabsorptive measures in the present experiment may reflect a lack of bioavailability, a lack of efficacy, or a combination of both. Additional measures of circulating PN and secondary metabolites are necessary to adequately assess bioavailability. Likewise, the use of in vitro models of hepatic metabolism are needed to directly assess the effect of PN on bovine hepatic metabolism to more fully determine the potential of PN to enhance metabolism in dairy cows.

Many studies have reported evidence that PN, such as the phenolics found in garlic, curcumin, and capsicum, elicit an antiinflammatory effect when consumed as dietary components. These effects are commonly attributed to the potent radical scavenging activity of phenolic compounds, which is thought to translate to antioxidant activity in vivo. Indeed, phenolics exhibit potent antioxidant activity in vitro; however, it has been much harder to demonstrate such activity in vivo. In fact, several studies have shown that the consumption of high levels (e.g., pharmacological doses) of polyphenols such as flavonoids can have deleterious consequences in monogastric species—hepatic and intestinal toxicity, DNA damage, and leukemogenic effects (Lambert et al., 2007);

however, we are unaware of any study that demonstrates such an effect in ruminant species. A likely and generally well-accepted mechanism for the proinflammatory and pro-oxidative effect of dietary phenolics is hydrogen peroxide generation (i.e., oxygen reduction) coupled with phenolic oxidation. Hydrogen peroxide and its metal-catalyzed reduction products (e.g., hydroxyl radicals) are capable of damaging biological molecules such as membrane components, proteins, and DNA (Lambert and Elias, 2010). A second potential mechanism that could explain the absence of an observed antioxidant effect of PN in this study and, in the case of 8-isoprostane levels, a slight increase in pro-oxidant activity, is the generation of o-quinones from phenolic oxidation. As highly electrophilic compounds, quinones are known to react with and consume nucleophiles in vivo (Sang et al., 2005), some of which are important antioxidant factors (e.g., glutathione).

Conclusion

The PN used in this study—curcumin, garlic extract, and capsicum—when administered postruminally at 2 g/cow per day had subtle or no effects on blood cells and chemistry, nutrient digestibility, fecal bacterial diversity, antioxidant status, or expression of mRNA in liver for key enzymes in dairy cows. These PN, however, appeared to have an immune-stimulatory effect by activating and inducing the expansion of CD4 cells. The production and other effects observed in the study should be interpreted with caution because of the short duration of the PN treatment.

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Table 3-1. Ingredient and chemical composition of the basal diet fed during the trial

Item	% of diet DM
Ingredients	
Corn silage[1]	39.7
Alfalfa haylage[2]	16.7
Grass hay[3]	5.8
Bakery byproduct meal[4]	7.4
SoyPLUS[5]	6.0
Corn grain, ground	5.7
Soybean seeds, whole heated	5.5
Canola meal	5.0
Sugar blend[6]	4.2
Vitamin and mineral premix[7]	2.9
Cotton seed, hulls	1.1
Composition,[8] % of DM (or as indicated)	
CP	15.7
NDF	29.3
ADF	20.4
NE_L, Mcal/kg	1.56
NFC	46.0
Ether extract	4.1
Ca	0.98
P	0.36
Average NE_L balance,[9] Mcal/d	(-2.4, -2.1, -3.0, -0.5)
Average MP balance,[9] g/d	(-264, -255, -322, -183)

[1]Corn silage was 42.9% DM and contained (DM basis): 7.6% CP and 35.5% NDF.
[2]Alfalfa haylage was 45.4% DM and (DM basis): 19.5% CP and 43.0% NDF.
[3]Grass hay contained (DM basis): 6.6% CP and 66.0% NDF.
[4]Bakery byproduct meal (Bakery Feeds Inc., Honey Brook, PA).
[5]SoyPLUS® (West Central®, Ralston, IA); contained 45.3% CP.
[6]Molasses (Westway Feed Products, Tomball, TX).
[7]The premix contained (%, as-is basis) trace mineral mix (Cargill Animal Nutrition, Cargill Inc., Roaring Spring, PA), 0.86; MgO (56% Mg), 8.0; NaCl, 6.4; vitamin ADE premix (Cargill Animal Nutrition, Cargill
Inc.), 0.48; limestone, 37.2; selenium premix (Cargill Animal Nutrition, Cargill Inc.), 0.07; and dry corn distillers grains with solubles, 46.7. Ca, 14.1%; P, 0.39%; Mg, 4.59%; K, 0.44%; S, 0.39%; Se, 6.91 mg/kg; Cu, 362 mg/kg; Zn, 1,085 mg/kg; Fe, 186 mg/kg, vitamin A, 276,717 IU/kg; vitamin D, 75,000 IU/kg; and vitamin E, 1,983 IU/kg.
[8]Analyzed (or estimated; NE_L, NFC) by Cumberland Valley Analytical Services (Maugansville, MD).
[9]Estimated based on NRC (2001) using actual DMI, milk yield, milk composition, and BW of the cows (Control, Curcumin, Garlic, and Capsicum diets, respectively).

Table 3-2. Effect of phytonutrients on milk yield and composition in dairy cows (means are least squares means)

	Treatment[1]					
Item	Control	Curcumin	Garlic	Capsicum	SEM[2]	*P*-value[3]
DMI, kg/d	21.2	21.2	19.9	21.3	1.89	0.09[4]
Milk yield, kg/d	37.4[a]	36.4[ab]	35.8[ab]	35.2[b]	2.82	0.05
Milk ÷ DMI, kg/kg	1.79	1.80	1.87	1.69	0.100	0.15
Milk fat, %	3.69	3.77	3.64	3.56	0.210	0.58
Yield, kg/d	1.37	1.39	1.29	1.26	0.121	0.27
4.0% FCM, kg/d	35.3	35.6	33.5	33.0	2.70	0.21
Milk true protein, %	3.18	3.25	3.24	3.24	0.079	0.59
Yield, kg/d	1.17	1.20	1.15	1.14	0.130	0.94
MPN yield, g/d[5]	185	186	182	179	21.5	0.97
MPN efficiency[5]	35.4	36.8	37.3	33.6	1.75	0.56
Milk lactose, %	4.84	4.90	4.88	4.93	0.061	0.32
Yield, kg/d	1.80	1.81	1.72	1.73	0.117	0.41
Milk NE_L, Mcal/d[6]	26.5	26.5	25.5	25.0	1.99	0.88
NE_L intake, Mcal/d	35.6	35.5	33.2	35.9	3.14	0.26
NE_L efficiency[7]	74.9	76.9	78.2	69.9	3.18	0.25
MUN, mg/100 ml	13.0	11.7	11.9	11.9	0.49	0.27
SCC, $\times 10^3$ cells/ml[8]	516	134	432	407	202.7	0.21

[a,b]Within a row, means without a common superscript letter differ ($P < 0.05$).

[1]Control, abomasal administration of 290 mL/cow per day of ethanol solution; Curcumin, abomasal administration of 2 g/cow per day *Curcuma* oleoresin dissolved in 290 mL ethanol solution; Garlic, abomasal administration of 2 g/d garlic extract dissolved in 290 mL ethanol solution; Capsicum, abomasal administration of 2 g/d *Capsicum* oleoresin dissolved in 290 mL ethanol solution.

[2]Highest SEM shown; n = 232 to 246 for milk yield, DMI, and milk ÷ DMI, n = 29 to 32 for all other variables (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment.

[4]Garlic vs. all other treatment, $P < 0.04$.

[5]MPN = milk protein N (milk true protein ÷ 6.38). MPN efficiency = (MPN yield ÷ N intake) × 100.

[6]Milk NE_L (Mcal/d) = kg of milk × (0.0929 × % fat + 0.0563 × % true protein + 0.0395 × % lactose) (NRC, 2001).

[7]Milk NE_L ÷ NE_L intake (Mcal/Mcal).

[8]SCC data were processed for outlier identification based on an absolute studentized residual value > 2 (PROC REG of SAS).

Table 3-3. Effect of phytonutrients on fecal microbial population (LSM; % copies) in dairy cows

	Treatment[1]					
Genus	Control	Curcumin	Garlic	Capsicum	SEM[2]	*P*-value[3]
Clostridium	10.2	10.6	10.4	10.6	0.44	0.79
Eubacterium	10.7	9.56	9.79	10.7	0.761	0.55
Ruminococcus	9.62	9.40	10.5	9.74	0.569	0.21
Bacteroides	8.55	7.89	7.88	9.31	0.726	0.44
Oscillospira	9.62	8.17	7.88	7.75	0.743	0.19
Odoribacter	8.31	7.09	7.97	9.00	1.198	0.68
Faecalibacterium	7.58	9.15	7.25	7.36	1.238	0.52
Blautia	3.19	3.67	3.69	3.43	0.331	0.67
Alistipes	3.62	2.88	2.96	3.61	0.451	0.45
Anaerotruncus	2.58	3.24	3.01	3.03	0.349	0.67
Roseburia	3.04	2.85	2.96	2.73	0.276	0.86
Sarcina	1.94	2.58	2.46	2.08	0.473	0.74
Prevotella	1.70	1.99	1.56	1.73	0.181	0.23
Coprococcus	1.80	1.28	1.36	1.54	0.221	0.41
Candidate Division TM7	1.31	1.39	1.37	1.38	0.444	1.00
Porphyromonas	0.94	0.84	1.01	1.15	0.140	0.55
Bulleidia	0.78	1.01	1.10	0.79	0.199	0.62
Anaerovorax	0.84	0.90	0.76	1.00	0.108	0.47
Treponema	0.80	0.90	0.96	0.79	0.193	0.70
Tannerella	0.83	0.88	0.71	0.68	0.156	0.75

[1] Control, abomasal administration of 290 mL/cow per day of ethanol solution; Curcumin, abomasal administration of 2 g/cow per day *Curcuma* oleoresin dissolved in 290 mL ethanol solution; Garlic, abomasal administration of 2 g/d garlic extract dissolved in 290 mL ethanol solution; Capsicum, abomasal administration of 2 g/d *Capsicum* oleoresin dissolved in 290 mL ethanol solution..

[2] n = 32 (n represents number of observations used in the statistical analysis).

[3] Main effect of treatment.

Table 3-4. Effect of phytonutrients on fecal and urinary nitrogen excretion (LSM) in dairy cows

	Treatment[1]					
Item	Control	Curcumin	Garlic	Capsicum	SEM[2]	*P*-value[3]
N intake, g/d	528	526	497	539	48.1	0.34
N excretion, g/d						
Urine N	148	160	159	153	15.0	0.85
Urea N	93.6	93.1	97.7	89.6	10.10	0.90
Fecal N	204	199	186	202	14.7	0.18
Total excreta N	351	359	346	354	25.2	0.87
As proportion of N intake, %						
Urine N	29.3	32.3	32.5	28.6	3.09	0.71
Fecal N	38.8	38.3	38.4	37.6	1.24	0.89
Total excreta N	68.0	70.6	70.9	66.2	3.64	0.76

[1] Control, abomasal administration of 290 mL/cow per day of ethanol solution; Curcumin, abomasal administration of 2 g/cow per day *Curcuma* oleoresin dissolved in 290 mL ethanol solution; Garlic, abomasal administration of 2 g/d garlic extract dissolved in 290 mL ethanol solution; Capsicum, abomasal administration of 2 g/d *Capsicum* oleoresin dissolved in 290 mL ethanol solution..
[2]n = 32 (n represents number of observations used in the statistical analysis).
[3]Main effect of treatment.

Table 3-5. Effect of phytonutrients on blood cell counts and hemoglobin (LSM) in dairy cows

	Treatment[1]					
Item	Control	Curcumin	Garlic	Capsicum	SEM[2]	*P*-value[3]
White blood cell, $10^3/\mu L$	9.46	9.14	9.37	9.16	0.588	0.69
Neutrophils	5.09	4.94	5.36	4.89	0.587	0.19
Lymphocytes	3.73	3.67	3.43	3.72	0.284	0.19
Monocytes	0.32	0.26	0.26	0.28	0.029	0.12
Eosinophils	0.31	0.26	0.28	0.25	0.063	0.68
Basophils	0.03	0.02	0.03	0.02	0.007	0.33
As % of total						
Neutrophils	54.1	53.3	55.4	52.2	3.11	0.09
Lymphocytes	39.2[b]	40.8[ab]	38.6[b]	41.8[a]	3.33	0.04
Monocytes	3.23	2.84	2.84	3.10	0.224	0.24
Eosinophils	3.21	2.86	2.84	2.67	0.601	0.80
Basophils	0.26	0.26	0.30	0.21	0.063	0.56
Red blood cells, $10^6/\mu L$	5.91	5.86	6.01	6.01	0.186	0.22[4]
Hematocrit, %	26.5	25.9	26.6	26.9	0.59	0.11
Platelet count, $10^3/\mu L$	394	396	385	363	33.7	0.57
Hemoglobin, g/dL	9.24	9.08	9.34	9.31	0.210	0.11

[a,b]Within a row, means without a common superscript letter differ ($P < 0.05$).

[1] Control, abomasal administration of 290 mL/cow per day of ethanol solution; Curcumin, abomasal administration of 2 g/cow per day *Curcuma* oleoresin dissolved in 290 mL ethanol solution; Garlic, abomasal administration of 2 g/d garlic extract dissolved in 290 mL ethanol solution; Capsicum, abomasal administration of 2 g/d *Capsicum* oleoresin dissolved in 290 mL ethanol solution..

[2]n = 126 (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment.

[4]Treatment × time of sampling interaction, $P < 0.01$. PN means were not different ($P > 0.05$) from the Control at both sampling time-points.

Table 3-6. Effect of phytonutrients on T cell phenotypes, proliferation, viability, and cytokine production of peripheral blood mononuclear cells (PBMC) in dairy cows (LSM)

	Treatment[1]					
Item	Control	Curcumin	Garlic	Capsicum	SEM[2]	*P*-value[3]
T cell phenotypes, %						
$\gamma\delta^{+}WC1^{-}$	4.05	4.73	5.19	4.16	0.734	0.36
$\gamma\delta^{-}WC1^{+}$	0.75	0.59	0.61	0.48	0.205	0.84
$\gamma\delta^{+}WC1^{+}$	8.69	8.92	9.56	9.04	1.963	0.90
Total $WC1^{+}$	9.44	9.51	10.1	9.52	2.024	0.92
Total $\gamma\delta^{+}$	12.7	13.7	14.7	13.2	2.47	0.55
$CD4^{+}CD25^{-}$	10.3[b]	16.9[a]	14.8[a]	14.4[a]	1.55	0.03
$CD4^{-}CD25^{+}$	3.12	3.19	2.43	4.15	1.325	0.86
$CD4^{+}CD25^{+}$	1.57	3.19	3.55	3.36	0.717	0.28
Total $CD4^{+}$	11.9[b]	20.3[a]	18.4[a]	17.8[a]	1.86	0.02
Total $CD25^{+}$	4.71	6.52	6.00	7.54	1.729	0.75
$\gamma\delta^{+}ACT2^{-}$	10.4	10.5	9.52	7.82	2.159	0.66
$\gamma\delta^{-}ACT2^{+}$	7.29	6.38	5.58	7.18	2.126	0.95
$\gamma\delta^{+}ACT2^{+}$	5.52	5.00	4.17	4.69	0.923	0.77
Total $ACT2^{+}$	12.8	11.4	9.75	11.9	2.409	0.87
$CD8\alpha^{+}ACT2^{-}$	8.08	9.40	8.45	7.25	1.662	0.83
$CD8\alpha^{-}ACT2^{+}$	7.04	8.39	7.73	10.5	2.321	0.74
$CD8\alpha^{+}ACT2^{+}$	3.90	3.08	3.43	4.17	1.487	0.95
Total $CD8\alpha^{+}$	12.0	12.5	11.9	11.4	2.68	0.99
$CD8\alpha^{+}\gamma\delta^{-}$	6.67	7.75	7.11	6.36	1.011	0.68
$CD8\alpha^{-}\gamma\delta^{+}$	11.4	12.2	12.7	12.7	2.32	0.74
$CD8\alpha^{+}\gamma\delta^{+}$	1.98	1.82	1.81	1.72	0.408	0.91
Total $CD8^{+}$	8.65	9.57	8.92	8.08	1.325	0.78
$CD8\alpha^{+}CD8\beta^{-}$	5.05	5.68	5.27	6.27	1.700	0.95
$CD8\alpha^{-}CD8\beta^{+}$	1.80	1.27	1.15	1.54	0.635	0.84
$CD8\alpha^{+}CD8\beta^{+}$	6.25	8.93	8.59	7.96	1.130	0.24
Total $CD8\beta^{+}$	8.03	10.2	9.73	9.47	1.085	0.39
$WC1^{+}CD25^{-}$	8.40	7.34	8.49	8.27	1.797	0.85
$WC1^{-}CD25^{+}$	3.53	4.50	4.75	3.75	0.948	0.81
$WC1^{+}CD25^{+}$	1.11	2.14	1.29	1.90	0.713	0.71
PBMC proliferation, %						

No Con A[4]	4.62	11.2	15.0	16.6	4.21	0.24
Con A	41.9	41.2	48.4	48.6	10.96	0.92
PBMC viability, %						
No Con A	82.2	82.0	81.7	79.8	7.00	0.99
Con A	84.3	86.9	88.5	86.9	4.15	0.88
Cytokines production[5]						
IL6, ng/ml						
IL6, no LPS[6]	0.13	0.13	0.11	0.12	0.019	0.59
IL6, LPS	0.33	0.34	0.31	0.27	0.031	0.49
IFNG, ng/ml						
IFNG, no LPS	0.78	0.84	0.67	0.68	0.094	0.54
IFNG, LPS	0.80	0.95	1.18	0.85	0.172	0.46
TNF, pg/ml						
TNF, no LPS	1.04	1.17	0.96	0.88	0.094	0.27
TNF, LPS	1.41	1.98	1.91	1.97	0.234	0.35

[a,b]Within a row, means without a common superscript letter differ ($P < 0.05$).

[1] Control, abomasal administration of 290 mL/cow per day of ethanol solution; Curcumin, abomasal administration of 2 g/cow per day *Curcuma* oleoresin dissolved in 290 mL ethanol solution; Garlic, abomasal administration of 2 g/d garlic extract dissolved in 290 mL ethanol solution; Capsicum, abomasal administration of 2 g/d *Capsicum* oleoresin dissolved in 290 mL ethanol solution..

[2]Highest SEM shown; n = 24 to 32 (n represents number of observations used in the statistical analysis).

[3]Main effect of treatment.

[4]Concanavalin A.

[5]Interleukin 6, interferon gamma, and tumor necrosis factor alpha.

[6]Lipopolysaccharide.

Table 3-7. Effect of phytonutrients on expression of mRNA in liver for key enzymes (LSM) in dairy cows

	Treatment[1]					
Item[2]	Control	Curcumin	Garlic	Capsicum	SEM[3]	*P*-value[4]
Pyruvate carboxylase	0.82	1.17	1.28	1.37	0.686	0.95
Phosphoenolpyruvate carboxykinase-C	1.88	2.86	2.12	1.81	0.816	0.74
Glucose 6-phosphatase	1.03	1.61	1.38	1.71	0.379	0.63
Carnitine palmitoyltransferase-1A	1.22	0.90	1.01	1.36	0.378	0.84
Catalase	1.49	1.48	1.42	1.53	0.470	1.00
Glutathionine peroxidase	2.09	2.52	3.03	3.56	0.718	0.57
Alpha-2-macroglobulin	0.70	0.40	0.46	0.39	0.208	0.65

[1] Control, abomasal administration of 290 mL/cow per day of ethanol solution; Curcumin, abomasal administration of 2 g/cow per day *Curcuma* oleoresin dissolved in 290 mL ethanol solution; Garlic, abomasal administration of 2 g/d garlic extract dissolved in 290 mL ethanol solution; Capsicum, abomasal administration of 2 g/d *Capsicum* oleoresin dissolved in 290 mL ethanol solution..

[2]Arbitrary units; normalized to 18S mRNA abundance. Total copies of each mRNA were divided by the total copies of 18S mRNA (White et al., 2011).

[3]Highest SEM shown; n = 31 to 32 (n represents number of observations used in the statistical analysis).

[4]Main effect of treatment.

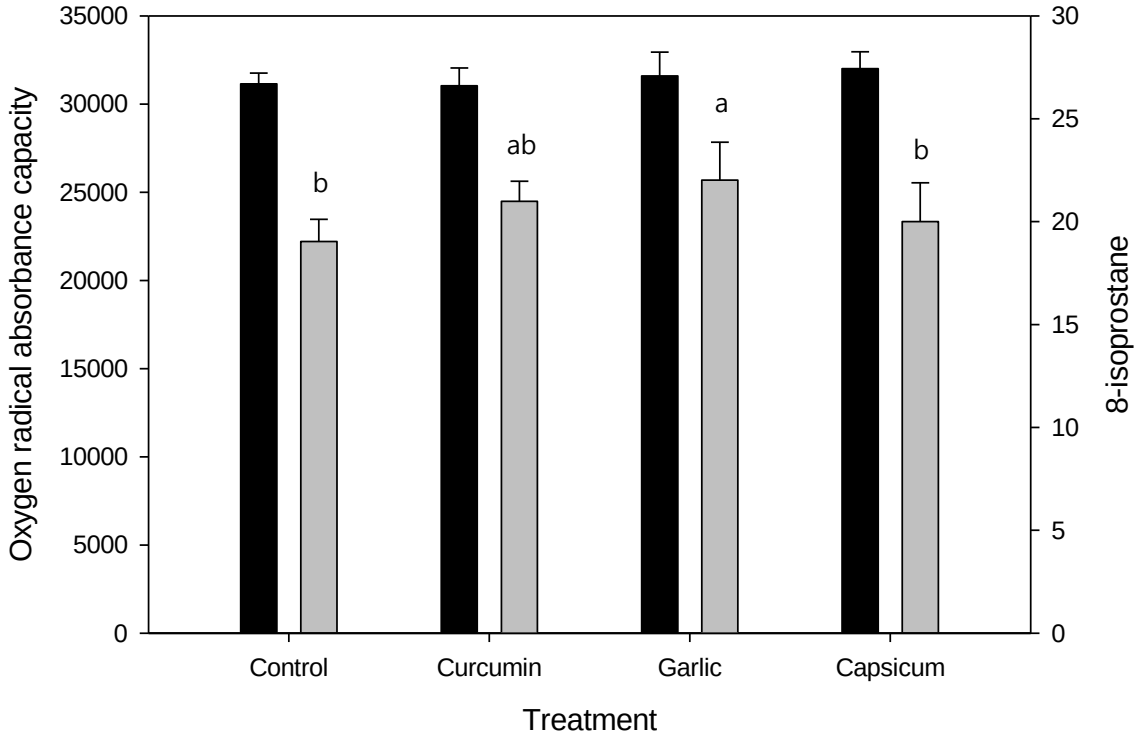

Figure 3-1. Effect of phytonutrients on oxidative stress markers in blood of dairy cows (means ± SE). Control, abomasal administration of 290 mL/cow per day of ethanol solution; Curcumin, abomasal administration of 2 g/cow per day Curcuma oleoresin dissolved in 290 mL ethanol solution; Garlic, abomasal administration of 2 g/d garlic extract dissolved in 290 mL ethanol solution; Capsicum, abomasal administration of 2 g/d Capsicum oleoresin dissolved in 290 mL ethanol solution. Trolox equivalent units for oxygen radical absorbance capacity (ORAC, black bars) and pg/ml for 8-isoprostane (gray bars). Overall effect of treatment (n = 64; n represents number of observations used in the statistical analysis), P = 0.84 and 0.02 (ORAC and 8-isoprostane, respectively; SEM = 993.0 and 1.99, respectively). a,b Within variable, bars without a common superscript letter differ (P < 0.05).

Chapter 4

Effects of dietary *Capsicum* oleoresin on productivity and immune responses in lactating dairy cows

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Abstract

This study investigated the effect of *Capsicum* oleoresin in granular form (CAP) on nutrient digestibility, immune responses, oxidative stress markers, blood chemistry, rumen fermentation, rumen bacterial populations, and productivity of lactating dairy cows. Eight multiparous lactating Holstein dairy cows, including 3 ruminally-cannulated, were used in a replicated 4 × 4 Latin square design experiment. Experimental periods were 25 d in duration, including a 14-d adaptation and a 11-d data collection and sampling period. Treatments included control (no CAP) and daily supplementation of 250, 500, or 1,000 mg CAP/cow. Dry matter intake was not affected by CAP (average 27.0 ± 0.64 kg/d), but milk yield tended to quadratically increase with CAP supplementation (50.3 to 51.9 ± 0.86 kg/d). *Capsicum* oleoresin quadratically increased energy-corrected milk yield, but had no effect on milk fat concentration. Rumen

fermentation variables, apparent total tract digestibility of nutrients, and N excretion in feces and urine were not affected by CAP. Blood serum β-hydroxybutyrate was quadratically increased by CAP, whereas the concentration of nonesterified fatty acids was similar among treatments. Rumen populations of *Bacteroidales*, *Prevotella*, and *Roseburia* decreased and *Butyrivibrio* increased quardratically with CAP supplementation. T cell phenotypes were not affected by treatment. Mean fluorescence intensity for phagocytic activity of neutrophils tended to be quadratically increased by CAP. Numbers of neutrophils and eosinophils and the ratio of neutrophils to lymphocytes in peripheral blood linearly increased with increasing CAP. Oxidative stress markers were not affected by CAP. Overall, in the conditions of this experiment, CAP did not affect feed intake, rumen fermentation, nutrient digestibility, T cell phenotypes, and oxidative stress markers. However, energy-corrected milk yield was quadratically increased by CAP, possibly as a result of enhanced mobilization of body fat reserves. In addition, CAP increased neutrophil activity and immune cells related to acute phase immune response.

Keywords: capsicum oleoresin, milk production, immune responses, dairy cow

Introduction

Capsicum is a genus of flowering plants containing capsaicinoids as its active compounds. As a feed additive in beef and dairy production system, original interest in *Capsicum* was based on its potential as modifier of rumen fermentation to optimize

rumen function and its pungency to increase feed intake (Calsamiglia et al., 2007; Rodríguez-Prado et al., 2012). Recent findings suggested, however, that the improvement in animal performance may be due to a host response (physiological and immunological) rather than direct antimicrobial or sensory effects. For example, *Capsicum* oleoresin or its mixture with other plant extracts have reduced oxidative stress (Karadas et al., 2014), prevented disease symptoms (Lee et al., 2011; Liu et al., 2012; Lee et al., 2013), and improved gut health during normal or disease conditions (Liu et al., 2014a; Liu et al., 2014b) in both poultry and swine. In addition, studies using rats showed that capsaicinoids, active compounds in *Capsicum*, had modulatory effects on immune cells such as neutrophils and T cells (Franco-Penteado et al., 2006; Takano et al., 2007), and decreased oxidative stress markers and adipose tissue (Yoshioka et al., 2000; Abdel-Salam et al., 2012). With respect to ruminants, Oh et al., (2013) reported that a short-term (9-d) abomasal infusion of *Capsicum* oleoresin increased lymphocyte proportion and CD4+ T cells and did not affect oxidative stress markers in blood of dairy cows. However, due to the possibility of inactivation in the rumen, the host-based physiological effects of *Capsicum* may be different when supplementation is through the feed vs. post-ruminal delivery.

Based on the existing data, we hypothesized that dietary supplementation of *Capsicum* oleoresin in unprotected granular form (**CAP**) could potentially modify rumen fermentation, facilitate immune response, and reduce oxidative stress in dairy cows. The CAP product could also stimulate feed intake and animal productivity. Thus, the objective of the experiment was to investigate the physiological effects of CAP in

relation to feed intake, rumen function, blood chemistry, hematology, immune responses, oxidative stress markers, and productivity of lactating dairy cows.

Materials and Methods

Animals and treatments

The Pennsylvania State University Animal Care and Use committee approved all procedures in this experiment. The design of the experiment was a replicated 4 × 4 Latin square with 8 multiparous Holstein cows averaging 590 ± 32.6 kg BW, 50 ± 9.6 DIM, and 52 ± 2.4 kg/d milk yield at the beginning of the experiment. Three cows were fitted with soft plastic rumen cannulas (10 cm internal diameter; Bar Diamond Inc., Parma, ID) and constituted one incomplete square of the experiment. Experimental periods were 25 d including a 14-d adaptation and an 11-d data collection periods. Treatments were **Control** (0 mg/d per cow CAP) and 3 application rates of CAP: 250 mg (**C250**), 500 mg (**C500**), and 1,000 mg/d per cow (**C1000**). The CAP product used in the experiment was CapsXL (X60-7035; 20% *Capsicum* oleoresin; 1.2% capsaicinoids; Pancosma, S. A., Geneva, Switzerland). The product was top-dressed daily mixed with a small amount of TMR during feeding. Cows were housed in a tie-stall barn, fed once daily at approximately 08:00 h, and had free access to fresh water. The basal diet (Table 4-1) was fed ad libitum as a TMR, targeting approximately 10% refusals. The diet was formulated to meet or exceed the nutrient requirements of a lactating Holstein cow yielding 50 kg of milk/d with 3.80% fat and 3.20% true protein at 27 kg/d DMI and 650 kg of BW (NRC,

2001). Cows were milked twice daily at 05:00 and 17:00 and treated with rbST (500 mg, i.m., Posilac; Elanco Co., Greenfield, IN) on d 1 and d 13 of each experimental period.

Sampling and analysis

Feed intake was measured daily, TMR samples were collected twice weekly, and individual ingredients of forage and concentrate were sampled once weekly. Composite samples of the TMR, forages, and concentrate feeds were processed and analyzed for OM, CP, NDF, ADF, Ca, P, indigestible NDF (**iNDF**), and NE_L as described in Oh et al. (2013).

Milk production of the cows was recorded daily and samples for milk composition were collected on d 20 and d 24 of each experimental period, from p.m. and a.m. milkings, preserved with 2-bromo-2-nitropropane-1,3 diol, and submitted to Dairy One laboratory for analysis (Pennsylvania DHIA, University Park, PA). Milk was analyzed for fat, true protein, lactose, and MUN using infrared spectroscopy (MilkoScan 4000; Foss Electric, Hillerød, Denmark). Another aliquot was collected in tube containing no preservative, kept at -20℃, and analyzed for milk FA composition as described in Hristov et al. (2010).

Samples of whole ruminal contents were collected from the cannulated cows on d 24 and d 25 of each experimental period at 2, 4, and 6 h after feeding, processed as described elsewhere (Hristov et al., 2011), and analyzed for pH (59000-60 pH Tester, Cole-Parmer Instrument Company, Vernon Hills, IL), VFA (Yang and Varga, 1989), and ammonia concentration (Chaney and Marbach, 1962). Aliquots of whole ruminal

contents were composited (on an equal wet weight basis), per cow and period, and stored frozen at -80℃ for bacterial population analysis. The 16S rRNA gene V4 variable region PCR primers 515/806 (Caporaso et al., 2011) were used in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, Germantown, MD) under the following conditions: 94℃ for 3 min, followed by 28 cycles (5 cycle used on PCR products) of 94℃ for 30 s, 53℃ for 40 s and 72℃ for 1 min, after which a final elongation step at 72℃ for 5 min was completed. Sequencing was performed at Molecular Research DNA (www.mrdnalab.com; Shallowater, TX) on an Ion Torrent PGM (Life Technologies, Carlsbad, CA) following the manufacturer's guidelines. Sequence data were processed using a proprietary analysis pipeline (MR DNA). In summary, sequences were depleted of barcodes and primers, then sequences < 150 bp were removed, and sequences with ambiguous base calls and with homopolymer runs exceeding 6 bp were also removed. Sequences were denoised, operational taxonomic units generated, and chimeras removed. Operational taxonomic units were defined by clustering at 3% divergence (97% similarity). Final operational taxonomic units were taxonomically classified using BLASTn against a curated database derived from GreenGenes, RDPII and NCBI (www.ncbi.nlm.nih.gov; DeSantis et al., 2006, http://rdp.cme.msu.edu; accessed December 24, 2014).

Spot fecal and urine samples were collected by stimulating defecation or from the rectum and by massaging the vulva, respectively, at 10:00, 16:00, and 22:00 on d 22; 04:00, 13:00, and 19:00 on d 23; and 01:00, and 07:00 h on d 24 of each experimental period. Urine and fecal samples were collected, processed and analyzed for OM, CP,

NDF, and ADF (fecal samples) and total N, UUN, creatinine, and the purine derivatives (**PD**) allantoin and uric acid (urine samples) as described in Oh et al. (2013). Apparent total tract digestibility of nutrients was estimated using iNDF as an intrinsic digestibility marker (Schneider and Flatt, 1975). Fecal and TMR samples were analyzed for iNDF according to Huhtanen et al. (1994), with the exception that 25-µm pore size Ankom filter bags (Ankom Technology) were used for the rumen incubation.

Blood samples were collected from the coccygeal vein or artery at 2 and 4 h after feeding on d 22 of each period for hematology analyses. Samples (approximately 10 mL) were collected into vacuumed tubes containing EDTA (BD Biosciences, Franklin Lakes, NJ), kept refrigerated (4°C), and analyzed the same day. The analysis included: red blood cell count, hemoglobin, hematocrit, platelet count, mean platelet volume, and total white blood cell count, including total count for neutrophils, eosinophils, lymphocytes, monocytes, and basophils using an automated hematology analyzer (HemaVet® ; Drew Scientific, Oxford, CT). A separate set of blood samples was collected into vacuumed tubes containing silica clot activator (SST™ Tube; BD Biosciences) at 2 and 4 h after feeding on d 22 and d 23 of each experimental period. Blood serum was separated (after clotting) through centrifugation at 3,000 × *g* at room temperature for 15 min, composited on an equal volume basis per cow, period, and sampling time-point and stored at -20°C. Serum was analyzed for albumin, amylase, BUN, Ca, cholesterol, Cl, creatinine, globulin, glucose, K, Na, P, and total protein (IDEXX VetTest® and VetLyte® Chemistry and Electrolyte Analyzers, Idexx Laboratories, Inc., Westbrook, ME). Serum samples were also analyzed for BHBA and NEFA using biochemistry analyzer (Cobas 6000; Roche,

Germany) and for insulin using radioimmunoassay (Coat-a-count insulin kit TKIN5; Siemens Healthcare Diagnostics, Los Angeles, CA). A third set of blood samples was collected into vacuumed tubes containing EDTA (BD Biosciences) at 2 and 4 h after feeding on d 22 and d 23 of each experimental period for oxidative stress markers. Plasma was obtained by centrifugation at 1,500 × *g* at 4℃ for 10 min, composited on an equal volume basis per cow, period, and sampling time-point and stored frozen at -80℃. Samples were analyzed for thiobarbituric acid reactive substances using colorimetric assay kits (Cayman Chemical, Ann Arbor, MI) and 8-isoprostane using ELISA kits (Cayman Chemical) and for oxygen radical absorbance capacity (**ORAC**) as described elsewhere (Cao and Prior, 1999).

Whole blood (approximately 50 mL) samples were collected via tail vein or artery before feeding from one experimental square (4 cows) on d 24 and from the other square (4 cows) on d 25 of each period for T cell phenotype analysis. Whole blood was transferred to borosilicate glass tubes and centrifuged at 1,500 × *g* at 4℃ for 10 min to obtain a lymphocyte-rich white blood cell layer. Peripheral blood mononuclear cells (**PBMC**) were isolated by centrifugation at 650 × *g* at 25℃ for 30 min over Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, Piscataway, NJ). A flow cytometer (GuavaEasyCyte Plus; Millipore, Billerica, MA) was used for counting and analysis of T lymphocytes. Peripheral blood mononuclear cells (1×10^6) were directly labeled with fluor-conjugated primary antibodies against T lymphocyte-surface antigens following a protocol described by Poole and Pate (2012). The antibodies used were against cluster of differentiation antigen (**CD**) 4 (MCA1653F; AbD Serotech, Raleigh, NC), CD25α

(CACT116A; VMRD, Pullman, WA), CD8α (MCA837F; AbD Serotech), and δ T cell receptor (δTCR; CACT61A, VMRD). The primary antibodies for CD25α and δTCR were conjugated to phycoerythrin using IgG1 and IgM goat anti-mouse antibodies (IgG1 [STAR132P] and IgM [102009], respectively (AbD Serotech) following the manufacturers guidelines. The following antibodies were used as controls: IgG2a negative control (MCA929F; AbD Serotech), IgG1 negative control (MCA928PE and MCA928F; AbD Serotech), and IgM negative control (MCA692; AbD Serotech). Samples were analyzed in duplicate, CD4 was paired with CD25α, and CD8 was paired with δTCR. Flow cytometer (GuavaEasyCyte Plus; Millipore) was used to determine the proportion of single- and dual-stained cells.

The phagocytic capacity of neutrophils was measured by a flow cytometric assay (Smits et al., 1997). Killed bacteria, *Streptococcus uberis*, were provided by the Animal Diagnostic Laboratory at the Pennsylvania State University, suspended in 1 mL phosphate buffered saline, and labeled with propidium iodide (**PI**) for 60 min at room temperature. The PI-labeled bacteria were washed 3 times with phosphate buffered saline to remove excess PI, suspended in Roswell Park Memorial Institute 1640 medium (Gibco Laboratories, Grand Island, NY), and counted using a flow cytometer (GuavaEasyCyte Plus, Millipore). Aliquots were stored at -80°C and thawed immediately before use. Whole blood (10 mL) was collected from the coccygeal vein or artery into heparinized-vacutainers (BD Biosciences) on d 23 of each experimental period. The blood was centrifuged at 4°C and 1,500 × *g* for 10 min, the supernatant and buffy coat layers were aspirated and discarded, and the packed red blood cells were lysed

by the addition of 2 volumes of red blood cell lysis buffer (0.14 *M* NH_4Cl, 10 m*M* $KHCO_3$). Lysis was stopped by adding a volume of Hank's balanced salt solution after 1 min. The lysate was centrifuged at 400 × *g* at 4℃ for 10 min with no brake, washed with Hank's balanced salt solution once at 300 × *g* at 4℃ for 10 min, and finally suspended in Roswell Park Memorial Institute 1640 medium. Total neutrophils were counted by flow cytometer (GuavaEasyCyte Plus, Millipore). Bacteria labeled with PI were added into 96-well plates at a ratio of the bacteria to neutrophils of 15:1 (bacteria:neutrophils = $15 \times 10^5 : 1 \times 10^5$). After 20 min incubation at room temperature, the plates were read on a flow cytometer (GuavaEasyCyte Plus, Millipore) and the proportion of neutrophils that had phagocytized bacteria was determined. Also, histogram analysis for mean fluorescence intensity (**MFI**) of PI was conducted to estimate mean phagocytic activity of total gated neutrophils, which indicated mean number of engulfed bacteria per neutrophil (Silvestre et al., 2011). The assay was run in duplicate.

Statistical analysis

All data were analyzed using the MIXED procedure of SAS 9.4 (2003; SAS Institute Inc., Cary, NC). Milk yield, DMI, and estimated feed efficiency data for the last 11 d and milk composition data for the 2 sampling days of each experimental period were averaged and the average values were used in the statistical analysis. The averaged milk yield, DMI, and milk composition data were used to calculate yields of milk fat, protein, lactose, 4.0% FCM, and ECM.

Nutrient intake, digestibility, rumen microbial population, urinary and fecal N excretions, milk composition, hematology, blood chemistry, BHBA, NEFA, T cell

phenotypes, and neutrophil phagocytosis data were analyzed by analysis of variance Latin square. The model used was as follows:

$$Y_{ijkl} = \mu + S_i + C(S)_{ij} + P_k + T_l + e_{ijkl},$$

where Y_{ijkl} is the dependent variable, μ is the overall mean, S_i is the square, $C(S)_{ij}$ is the cow within square, P_k is the kth period, and T_l is the lth treatment with the error term e_{ijkl}. Square and cow within square were random effects and all others were fixed.

Rumen fermentation data (pH and ammonia and VFA concentrations), DMI, milk yield, SCC, and feed efficiency data were analyzed as repeated measures assuming an AR(1) covariance structure. The model used was as follows:

$$Y_{ijklm} = \mu + S_i + C(S)_{ij} + P_k + T_l + D_m + TD_{lm} + e_{ijklm},$$

where Y_{ijklm} is the dependent variable, μ is the overall mean, S_i is the square, $C(S)_{ij}$ is the cow within square, P_k is the kth period, T_l is the lth treatment, D_m is the time effect, and TD_{lm} is the treatment × time of sampling interaction, with the error term e_{ijklm}. Square and cow within square were random effects and all others were fixed.

Data were tested for normality using the UNIVARIATE procedure of SAS. Log-transformed data were analyzed when the W statistic of the Shapiro-Wilk test was less than 0.05. Orthogonal contrasts were used to evaluate CAP treatments vs. control, linear, and quadratic effects of CAP supplementation. Statistical differences were considered significant at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$.

Results

The basal diet used in this experiment was formulated to meet NE_L and MP requirements of cows milking 50 kg/d. Due to higher than expected milk yield and BW deposition during the experiment, however, the diet provided NE_L slightly below NRC (2001) requirements (-2.0 and -2.1 % for C250 and C500, respectively) and MP from -1.8 to -5.0 % below the requirements (Table 4-1). Cows gained on average 34 ± 10.3 kg BW during the experiment ($P = 0.26$).

Dry matter intake was not affected by CAP supplementations (Table 4-2). Milk yield tended to quadratically increase ($P = 0.09$) with CAP. Cows in this experiment were experiencing milk fat depression. Despite numerical differences, milk fat concentration was not affected by treatments, but milk fat yield was higher ($P = 0.05$) for CAP than the control and tended to be quadratically increased ($P = 0.09$) by CAP supplementation. Compared with the control, 4% FCM and ECM were increased ($P = 0.04$ and $P = 0.06$, respectively) by CAP and quadratically increased ($P = 0.04$) with increasing CAP supplementation rate. There was no effect of CAP supplementation on milk true protein content and yield. Concentration of lactose linearly decreased ($P < 0.01$), but concentration of total solids and MUN were not affected by CAP. Milk NE_L increased quadratically ($P = 0.05$) with CAP supplementation. As dietary NE_L intake remained constant, milk NE_L efficiency was higher ($P = 0.05$) for CAP than the control. Treatment did not affect milk SCC and BW of the cows.

The milk FA data from this experiment are shown in Table 4-3. Saturated FA in milk were not affected by CAP except 18:0, and 20:0. Concentration of 18:0 tended to

linearly increase (P = 0.09) and that of 20:0 was linearly increased (P = 0.03) by CAP, compared with the control. Milk *cis*-9, cis-12 18:2, and *trans*-11 18:1 tended to linearly decrease (P = 0.10 and 0.07, respectively), whereas other *trans* and *cis* 18:1 FA were not affected by CAP. Supplementation with CAP linearly increased (P = 0.04) concentration of 18:3. Compared with the control, concentration of *cis* 9, *trans* 11 CLA was decreased (P = 0.03) by CAP. The sum of saturated FA, total *trans* FA, and MUFA were not affected by CAP. Concentration of PUFA was linearly decreased (P = 0.05) by CAP.

Rumen ammonia and VFA concentrations were not affected by CAP supplementation (Table 4-4). Rumen pH tended to decrease (P = 0.08) with CAP compared with the control. Intake and apparent total tract digestibility of dietary nutrients were not affected by treatment (Table 4-5). Supplementation with CAP also did not affect N excretion in feces or urine, urine volume, urinary urea N excretion, total excreta N, milk N secretion, and urinary PD excretion (Table 4-6).

Relative to the control, plasma BHBA concentration was quadratically increased (P = 0.02) by CAP, but there was no effect of CAP on NEFA and insulin concentrations (Table 4-7). Plasma BHBA concentration in our experiment was below the level for subclinical ketosis (1,200 to 2,900 μM; Oetzel, 2004). Supplementation of the diet with CAP increased (P = 0.02) BUN compared with the control. Concentrations of glucose, creatinine, total protein, albumin, globulin, amylase, Na, and Cl in blood plasma were not affected by CAP except cholesterol that tended to quadratically increase (P = 0.07). Plasma K concentration linearly decreased (P < 0.01) with CAP supplementation.

As revealed by the sequencing results, the predominant bacterial orders in whole ruminal contents were *Clostridiales* and *Bacteroidales* (Table 4-8). *Bacteroidales* decreased quadratically ($P = 0.03$) and *Bifidobacteriales* tended to be linearly increased ($P = 0.10$) by CAP. Relative to the control, *Synergistales* increased quadratically ($P = 0.03$) and *Spirochaetales* tended to be quadratically increased ($P = 0.09$) by CAP. The relative abundance of *Ruminococcaceae*, which was the most predominant genus in the rumen, was not affected by treatment (Table 4-9). *Prevotella* and *Roseburia* were quardratically decreased ($P \leq 0.04$) and the proportion of *Butyrivibrio* was increased quadratically ($P < 0.01$) by CAP supplementation. *Capsicum* quadratically increased ($P \leq 0.04$) the abundance of *Faecalibacterium*, *Syntrophococcus,* and *Dorea.*

T cell phenotypes were not affected by CAP supplementation (Table 4-10). Although CAP did not affect numbers of neutrophils positive for phagocytosis, MFI tended to be quadratically increased ($P = 0.08$). Supplementation with CAP linearly increased total white blood cells, neutrophils, and eosinophils ($P \leq 0.04$; Table 4-11). The proportion of lymphocytes in total white blood cells decreased linearly ($P < 0.01$) and that of neutrophils increased linearly ($P < 0.01$) with increasing CAP supplementation, therefore, the ratio of neutrophils to lymphocytes was linearly increased ($P < 0.01$) by CAP. Treatment did not affect concentrations of monocytes and basophils in blood. Red blood cells and hemoglobin concentration quadratically increased ($P \leq 0.04$) and platelets linearly decreased ($P = 0.04$) with CAP, even though mean platelet volume was not affected. Hematocrit percentage tended to be increased (*P*

= 0.07) by CAP. Oxidative stress markers were not affected by CAP supplementation (Table 4-12).

Discussion

Supplementation on the diet with *Capsicum* oleoresin increased feed intake in beef cattle (Cardozo et al., 2006; Rodríguez-Prado et al., 2012). In these experiments, the pungent property of *Capsicum* led to high consumption of water followed by increased feed intake. However, CAP in the current experiment had no effect on DMI. The CAP product contained fat to reduce its pungent taste, which may have resulted in lack of effect on DMI. The application rate was also somewhat higher in the beef studies compared with the current experiment. For example, the amount of *Capsicum* oleoresin used in the study of Rodríguez-Prado et al. (2012) was 2.5 to 10.6 mg/kg DMI, whereas application rate of *Capsicum* oleoresin was 1.8 to 7.5 mg/kg DMI in the current study. Tager and Krause (2011) also reported no effect of *Capsicum* at 250 mg/d on DMI in dairy cows.

The combination of dietary nutrient supplies and quadratic increases of milk, 4% FCM, and ECM yields with CAP supplementation in the current experiment resulted in negative NE_L balances (-0.9 Mcal/d) for both C250 and C500 treatment. The negative energy balance may have led to mobilization of body fat reserves, which may be responsible for the observed quadratic increase in serum BHBA concentration with the CAP treatments (McArt et al., 2012). This potentially enhanced fat mobilization in the

CAP-treated cows may explain the numerical trend for increased milk fat concentration and the quadratic increase in 4% FCM and ECM, compared with the control cows. This hypothesis, however, was not supported by the similar serum NEFA concentrations between the CAP-treated and control cows.

Milk fat concentration was on average 3.03% in this experiment, which is lower than typical values for Holstein cows (CDCB, 2013) and is indicative of milk fat depression. This may have been caused by the high starch content of the corn silage (41.4%) and high proportion of corn silage in the basal diet. Diets rich in starch are known to cause milk fat depression by decreasing mammary lipogenesis (Shingfield et al., 2010) and an increase of milk fat *trans*-10 18:1 has been consistently associated with milk fat depression (Shingfield and Griinari, 2007). Concentrations of milk fat *trans*-10 18:1 in the current study were 2.19 to 2.53% for all treatment, which could cause 20 to 22% reduction in milk fat in lactating cows according to Shingfield et al. (2010). The relatively low rumen pH for all treatments is also reflective of high level of starch fermentation in the rumen. A decrease by CAP in *cis*-9, *trans*-11 CLA is consistent with the results for *trans*-11 18:1 in the current experiment because *cis*-9, *trans*-11 CLA is synthesized in the mammary gland from *trans*-11 18:1 by Δ^9-desaturase (Griinari et al., 2000).

Overall, rumen fermentation parameters were not affected by CAP in the current experiment although there was a slight decrease (P = 0.06) in pH. These results are consistent with those reported by Tager and Krause (2011), in which ruminal ammonia and VFA concentrations were not affected by 250 mg/d of a *Capsicum* product in

lactating dairy cows. Others, however, have reported decreased pH and acetate concentration and increased ammonia concentration in the rumen of beef cattle supplemented with encapsulated *Capsicum* oleoresin (Rodríguez-Prado et al., 2012).

Explanation of the increased BUN concentration by CAP in the current experiment remains uncertain because ruminal ammonia concentration and MUN were not different from the control. None of the other blood chemistry variables analyzed, with the exception of BHBA, were affected by CAP. The BUN results were not consistent with previous data from experiments with non-ruminant species. For example, capsaicin or *Capsicum* extract were reported to decrease BUN concentration in rats (Monsereenusorn, 1983; Jung et al., 2014).

Antimicrobial properties of capsaicin or *Capsicum* oleoresin against *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Helicobacter pylori*, *Botrytis cinerea*, and *Aspergillus niger* were reported in vitro (Al-Delaimy and Ali, 1970; Cichewicz and Thorpe, 1996; Jones et al., 1997; Xing et al., 2006). Therefore, some antimicrobial effects of *Capsicum* in the rumen were expected in the current experiment. In a related experiment, we determined that in situ disappearance rate of capsaicinoids in CAP was 43.8% in 2 h and 69.0% after 4 h of incubation (J. Oh and A. N. Hristov, unpublished data). Supplementation of CAP decreased *Prevotella* species in the rumen, gram-negative bacteria that degrade proteins and are involved in the uptake and fermentation of peptides (Stewart et al., 1997). Ruminal ammonia concentration, however, was not affected by CAP in the current experiment. *Butyrivibrio* species are associated with biohydrogenation of FA in the rumen (Jenkins et al., 2008). It is known

that milk fat *trans*-11 18:1 and *cis*-9, *trans*-11 CLA are formed from linoleic acid by *B. fibrisolvens.* The observed quadratic increase in the abundance of *Butyrivibrio* species and *B. fibrisolvens* (*P* = 0.08; 0.01, 0.03, 0.03, and 0.02 % for Control, C250, C500, and C1000, respectively; data not shown in tables) is consistent with the decrease in PUFA, but did not result in increased milk fat *trans*-11 18:1 and *cis*-9, *trans*-11 CLA.

Capsaicinoids have been shown to have immunomodulatory effects in vitro and in vivo in rats (Sancho et al., 2002; Park et al., 2004; Liu et al., 2012). Capsaicin and *Capsicum* oleoresin reduced interleukin (IL) -2 and interferon gamma production in murine Peyer's patches, lymphoid tissues where systemic immune responses are induced in the intestine (Takano et al., 2007). Takano et al. (2007) also reported that capsaicin and *Capsicum* oleoresin decreased proportion of CD4+ cells in Peyer's patches. These anti-inflammatory effects of capsaicin were shown by Park et al. (2004) who reported that capsaicin suppressed tumor necrosis factor (TNF) production in murine macrophage. In food animals, *Capsicum* oleoresin linearly decreased TNF, IL1B, and transforming growth factor production of LPS-induced porcine macrophages *in vitro* (Liu et al., 2012), suggesting an anti-inflammatory effect of capsaicin on macrophages in pigs. Oh et al. (2013) infused *Capsicum* oleoresin (2 g/d) into the abomasum of lactating dairy cows and measured inflammatory cytokines production of LPS-induced PBMC *ex vivo* and subpopulations of T cells in blood. In that experiment, the abomasal infusion of *Capsicum* oleoresin did not affect inflammatory cytokines such as interferon gamma, IL6, and TNF, but increased the proportion of CD4+ cells in blood. In the current experiment, however, we did not observe any effect of dietary CAP on populations of T cells. It is

possible that the different doses used in the two experiments influenced the T cell response. In addition, the mode of supplementation (abomasal infusion vs. TMR top-dressing) might have influenced the effects of CAP.

Neutrophils are the most abundant type of white blood cell and play critical roles in innate immunity by killing bacteria and inducing an adaptive immune response (Witko-Sarsat et al., 2000). Supplementation of the diet with CAP in the current experiment increased neutrophil counts in blood and neutrophil activity in phagocytosis. Neutrophils may be directly affected by CAP because they have a transient receptor potential cation channel subfamily V member 1 (TRPV 1) channel (Heiner et al., 2003), or indirectly affected via release of neuronal peptides (Zimmerman et al., 1992). In the current study, it is possible that neurons activated by CAP facilitated neutrophil function through secretion of neuropeptides. Zhukova and Makarova (2002) indicated that neuropeptides regulated by capsaicin increased count and activity of neutrophils in rats. Franco-Penteado et al. (2006) reported that capsaicin increased neutrophil production in rat bone marrow. An increase of the neutrophil to lymphocyte ratio was suggested as an indicator of an acute stress response (Weiss and Wardrop, 2010). In the current experiment, CAP increased the ratio of neutrophils to lymphocytes as well as the counts of neutrophils and eosinophils. Eiosinophils are granulocytes and are involved in acute phase responses such as host protection against parasites (Rothenberg and Hogan, 2006).

In our previous study, we reported that abomasal infusion of *Capsicum* oleoresin for 5 d did not affect 8-isoprostane and ORAC in blood of dairy cows (Oh et al., 2013). This is consistent with data from the current experiment, although CAP was supplied to

the cows for longer period (21 d) and through the TMR. In studies with rats, however, capsaicin reduced oxidative stress markers such as TBARS or malondialdehyde in serum and (or) the animals' liver, lung, kidney, and muscle (Lee at al., 2003; Manjunatha and Srinivasan, 2006).

Conclusions

Our results suggest that dietary supplementation of CAP has subtle or no effects on feed intake, rumen fermentation, nutrients digestibility, blood chemistry, T cell phenotypes, and antioxidant status in lactating dairy cows. However, CAP appeared to have a positive effect, perhaps through enhanced mobilization of body fat reserves, on the energy balance of the cows, resulting in increased ECM yield. *Capsicum* facilitated neutrophil activity and immune cells related to the acute phase response.

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Table 4-1. Ingredient and chemical composition of the diet fed during the experiment

Item	% of diet DM
Ingredients	
Corn silage[1]	44.0
Grass hay[2]	7.0
Cottonseed, hulls	5.5
Corn grain, ground	9.5
Candy by-product meal[3]	1.8
Soybean seeds, whole heated[4]	8.0
Canola meal[5]	12.0
Molasses[6]	5.4
Vitamin and mineral premix[7]	3.0
SoyPLUS[8]	3.4
Optigen[9]	0.4
Composition,[10] % of DM (or as indicated)	
CP[10]	15.8
RDP[11]	10.2
RUP[11]	7.0
NDF[10]	30.7
ADF[10]	18.8
NE_L, Mcal/kg[10]	1.58
Ca[10]	0.70
P[10]	0.40
NFC[11]	43.2
Average NE_L balance,[12] Mcal/d	0.2, -0.9, -0.9, and 0.4
Average MP balance,[12] g/d	-140, -137, -163, and -57

[1]Corn silage was 46.5% DM and contained (DM basis): 8.5% CP, 32.1% NDF, and 41.4% starch.
[2]Grass hay was 90.7% DM and contained (DM basis): 7.0% CP and 73.8% NDF.
[3]Candy by-product meal (Graybill Processing, Elizabethtown, PA) contained (DM basis) 16.6% CP and 27.8% NDF.
[4]Soybean seeds contained (DM basis) 40.0% CP.
[5]Canola meal contained (DM basis) 41.8% CP.
[6]Molasses (Westway Feed Products, Tomball, TX) contained (DM basis) 3.9% CP and 66% total sugar.
[7]The premix (Cargill Animal Nutrition, Cargill Inc., Roaring Spring, PA) contained (%, as-is basis) trace mineral mix, 0.86; MgO (56% Mg), 8.0; NaCl, 6.4; vitamin ADE premix (Cargill Animal Nutrition, Cargill Inc.), 0.48; limestone, 37.2; selenium premix (Cargill Animal Nutrition, Cargill Inc.), 0.07; and dry corn distillers grains with solubles, 46.7. Ca, 14.1%; P, 0.39%; Mg, 4.59%; K, 0.44%; S, 0.39%; Se, 6.91 mg/kg; Cu, 362 mg/kg; Zn, 1,085 mg/kg; Fe, 186 mg/kg, vitamin A, 276,717 IU/kg; vitamin D, 75,000 IU/kg; and vitamin E, 1,983 IU/kg.
[8]SoyPLUS (West Central Cooperative, Ralston, IA) contained (DM basis) 46.6% CP.
[9]Optigen is a slow-release urea (Alltech Inc., Nicholasville, Kentucky).

[10]Values calculated using the chemical analysis (Cumberland Valley Analytical Services Inc., Maugansville, MD) of the ingredients of the diet.
[11]Estimated by NRC (2001).
[12]Estimated based on NRC (2001) using actual DMI, milk yield, milk composition, and BW of the cows throughout the experiment (Control, C250, C500, and C1000, respectively).

Table 4-2. Effect of dietary *Capsicum* oleoresin (CAP) on DMI, milk yield and composition, and BW in dairy cows

	Treatment[1]					*P*-value[2]		
Item	Control	C250	C500	C1000	SEM[3]	Con vs. T	L	Q
DMI, kg/d	27.0	27.5	27.0	26.5	0.64	0.75	0.27	0.84
Milk yield, kg/d	50.5	51.9	51.5	50.3	0.86	0.37	0.54	0.09
Feed efficiency[4], kg/kg	1.90	1.93	2.02	1.96	0.047	0.16	0.27	0.25
Milk fat, %	2.89	3.12	3.03	3.07	0.010	0.12	0.36	0.33
Yield, kg/d	1.46	1.62	1.55	1.56	0.054	0.05	0.60	0.09
4% FCM, kg/d	42.3	45.4	44.2	43.4	1.01	0.04	0.79	0.04
ECM,[5] kg/d	46.6	49.5	48.3	47.3	1.02	0.06	0.98	0.04
Milk true protein, %	3.01	3.00	2.98	2.98	0.036	0.43	0.34	0.73
Yield, kg/d	1.53	1.56	1.54	1.50	0.038	0.85	0.30	0.26
Milk lactose, %	4.86	4.82	4.78	4.78	0.016	0.01	<0.01	0.13
Yield, kg/d	2.46	2.50	2.49	2.41	0.046	0.96	0.29	0.29
Total solids, %	11.8	12.0	11.8	11.8	0.13	0.48	0.99	0.65
MUN, mg/100 mL	16.6	16.1	16.7	15.8	0.69	0.34	0.21	0.63
Milk NE_L,[6] Mcal/d	32.0	33.9	33.0	32.3	0.78	0.09	0.83	0.05
Dietary NE_L intake, Mcal/d	42.7	43.1	42.4	41.7	1.02	0.76	0.24	0.65
Milk NE_L efficiency,[7] %	74.9	79.0	77.8	77.6	1.86	0.05	0.30	0.13
SCC, x 10^3 cells/ml	172	133	77.2	138	86.5	0.40	0.67	0.33
BW, kg	623	621	614	614	10.3	0.26	0.16	0.57

[1]Control, 0 mg/d CAP; C250, 250 mg/d CAP; C500, 500 mg/d CAP; C1000, 1000 mg/d CAP.

[2]Con vs. T, control vs. treatment; L, linear effect of CAP; Q, quadratic effect of CAP.

[3]Highest SEM shown; n = 359 for DMI and dietary NE_L intake, n = 353 for milk yield and feed efficiency, n = 339 for BW, n = 32 for all other variables (n represents number of observations used in the statistical analysis).

[4]Milk yield ÷ DMI.

[5]Energy-corrected milk (kg/d) = kg of milk × ((38.3 × % fat × 10 + 24.2 × % true protein × 10 + 16.54 × % lactose × 10 + 20.7) ÷ 3,140) (Sjaunja et al., 1990).

[6]Milk NE_L (Mcal/d) = kg of milk × (0.0929 × % fat + 0.0563 × % true protein + 0.0395 × % lactose) (NRC, 2001).

[7]Milk NE_L ÷ NE_L intake × 100.

Table 4-3. Effect of dietary *Capsicum* oleoresin (CAP) on milk fatty acids in dairy cows (% of milk fat)

	Treatment[1]					*P*-value[2]		
Item	Control	C250	C500	C1000	SEM[3]	Con vs. T	L	Q
4:0	3.31	3.38	3.54	3.47	0.211	0.62	0.24	0.19
6:0	1.76	1.85	1.84	1.84	0.105	0.73	0.21	0.30
8:0	1.03	1.09	1.06	1.06	0.047	0.82	0.31	0.50
10:0	2.42	2.57	2.45	2.47	0.098	0.97	0.38	0.61
12:0	2.85	2.95	2.80	2.86	0.083	0.99	0.61	0.90
14:0	9.13	9.26	8.90	9.06	0.310	0.77	0.52	0.62
14:1	1.18	1.05	0.99	1.07	0.108	0.79	0.30	0.25
15:0	0.95	0.96	0.86	0.88	0.077	0.20	0.41	1.00
16:0	22.7	23.2	22.9	23.1	1.69	0.45	0.69	0.70
16:1	1.69	1.52	1.50	1.58	0.116	0.74	0.30	0.36
17:0	0.42	0.43	0.42	0.42	0.027	0.57	0.95	0.57
18:0	9.40	9.54	9.85	10.0	0.378	0.21	0.09	0.77
18:1, *trans* 6-8	0.56	0.57	0.57	0.58	0.035	0.86	0.86	0.56
18:1, *trans* 9	0.45	0.44	0.42	0.46	0.024	0.58	0.85	0.18
18:1, *trans* 10	2.32	2.45	2.19	2.53	0.403	0.48	0.82	0.19
18:1, *trans* 11	1.33	1.26	1.12	1.09	0.181	0.78	0.07	0.50
18:1, *trans* 12	0.61	0.58	0.54	0.58	0.037	0.94	0.39	0.15
18:1, *cis* 9	24.9	24.3	25.5	24.5	1.84	0.12	0.87	0.54
18:1, *cis* 11	1.91	1.87	1.85	1.85	0.062	0.11	0.25	0.46
18:2, *cis* 9, *cis* 12	4.55	4.49	4.42	4.34	0.099	0.81	0.10	0.84
18:3	0.43	0.43	0.42	0.41	0.009	0.22	0.04	0.73
20:0	0.10	0.10	0.10	0.11	0.004	0.21	0.03	0.88
CLA- *cis* 9, *trans* 11	0.61	0.58	0.54	0.51	0.077	0.03	0.08	0.76
CLA- *trans* 10, *cis* 12	0.01	0.01	0.01	0.01	0.002	0.20	0.73	0.60
Σ unidentified	5.29	5.26	5.11	5.12	0.151	0.07	0.11	0.44
Σ saturated FA	54.1	55.3	54.8	55.3	2.29	0.57	0.46	0.67
Σ *trans* FA	5.33	5.16	4.90	5.31	0.337	0.93	0.52	0.25
Σ MUFA[4]	35.0	33.9	34.7	34.3	2.00	0.30	0.73	0.72
Σ PUFA[4]	5.60	5.51	5.40	5.27	0.173	0.62	0.05	0.80

[1]Control, 0 mg/d CAP; C250, 250 mg/d CAP; C500, 500 mg/d CAP; C1000, 1000 mg/d CAP.

[2]Con vs. T, control vs. treatment; L, linear effect of CAP; Q, quadratic effect of CAP.

[3]n = 32 for all variables (n represents number of observations used in the statistical analysis).

[4]MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

Table 4-4. Effect of dietary *Capsicum* oleoresin (CAP) on rumen fermentation in dairy cows

	Treatment[1]					*P*-value[2]		
Item	Control	C250	C500	C1000	SEM[3]	Con vs. T	L	Q
pH	5.86	5.78	5.76	5.78	0.075	0.08	0.22	0.23
Ammonia, m*M*	2.22	2.22	2.21	2.03	0.284	0.70	0.32	0.60
Total VFA, m*M*	140	139	147	138	6.3	0.86	0.88	0.32
As % of total VFA								
Acetate	50.8	51.3	50.5	49.8	0.81	0.59	0.10	0.53
Propionate	31.1	30.4	31.4	31.9	1.16	0.85	0.34	0.74
Butyrate	12.6	12.9	12.3	12.5	0.58	0.93	0.75	0.88
Isobutyrate	0.64	0.63	0.61	0.63	0.016	0.42	0.77	0.17
Valerate	3.35	3.37	3.56	3.48	0.474	0.52	0.49	0.58
Isovalerate	1.47	1.56	1.56	1.54	0.042	0.13	0.39	0.23
Acetate:propionate	1.66	1.70	1.62	1.60	0.083	0.71	0.22	0.76

[1]Control, 0 mg/d CAP; C250, 250 mg/d CAP; C500, 500 mg/d CAP; C1000, 1000 mg/d CAP.

[2]Con vs. T, control vs. treatment; L, linear effect of CAP; Q, quadratic effect of CAP.

[3]Highest SEM shown; n = 71 for VFA, n = 72 for all other variables (n represents number of observations used in the statistical analysis).

Table 4-5. Effect of dietary *Capsicum* oleoresin (CAP) on intake and apparent total tract digestibility of dietary nutrients in dairy cows

	Treatment[1]					*P*-value[2]		
Item	Control	C250	C500	C1000	SEM[3]	Con vs. T	L	Q
Intake, kg/d								
DM[4]	27.9	28.0	27.4	27.0	0.66	0.54	0.24	0.94
OM	26.3	26.4	25.8	25.4	0.62	0.54	0.23	0.93
CP	4.39	4.42	4.31	4.24	0.102	0.54	0.21	0.89
NDF	9.32	9.37	9.15	9.02	0.219	0.57	0.24	0.90
ADF	6.52	6.45	6.32	6.20	0.147	0.25	0.10	0.86
Starch	6.11	6.13	6.00	5.91	0.143	0.54	0.24	0.91
Apparent digestibility, %								
DM	66.3	66.1	66.9	67.2	0.49	0.52	0.16	0.91
OM	67.3	67.0	67.9	68.1	0.47	0.60	0.18	0.88
CP	65.7	65.7	67.2	67.0	2.11	0.53	0.36	0.72
NDF	42.8	41.2	42.5	41.3	0.80	0.28	0.41	0.89
ADF	38.1	38.2	37.7	37.1	1.15	0.79	0.54	0.89
Starch	95.8	95.4	96.3	96.4	0.39	0.68	0.14	0.82

[1]Control, 0 mg/d CAP; C250, 250 mg/d CAP; C500, 500 mg/d CAP; C1000, 1000 mg/d CAP.

[2]Con vs. T, control vs. treatment; L, linear effect of CAP; Q, quadratic effect of CAP.

[3]n = 32 for all variables (n represents number of observations used in the statistical analysis) except starch (n = 31).

[4]DMI data are for the fecal collection period.

Table 4-6. Effect of dietary *Capsicum* oleoresin (CAP) on nitrogen utilization and urinary purine derivatives excretion in dairy cows

	Treatment[1]					*P*-value[2]		
Item	Control	C250	C500	C1000	SEM[3]	Con vs. T	L	Q
N intake, g/d	703	707	689	679	16.3	0.54	0.21	0.89
N excretion or secretion, g/d								
Urine N	194	204	197	201	6.7	0.30	0.53	0.69
UUN[4], g/d	147	158	150	158	11.0	0.13	0.22	0.83
Fecal N	243	245	229	226	16.9	0.36	0.14	0.82
Total excreta N	438	448	426	428	13.2	0.76	0.29	1.00
Milk N	245	250	247	240	6.1	0.85	0.31	0.27
As proportion of N intake, %								
Urine N	28.1	28.9	29.8	29.9	1.75	0.35	0.30	0.66
Fecal N	34.3	34.3	32.8	33.0	2.11	0.53	0.36	0.72
Total excreta N	62.4	63.2	62.6	63.0	1.44	0.77	0.87	0.93
Milk N	35.3	35.7	37.3	36.0	0.96	0.43	0.58	0.32
Urine output, kg/d	16.1	16.8	16.3	16.4	0.33	0.38	0.93	0.55
Urinary PD[5] excretion, mmol/d								
Allantoin	422	431	407	389	27.6	0.63	0.25	0.80
Uric acid	69.7	68.3	74.8	64.4	2.88	0.88	0.29	0.11
Total PD	492	499	482	453	27.8	0.64	0.23	0.67

[1]Control, 0 mg/d CAP; C250, 250 mg/d CAP; C500, 500 mg/d CAP; C1000, 1000 mg/d CAP.

[2]Con vs. T, control vs. treatment; L, linear effect of CAP; Q, quadratic effect of CAP.

[3]n = 32 for all variables (n represents number of observations used in the statistical analysis).

[4]Urinary urea nitrogen.

[5]Purine derivatives.

Table 4-7. Effect of dietary *Capsicum* oleoresin (CAP) on blood chemistry in dairy cows

	Treatment[1]					*P*-value[2]		
Item	Control	C250	C500	C1000	SEM[3]	Con vs. T	L	Q
BHBA, µ*M*	767	863	948	827	84.9	0.07	0.49	0.02
NEFA, m*M*	0.14	0.13	0.16	0.13	0.009	0.71	0.90	0.55
Insulin, µIU/ml	10.8	13.5	10.0	11.0	1.23	0.57	0.59	0.78
Glucose, mg/dL	62.1	60.3	59.6	61.0	1.94	0.23	0.65	0.17
BUN, mg/dL	16.1	17.4	17.1	17.3	0.70	0.02	0.11	0.17
Creatinine, mg/dL	0.68	0.69	0.66	0.67	0.018	0.59	0.54	0.66
Total protein, g/dL	7.83	7.88	7.90	7.69	0.107	0.97	0.21	0.18
Albumin, g/dL	3.08	3.09	3.11	3.02	0.037	0.92	0.25	0.21
Globulin, g/dL	4.74	4.78	4.78	4.66	0.085	0.96	0.36	0.31
Cholesterol, mg/dL	282	285	288	272	5.6	0.69	0.24	0.07
Amylase, U/L	47.1	55.0	48.0	41.8	4.81	0.81	0.13	0.18
P, mg/dL	6.14	5.84	5.91	5.98	0.119	0.09	0.55	0.13
Ca, mg/dL	10.1	10.1	10.1	9.94	0.101	0.37	0.13	0.65
Na, m*M*	147	146	149	144	1.6	0.91	0.24	0.11
K, m*M*	4.38	4.18	4.16	4.01	0.132	<0.01	<0.01	0.39
Cl, m*M*	104	103	104	101	1.32	0.42	0.13	0.58

[1]Control, 0 mg/d CAP; C250, 250 mg/d CAP; C500, 500 mg/d CAP; C1000, 1000 mg/d CAP.

[2]Con vs. T, control vs. treatment; L, linear effect of CAP; Q, quadratic effect of CAP.

[3] Highest SEM shown; n = 57 for amylase, n = 64 for all other variables (n represents number of observations used in the statistical analysis).

Table 4-8. Effect of dietary *Capsicum* oleoresin (CAP) on relative abundance (as percentage[1] of total sequences) of rumen major bacterial order in dairy cows

	Treatment[2]					*P*-value[3]		
Item	Control	C250	C500	C1000	SEM[4]	Con vs. T	L	Q
Clostridiales	60.1	60.8	65.9	62.8	2.51	0.17	0.20	0.15
Bacteroidales	27.6	25.0	17.0	21.4	1.73	0.02	0.03	0.03
Erysipelotrichales	2.41	3.08	3.65	2.95	0.439	0.15	0.41	0.12
Bifidobacteriales	1.01	1.71	3.76	3.35	1.393	0.12	0.10	0.27
Coriobacteriales	1.49	1.27	1.98	2.10	0.560	0.50	0.19	1.00
Fibrobacterales	1.17	1.31	1.35	1.47	0.300	0.37	0.32	0.81
Methanobacteriales	1.02	0.91	1.56	1.22	0.235	0.54	0.45	0.45
Synergistales	0.97	1.22	1.39	1.08	0.070	0.06	0.51	0.03
Chromatiales	1.33	1.37	0.83	1.02	0.769	0.60	0.51	0.66
Spirochaetales	0.90	1.38	0.92	0.83	0.150	0.20	0.11	0.09
Bacillales	0.36	0.35	0.27	0.31	0.090	0.67	0.66	0.69
Desulfovibrionales	0.20	0.21	0.06	0.09	0.055	0.19	0.10	0.34
Sphingobacteriales	0.09	0.11	0.16	0.11	0.045	0.57	0.79	0.44
Planctomycetales	0.15	0.10	0.09	0.07	0.046	0.28	0.29	0.63
Aeromonadales	0.10	0.10	0.09	0.08	0.050	0.98	0.83	0.91
Lactobacillales	0.08	0.09	0.11	0.06	0.021	0.78	0.55	0.22

[1]The percentage represents the percentage of the total sequences analyzed within the sample.

[2]Control, 0 mg/d CAP; C250, 250 mg/d CAP; C500, 500 mg/d CAP; C1000, 1000 mg/d CAP.

[3]Con vs. T, control vs. treatment; L, linear effect of CAP; Q, quadratic effect of CAP.

[4]n = 12 for all variables (n represents number of observations used in the statistical analysis).

Table 4-9. Effect of dietary *Capsicum* oleoresin (CAP) on relative abundance (as percentage[1] of total sequences) of rumen major bacterial genera in dairy cows

	Treatment[2]					*P*-value[3]		
Item	Control	C250	C500	C1000	SEM[4]	Con vs. T	L	Q
Ruminococcaceae	21.4	22.2	22.4	22.6	2.83	0.74	0.77	0.87
Prevotella	18.8	16.4	11.3	15.0	1.28	0.04	0.08	0.04
Acetitomaculum	6.45	6.51	9.98	6.89	2.793	0.68	0.82	0.47
Butyrivibrio	5.07	6.72	6.97	6.20	1.080	<0.01	0.03	<0.01
Prevotellaceae	6.12	6.09	3.77	4.35	0.834	0.17	0.11	0.32
Ruminococcus	3.99	3.36	3.85	3.29	0.352	0.30	0.34	0.98
Roseburia	4.58	2.48	2.73	3.49	0.590	0.04	0.38	0.05
Blautia	3.28	3.41	3.10	3.15	0.204	0.68	0.28	0.85
Saccharofermentans	2.62	3.52	2.34	2.99	0.536	0.65	0.96	0.94
Bifidobacterium	0.66	1.47	3.14	2.70	1.249	0.15	0.15	0.28
Coprococcus	1.56	1.89	2.01	1.74	0.369	0.45	0.79	0.39
Faecalibacterium	1.48	1.74	1.96	1.42	0.134	0.21	0.59	0.04
Syntrophococcus	1.60	1.19	1.34	2.06	0.508	0.80	0.13	0.10
Fibrobacter	1.17	1.31	1.35	1.47	0.250	0.37	0.32	0.81
Synergistaceae	0.95	1.20	1.38	1.06	0.070	0.05	0.48	0.02
Dorea	0.89	1.17	1.41	1.00	0.123	0.10	0.64	0.04
Methanobrevibacter	0.89	0.82	1.45	1.12	0.223	0.46	0.40	0.40
Erysipelotrichaceae	0.85	0.96	1.18	1.25	0.284	0.13	0.08	0.49
Thioalkalibacter	1.22	1.27	0.77	0.96	0.716	0.61	0.52	0.65
Olsenella	1.00	0.62	1.16	1.40	0.443	0.87	0.28	0.61
Treponema	0.90	1.37	0.92	0.83	0.149	0.21	0.11	0.09

[1]The percentage represents the percentage of the total sequences analyzed within the sample.

[2]Control, 0 mg/d CAP; C250, 250 mg/d CAP; C500, 500 mg/d CAP; C1000, 1000 mg/d CAP.

[3]Con vs. T, control vs. treatment; L, linear effect of CAP; Q, quadratic effect of CAP.

[4]n = 12 for all variables (n represents number of observations used in the statistical analysis).

Table 4-10. Effect of dietary *Capsicum* oleoresin (CAP) on T cell phenotypes and phagocytosis of neutrophils in dairy cows

	Treatment[1]					*P*-value[2]		
Item	Control	C250	C500	C1000	SEM[3]	Con vs. T	L	Q
T cell phenotypes, %								
CD4+CD25-	9.50	8.03	6.98	7.83	1.297	0.15	0.32	0.21
CD4-CD25+	8.00	8.41	8.40	7.91	0.757	0.75	0.83	0.51
CD4+CD25+	10.8	12.5	11.9	11.3	1.32	0.19	0.93	0.15
Total CD4	20.3	20.5	18.9	19.2	1.24	0.64	0.46	0.79
Total CD25	18.8	20.9	20.3	19.2	1.95	0.31	0.95	0.20
CD8+γδ-	6.08	4.97	5.81	5.40	0.801	0.41	0.72	0.72
CD8-γδ+	8.62	9.34	9.38	8.73	0.589	0.49	0.95	0.31
CD8+γδ+	2.69	2.25	2.36	2.18	0.258	0.16	0.25	0.57
Total CD8	8.77	7.22	8.17	7.57	0.856	0.29	0.53	0.65
Total γδ	11.3	11.6	11.7	10.9	0.70	0.91	0.64	0.47
Neutrophil phagocytosis								
positive cells, %	77.8	81.2	82.3	75.9	4.09	0.48	0.78	0.20
MFI[4]	143	177	181	140	19.9	0.31	0.84	0.08

[1]Control, 0 mg/d CAP; C250, 250 mg/d CAP; C500, 500 mg/d CAP; C1000, 1000 mg/d CAP.

[2]Con vs. T, control vs. treatment; L, linear effect of CAP; Q, quadratic effect of CAP.

[3]n = 32 for all variables (n represents number of observations used in the statistical analysis).

[4]Mean fluorescence intensity, arbitrary units.

Table 4-11. Effect of dietary *Capsicum* oleoresin (CAP) on blood cell counts in dairy cows

	Treatment[1]					*P*-value[2]		
Item	Control	C250	C500	C1000	SEM[3]	Con vs. T	L	Q
White blood cells, $10^3/\mu L$	7.06	7.29	7.06	8.01	0.298	0.29	0.04	0.36
Neutrophils	3.59	3.75	3.62	4.56	0.252	0.19	0.01	0.33
Lymphocytes	2.99	3.00	2.91	2.85	0.076	0.47	0.16	0.89
Monocytes	0.22	0.21	0.23	0.22	0.019	0.92	0.94	0.68
Eosinophils	0.25	0.31	0.29	0.37	0.038	0.02	0.01	0.46
Basophils	0.01	0.02	0.01	0.02	0.005	0.27	0.30	0.99
As % of total								
Neutrophils	51.1	51.1	51.7	55.1	1.31	0.14	<0.01	0.43
Lymphocytes	42.3	41.6	41.0	37.0	1.20	0.04	<0.01	0.30
Monocytes	3.07	2.81	3.16	2.82	0.20	0.54	0.57	0.72
Eosinophils	3.41	4.25	4.02	4.78	0.496	0.03	0.04	0.34
Basophils	0.15	0.23	0.17	0.24	0.048	0.22	0.27	0.96
Neutrophil:lymphocyte	1.26	1.28	1.29	1.73	0.084	0.06	<0.01	0.24
Red blood cells, $10^6/\mu L$	5.66	5.70	5.85	5.66	0.254	0.30	0.90	0.04
Hemoglobin, g/dL	8.59	8.84	8.96	8.77	0.106	<0.01	0.13	<0.01
Hematocrit, %	24.7	24.9	25.5	24.7	0.67	0.36	0.97	0.07
Platelets, $10^3/\mu L$	405	371	380	352	48.9	0.05	0.04	0.72
Mean platelet volume, fL	6.21	6.20	6.18	6.14	0.193	0.76	0.59	0.94

[1]Control, 0 mg/d CAP; C250, 250 mg/d CAP; C500, 500 mg/d CAP; C1000, 1000 mg/d CAP.

[2]Con vs. T, control vs. treatment; L, linear effect of CAP; Q, quadratic effect of CAP.

[3]n = 64 for all variables (n represents number of observations used in the statistical analysis).

Table 4-12. Effect of dietary *Capsicum* oleoresin (CAP) on oxidative stress markers in dairy cows

	Treatment[1]					*P*-value[2]		
Item	Control	C250	C500	C1000	SEM[3]	Con vs. T	L	Q
8-isoprostane, pg/ml	16.5	14.2	15.3	14.6	3.72	0.25	0.44	0.99
TBARS[4], µ*M*	3.11	3.40	3.30	4.29	0.64	0.31	0.45	0.69
ORAC[5], µ*M*	8,967	9,336	9,411	9,414	322.0	0.31	0.42	0.52

[1]Control, 0 mg/d CAP; C250, 250 mg/d CAP; C500, 500 mg/d CAP; C1000, 1000 mg/d CAP.
[2]Con vs. T, control vs. treatment; L, linear effect of CAP; Q, quadratic effect of CAP.
[3]n = 64 for all variables (n represents number of observations used in the statistical analysis).
[4]Thiobarbituric acid reactive substances; trolox equivalents.
[5]Oxygen radical absorbance capacity.

Chapter 5

Effects of rumen-protected *Capsicum* oleoresin on feed intake, digestibility, milk production, and responses to intravenous lipopolysaccharide challenge and glucose tolerance test in lactating dairy cows

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Abstract

The objective of this experiment was to investigate the effects of rumen-protected *Capsicum* oleoresin (RPC) supplementation on feed intake, digestibility, nitrogen utilization, milk yield and composition, fat mobilization, and responses to an glucose tolerance test and immune challenge with lipopolysaccharide (LPS) in lactating dairy cows. Nine multiparous Holstein cows were used in a replicated 3 × 3 Latin square design balanced for residual effects with 3, 28-d periods. Each period consisted of 14 d for adaptation and 14 d for data collection and sampling including 3 d for recovery from an immune challenge. Treatments were 0 (control), 100, and 200 mg RPC/cow/d, which were mixed with a small portion of the TMR and top-dressed. Dry matter intake tended to quadratically increase with RPC: 29.4, 30.0, and 29.2 kg/d, respectively. Milk yield also tended to quadratically increase with RPC: 42.8, 44.7, and 43.9 kg/d, respectively.

Feed efficiency was linearly increased by RPC supplementation: 1.48, 1.52, and 1.57 kg/kg, respectively. Concentrations of fat, true protein, and lactose in milk were not affected by RPC. Apparent total tract digestibility of DM, OM, and CP and fecal N excretion, as proportion of N intake were linearly increased by RPC supplementation. Concentration of non-esterified fatty acids and beta-hydroxybutyrate in serum were not affected by RPC treatment. Glucose concentration in serum was not affected by RPC supplementation post glucose challenge. The area under the curve of insulin concentration in serum was decreased by RPC treatment. Serum insulin concentration was decreased at 5, 10, and 40 min with RPC supplementation. Dry matter intake, milk yield, and white blood cells were not affected by RPC after LPS challenge. Plasma thiobarbituric acid reactive substances as an oxidative stress marker tended to be increased by RPC supplementation compared with the control. Cortisol and haptoglobin concentration in serum were lower in RPC treatment than in the control. In conclusion, RPC tended to increase feed intake and milk production and increased feed efficiency in dairy cows. Additionally, RPC treatment increased insulin sensitivity during the glucose tolerance test and had anti-inflammatory effect on acute phase responses after LPS challenge. *Capsicum* may stimulate productivity and insulin activity and alleviate acute phase responses via the neurological pathways in lactating dairy cows.

Keywords: capsicum oleoresin, lipopolysaccharide challenge, glucose tolerance test, milk production, dairy cow

Introduction

We have demonstrated in this dissertation that *Capsicum* oleoresin supplementation had immunoregulatory effects in dairy cows. Abomasal infusion of *Capsicum* oleoresin increased $CD4^+$ T cells in Chapter 3 and dietary supplementation of unprotected *Capsicum* oleoresin increased numbers and activity of immune cells related to acute phase responses in Chapter 4 (Oh et al., 2013; Oh et al., 2015). However, cows used in these experiments were not experiencing immune challenges or immunosuppression which likely occurs in periparturient period (Sordillo, 2005). Average DIM of the cows was 175 and 50 d for Chapter 3 and 4, respectively, at the beginning of the experiments. According to Liu et al. (2012) and Oh et al. (2013), immune responses by *Capsicum* oleoresin supplementation could be different between activated and non-activated immune cells. In a study, *Capsicum* oleoresin inclusion in basal diets exhibited anti-inflammatory effects in weaned pigs challenged by *E. coli* (Liu et al., 2013). It is unknown how *Capsicum* oleoresin supplementation affects immune status in immunologically challenged dairy cows.

On the other hand, numerous studies in rats and humans reported regulatory effects of capsaicin, a major active compound in *Capsicum*, on feed intake, digestive enzyme secretion, hormone regulation, and fat mobilization (Luo, 2011; Srinivasan, 2015). Capsaicin treatment stimulated gastric emptying and decreased leptin levels, which resulted in higher feed (or food) intake in humans and rats (McCann et al., 1988; Debreceni et al., 1990; Hsu and Yen, 2007). Activity of digestive enzymes such as lipase and trypsin was increased by capsaicin in the pancreas of rats (Platel and Srinivasan,

2000). Interestingly, capsaicin treatment reportedly regulated pancreatic hormone secretion or increase insulin sensitivity in rats and humans (Koopmans et al., 1998; van de Wall et al., 2005; Chaiyasit et al., 2009).

Collectively, we hypothesized that *Capsicum* oleoresin supplementation had anti-inflammatory effects on immune parameters in immunologically challenged dairy cows. Additionally, feed intake, digestibility, and insulin sensitivity could be facilitated by *Capsicum* oleoresin in dairy cows. To focus on the post-ruminal effects of *Capsicum* oleoresin, we used rumen-protected *Capsicum* oleoresin (RPC) in the current experiment because dietary supplementation of unprotected *Capsicum* oleoresin did not affect rumen fermentation in Chapter 4 (Oh et al., 2015). Objectives of this experiment were to investigate the effects of RPC supplementation on white blood cell counts, oxidative stress markers, cortisol, and haptoglobin in dairy cows challenged with lipopolisaccharide (LPS), an acute inflammatory stimulus. In addition, RPC supplementation was assessed on feed intake, productivity, digestibility, BHBA, NEFA, and insulin sensitivity.

Materials and Methods

Animals and treatments

The Pennsylvania State University Animal Care and Use committee approved all procedures used in this experiment. The experiment was in a replicated 3 x 3 Latin square design, balanced for residual effects and was conducted at the tie-stall barn of the

Pennsylvania State University Dairy Center. The experiment involved 9 Holstein cows (milk yield, 47 ± 5.7 kg; DIM, 100 ± 9.1 d; BW, 665 ± 83.3 kg, at the beginning of the experiment). Cows were grouped in squares based on age and milk yield. Each period consisted of 28 d: 14 d for adaptation, and 14 d for sampling, data collection, and recovery period following LPS challenge. Cows were fed once a day ad libitum targeting 5 to 10% refusals. Milk yields and body weights were recorded daily. Recombinant bST was administrated at the beginning (i.e., d 1) and in the middle (d 15) of each experimental period. Rumen protected *Capsicum* oleoresin (RPC) was top-dressed on the TMR during feeding. Three treatments were tested in this experiment: 0 mg/d per cow RPC (**control**), 100 mg/d RPC (**C100**), and 200 mg/d RPC (**C200**). The RPC product used in the experiment was RP-Caps (X50-7035; 15.5% *Capsicum* oleoresin; 0.93% capsaicinoids; Pancosma, S. A., Geneva, Switzerland). All cows were fed the same basal TMR (Table 5-1). Intake and refusal weights were recorded daily.

Weekly composites samples of the TMR and refusals were prepared from sub-samples collected twice weekly. Forages were sampled weekly; a composite sample was made for each experimental period. Samples of the concentrate feeds were collected randomly during the experiment and composited for later analyses. Composite samples of the TMR, forages, concentrates, and orts were stored frozen, oven-dried to constant weight (65°C) and ground through a 1-mm sieve before being analyzed for CP (AOAC International, 2000), NDF (Van Soest et al., 1991), ADF (AOAC International, 2000), ether extract (AOAC International, 2006), Ca (AOAC International, 2000), P (AOAC International, 2000), NFC (NRC, 2001), and NE_L (NRC, 2001) by Cumberland Valley Analytical

Services (Maugansville, MD). Total mixed ration and fecal samples were ashed for 4 h at 600℃ for analysis of OM. Samples of the TMR were also analyzed for indigestible NDF (iNDF).

Sampling and analyses

Sampling occurred during the last week of each experimental period. Fecal, urine, and milk samples were collected and analyzed as follows. Total tract digestibility of nutrients was estimated using iNDF as an internal marker (Schneider and Flatt, 1975). Grab fecal samples were collected from the rectum or after stimulating defecation at the following time-points: D (day of experimental period) 20 - 10 am, 4 pm, and 10 pm; D21 - 4am, 1pm, and 7 pm; D22 - 1 am and 7 am. Approximately 300 g wet fecal samples were collected per sampling and immediately oven-dried at 65℃. After drying, the samples were ground through a 1 mm sieve and composited per cow and period. Apparent digestibility of feed DM, OM, N, NDF, and ADF was estimated using dietary and fecal concentrations of iNDF. Spot urine samples (approximately 300 ml each) were collected at the same time-points as for the fecal samples. Urine samples were collected by massaging the vulva. Urine was acidified (pH < 3) with H_2SO_4 solution. The acidic solution was made by adding 0.6 ml 2 M H_2SO_4 to 90 ml distilled water. The samples were composited on an equal volume basis and stored frozen at -20℃. One composite sample per cow per period was analyzed for total N, purine derivatives (allantoin and uric acid), urea-N, and creatinine. The analysis of urine samples are described in detail in Oh et al. (2013). Urine volume was estimated by urinary creatinine concentration,

assuming a creatinine excretion rate of 29 mg/kg of BW (Hristov et al., 2011). Daily urinary excretion of total N, urea-N and purine derivatives was calculated using the estimated urine output. Fecal and urine samples were collected only in the second and third period. Milk samples for composition analyses (fat, protein, lactose, SCC, MUN) were collected during the last week of each experimental period (PM and AM milkings) on D20. Milk samples were submitted to Dairy One laboratory for analysis (Pennsylvania DHIA, University Park, PA). Milk was analyzed for fat, true protein, lactose, and MUN using infrared spectroscopy (MilkoScan 4000; Foss Electric, Hillerød, Denmark).

Catheters (14-gauge X 13 cm MILA International Inc., Erlanger, KY) were inserted with an extension set into the jugular vein of all cows (n = 9) on the day before the glucose tolerance test (GTT), i.e. D22. An intravenous glucose challenge was conducted on D23. Catheters were flushed with 8 ml of heparinized saline (10 IU/ml) every 6 h and during blood sampling. Feeding was suspended for 12 h before the glucose challenge, but cows had access to water. Glucose (D-glucose 50%, wt/vol) was administered intravenously at 0.3 g/kg of BW at a constant rate within 3 min by syringes. The glucose tolerance test was performed once during each experimental period. Blood samples were collected from the jugular vein catheters at -20, 0, 5, 10, 15, 20, 30, 40, 50, 65, 80, and 110 min relative to administration of glucose on D23. Whole blood samples were collected at the time points above into vacuumed tubes containing silica clot activator (SST™ Tube; BD Biosciences, Franklin Lakes, NJ), placed at room temperature to allow clotting, and centrifuged at 3,000 × g at room temperature for 15

min to separate blood serum. Serum samples were separated into three 2ml vials and used for glucose, insulin, leptin, NEFA, and BHBA analysis. These samples were kept frozen in a -80℃ freezer until analysis. Serum samples at 0h were used for the basal concentration of NEFA and BHBA. Serum samples were analyzed for glucose using a chemistry analyzer (IDEXX VetTest® and VetLyte® Chemistry and Electrolyte Analyzers, Idexx Laboratories, Inc., Westbrook, ME), insulin with a radioimmunoassay (PI-12K, EMD Millipore, Billerica, MA), leptin with a radioimmunoassay (XL-85K, EMD Millipore), NEFA using an enzymatic colorimetric method (NEFA-HR(2), Wako Diagnostics, Mountain View, CA), and BHBA using a biochemistry analyzer (Cobas 6000; Roche, Germany).

Experimental immune challenge was conducted on d24 with bacterial LPS (*Escherichia coli* O111:B4, Sigma Chemical Co., St. Louis, MO). On the day of LPS infusion by the catheters, LPS was dissolved in 100 ml of 0.9 % sterile saline. The amount of LPS was 1.0 μg/kg BW. The LPS solution was infused into the jugular vein of all cows at a rate of 1 ml/min through sterile tubing by peristaltic pumps for 100 min. The infusion began at the feeding time and three sets of blood samples were collected at 0, 2, 4, 8, and 24 h after the end of the infusion. The first set of samples (approximately 10 mL) was collected into Vacutainer tubes containing EDTA (BD Biosciences, Franklin Lakes, NJ), kept refrigerated (4℃), and analyzed on the same or next day for hematology analysis. The analysis included total white blood cell count, including total count for neutrophils, eosinophils, lymphocytes, monocytes, and basophils using an automated hematology analyzer (HemaVet® ; Drew Scientific, Oxford, CT). Another set

of blood samples was collected from the catheters into vacuumed tubes (SST™ Tube; BD Biosciences) for blood serum at the same time points after LPS infusion and allowed to be clotted at room temperature. Blood serum were obtained by centrifugation at 3,000 × *g* at room temperature for 15 min and kept frozen at -80℃ until analysis for cortisol using a radioimmunoassay kit (ImmuChem Coated Tube, MP Biomedicals, NY) and haptoglobin using a biochemistry analyzer (Cobas 6000). The other set of blood samples was collected into vacuumed tubes containing EDTA (BD Biosciences). Plasma was obtained by centrifugation at 1,500 × *g* at 4℃ for 10 min and stored frozen at -80℃. Samples were analyzed for thiobarbituric acid reactive substances (**TBARS**) using a colorimetric assay (Cayman Chemical, Ann Arbor, MI). Rectal temperature was measured at 0, 2, 4, 8, and 24h post LPS challenge.

In situ disappearance rate of RPC

To compare the ruminal bypass rate of RPC with that of unprotected *Capsicum* oleoresin (CAP) in Chapter 4, an *in situ* incubation was conducted to determine the ruminal disappearance rate of RPC. Two Holstein cows fitted with 10-cm rumen cannulas (Bar Diamond Inc., Parma, ID) were utilized for this experiment. Polyester bags (5 x 10 cm, 50 μm porosity; Ankom Technology, Macedon, NY) containing 5 g RPC/bag were incubated in triplicates in each cow for 0, 1, 3, 6, 12, and 24 h. Bags were inserted in the rumen sequentially and removed simultaneously. After the incubation, the bags were washed with cold tap water and freeze-dried. The residues were analyzed for

capsaicinoids by UPLC-MS/MS on a triple quadrupole mass spectrometer (Waters Xevo; Waters Corporation, Midford, MA).

Kinetic parameters of capsaicinoids disappearance rate in the rumen were estimated using the following model (Ø rskov and McDonald, 1979):

$$y = a + b\,(1 - e^{-ct})$$

where y was the percentage of capsaicinoids that disappeared at time t, a was the fraction of capsaicinoids rapidly soluble (%), b was the fraction of capsaicinoids potentially degradable (%), c was the fractional rate constant for the disappearance of fraction b, and t was the time of incubation (h). The potentially degradable fraction was a + b. Effective ruminal degradability (ED) was calculated from the following equation:

$$ED = a + (b \times c) / (c + k)$$

where k was the fractional passage rate from the rumen and constants a, b, and c were defined previously. Effective ruminal degradability of capsaicinoids in RPC was estimated for hypothetical fractional passage rates of 0.08, 0.10, 0.12, and 0.15/h.

Calculations and statistical analysis

Microsoft EXCEL was used to fit exponential curves for glucose and insulin data using the following equation (Pires et al., 2007):

$$F(t) = A \times e^{(-k \times t)}$$

where F(t) is the concentration at time t; A is the maximum concentration; t is the time after the glucose challenge; and k is the regression coefficient. Clearance rate (CR), time to half maximal concentration ($T_{1/2}$), and time to reach basal concentration (T_{basal}) were calculated by the following equations :

$$CR\ (\%/min) = [\{\ln (ta) - \ln (tb)\} / (tb - ta)] \times 100$$

$$T_{1/2} = [\{\ln (2)\} / CR] \times 100$$

$$T_{basal} = [\{\ln (ta) - \ln (tb)\} / CR] \times 100$$

where ta is the concentration at time a, and tb is the concentration at time b.

The trapezoidal method was used for calculating the area under the curve (AUC) calculation of glucose and insulin post glucose challenge after subtracting the baseline concentrations. Concentrations at 0h (before the glucose challenge) were used for the baseline concentration.

All data were analyzed using the MIXED procedure of SAS 9.4 (2003; SAS Institute Inc., Cary, NC). Milk yield, DMI, and estimated feed efficiency data for 9 d (i.e. D15 to D23) and milk composition data for the 2 milk sampling days (D20 and D21) of each experimental period were averaged and the average values were used in the statistical analysis. The averaged milk yield, DMI, and milk composition data were used to calculate yields of milk fat, protein, lactose, and ECM. Data were tested for normality using the UNIVARIATE procedure of SAS. Log-transformed data were analyzed when the W statistic of the Shapiro-Wilk test was less than 0.05.

Nutrient intake, digestibility, urinary and fecal N excretions, milk composition, NEFA, BHBA, GTT data (glucose, insulin, and leptin) for each time point post glucose administration including AUC, peak and basal concentration, CR, $T_{1/2}$, and T_{basal}, and LPS data (white blood cells, cortisol, haptoglobin) for each time point were analyzed by analysis of variance Latin square. The model used was as follows:

$$Y_{ijkl} = \mu + S_i + C(S)_{ij} + P_k + T_l + e_{ijkl},$$

where Y_{ijkl} is the dependent variable, μ is the overall mean, S_i is the square, $C(S)_{ij}$ is the cow within square, P_k is the kth period, and T_l is the lth treatment with the error term e_{ijkl}. Square and cow within square were random effects and all others were fixed.

DMI, milk yield, feed efficiency, ruminal degradability parameters and GTT data (glucose, insulin, and leptin) and LPS data (white blood cells, cortisol, haptoglobin, TBARS) combining all time points were analyzed as repeated measures assuming an AR(1) covariance structure. The model used was as follows:

$$Y_{ijklm} = \mu + S_i + C(S)_{ij} + P_k + T_l + D_m + TD_{lm} + e_{ijklm},$$

where Y_{ijklm} is the dependent variable, μ is the overall mean, S_i is the square, $C(S)_{ij}$ is the cow within square, P_k is the kth period, T_l is the lth treatment, D_m is the time effect, and TD_{lm} is the treatment × time of sampling interaction, with the error term e_{ijklm}. Square and cow within square were random effects and all others were fixed.

Orthogonal contrasts were used to evaluate RPC treatments vs. control, linear, and quadratic effects of RPC supplementation. Statistical differences were considered significant at $P \leq 0.05$ and a trend at $0.05 < P \leq 0.10$.

Results

The basal diet used in this experiment was formulated to meet NE_L and MP requirements of cows milking 45 kg/d (Table 5-1). The experimental diet supplied NE_L in excess of cow requirements (NRC, 2001).

As results from *in situ* incubation, rapidly soluble fraction and potentially degradable fraction of RPC in capsaicinoids were not different from those of CAP (Table 5-2). However, rate of capsaicinoids degradation was significantly higher ($P = 0.03$) in CAP than RPC. Compared with RPC, effective degradability also tended to be higher ($P \leq 0.07$) in CAP. Assuming fractional passage rate (k) was 0.15, capsaicinoids amounts bypassing the rumen were from 80.0 to 320.0 g/d for CAP in Chapter 4, and from 43.5 to 86.9 g/d for RPC in the current experiment.

Dry matter intake and milk yield tended to be quadratically increased ($P \leq 0.09$) by RPC supplementations (Table 5-3). A linear increase ($P < 0.01$) was observed in feed efficiency with RPC supplementations. Concentration of milk fat, milk true protein, milk lactose, and total solid was not affected by treatments. The RPC treatments tended to quadratically decrease ($P = 0.06$) MUN and there was no effect on SCC. Total tract digestibility of DM, OM, and CP was linearly increased ($P \leq 0.03$) while that of NDF

and ADF tended to be linearly increased ($P \leq 0.10$) with treatments (Table 5-4). Although excretion of urine N was not affected by RPC, fecal N excretion and total excreta N tended to be linearly decreased ($P \leq 0.07$) by RPC (Table 5-5). Amount of UUN excreted tended to be quadratically decreased ($P = 0.09$) by RPC. The proportion of fecal N excreted to N intake was linearly decreased ($P = 0.03$) by RPC. Compared with the control, urine output tended to be decreased ($P = 0.08$) by RPC supplementations. The RPC treatments tended to decrease uric acid, but allantoin and total PD excretion in urine were not affected by treatment. Serum concentration of NEFA and BHBA were also not affected by RPC (Table 5-6). Serum BHBA concentration in this experiment was below the level for subclinical ketosis (1,200 to 2,900 μmol/L; Oetzel, 2004).

Serum glucose concentration peaked at 5 min and returned to the basal level at 80 min after glucose infusion (Figure 5-1). Glucose concentration in serum during GTT was not affected by RPC treatments when analyzed as repeated measure (Table 5-7). Basal and peak levels, CR, $T_{1/2}$, T_{basal}, and AUC of glucose also did not differ among the treatments. Glucose concentration tended to be quadratically decreased ($P = 0.06$) by RPC at 40 min, and RPC supplementations tended to decrease ($P = 0.07$) glucose concentration compared with the control at 110 min post glucose challenge. Insulin concentration peaked at 10 min for the control and at 5 min for C100 and C200, and returned to the basal level at 65 min after glucose administration (Figure 5-2). Serum insulin concentration during GTT was not affected by RPC when analyzed as repeated measure (Table 5-8). Peak concentration of insulin tended to be decreased ($P = 0.07$) by

RPC, but basal concentration, CR, $T_{1/2}$, and T_{basal} were not affected by the treatments. The RPC supplementations significantly decreased (P = 0.04) AUC of insulin concentration compared with the control. After glucose infusion, insulin concentration in serum was significantly decreased ($P \leq 0.04$) at 5, 10, and 40 min and tended to be decreased ($P \leq 0.08$) at 30 and 50 min for RPC treatments compared with the control. There was a tendency of linear decrease (P = 0.10) at 20 min post glucose challenge with RPC. Leptin started increasing at 10 or 15 min after glucose infusion (Figure 5-3). Whereas the control had a peak level of leptin at 30 min, C100 and C200 had peak levels at 50 and 80 min, respectively, after glucose administration. Leptin concentration returned to the basal level at 110 min after glucose infusion for all the groups. Serum leptin concentration post glucose challenge was quadratically increased (P = 0.04) by RPC treatments when analyzed as repeated measure (Table 5-9). However, a treatment × period interaction ($P < 0.01$) was observed in leptin data (Figure 5-4). After glucose infusion, leptin concentration in serum was linearly decreased (P = 0.03) at 40 min, and tended to be increased (P = 0.07) at 50 min by RPC.

Rectal temperature had peaks at 2h for the control and C100, and 4h for C200 and returned to the basal temperature at 8h after LPS challenge (Figure 5-5). Treatments tended to linearly increase (P = 0.10) rectal temperature at 4h and also linearly increased (P = 0.03) at 8h post LPS administration. Following the challenge, DMI, milk yield, and milk efficiency were not affected by RPC treatments although there were numerical differences in milk yield (1.8 to 3.4 kg/d) between the control and RPC supplementation (Table 5-10). Numbers of white blood cells including neutrophils, lymphocytes,

monocytes, and eosinophils immediately decreased following LPS infusion and were the lowest at 2 h in all treatments (Figure 5-6). Overall, the RPC supplementation did not affect white blood cell numbers although there was a quadratic increase (P = 0.02) with RPC treatments in lymphocyte numbers at 0h. White blood cells were not affected by RPC supplementation in LPS challenge test when analyzed as repeated measure (Table 5-10). Treatments tended to increase (P = 0.07) plasma TBARS concentration compared with the control (Table 5-10) and there was no time effect (P = 0.21) in TBARS concentration. Serum cortisol concentration had peaks at 2h and returned to the basal at 8h after LPS challenge for all treatments (time effect; 0h vs. 8h, P = 0.33). Cortisol concentration was lower with a trend (P = 0.06) in RPC treatments compared with the control at 2h (Figure 5-7) and was decreased (P = 0.03) by RPC supplementation relative to the control when analyzed as repeated measure at 2h and 4h (Table 5-11). The RPC supplementation decreased (P = 0.04) haptoglobin concentration compared with the control during the challenge (Table 5-11).

Discussion

Effect of *Capsicum* oleoresin on feed intake

Possible explanations for the trend of quadratic increase in DMI with RPC in the current experiment include the pungency and the alteration of appetite-related hormones by *Capsicum*. In studies with beef cattle, inclusion of *Capsicum* in a high-concentrate diet increased DMI in a dose-dependent manner (Cardozo et al., 2006; Rodriguez et al.,

2012). Water intake was also increased by *Capsicum* supplementation in these studies and there was a strong relationship (R^2 = 0.98) between DMI and water intake (Rodriguez et al., 2012). It is likely that pungency of *Capsicum* caused higher water intake which was followed by increased DMI. In the current experiment, however, it is not certain if water intake by the pungency induced the increase in DMI because water intake data were not collected. In addition, other studies in dairy cows reported that *Capsicum* supplementation did not affect DMI (Tager and Krause, 2011; Oh et al., 2015). *Capsicum* could also increase DMI by altering appetite-related hormone secretions, although there are conflicting data in the literature (Smeets and Westerterp, 2009). McCann et al. (1988) reported that the inhibiting effect of cholecystokinin (CCK) on food intake was alleviated in rats having desensitized afferent nerves by capsaicin. Van de Wall et al. (2006) also demonstrated that a reduction in food intake by CCK injection was attenuated in rats having desensitized nerves by neonatal treatment with capsaicin. Neonatal treatment of capsaicin in rat has been reported to cause ablation of sensory afferent neurons (Camara et al., 2008). The results of van de Wall et al. (2006) indicate that CCK acts through vagal nerves on food intake and capsaicin treatment could inhibit the effect of CCK by desensitization of vagal nerves. In addition, capsaicin has been reported to decrease leptin, a protein hormone inhibiting hunger, in adipocytes (Klok et al., 2007; Hsu and Yen, 2007). However, basal leptin concentration in blood in the current experiment was not different between the RPC treatments and the control. On the other hand, it has been reported that the stimulatory effects of *Capsicum* depend on dose amount (Abdel-Salam et al., 1995; Rao et al., 2003; Demirbilek et al., 2004). This property of *Capsicum* might cause quadratic effect on DMI in the current experiment.

Effect of *Capsicum* oleoresin on milk yield, total tract digestibility, N utilization and fat mobilization

The trend of quadratic increase in milk yield by RPC treatments in the current experiment might have resulted from the trend of quadratic increase in DMI. In our previous experiment, milk production also tended to be quadratically increased by *Capsicum* oleoresin supplementation in dairy cows (Oh et al., 2015). The increase in total tract digestibility of dietary nutrients by RPC in the current experiment might be due to stimulatory effects of capsaicin on digestive enzymes in the lower gut. Dietary supplementation of capsaicin has been reported to increase secretion of digestive enzymes such as lipase, amylase, trypsin, and chymotrypsin in the pancreas of rats (Platel and Srinivasan, 1996; Platel and Srinivasan, 2000). Rao et al. (2003) also found an increase of amylase activity in pancreas homogenate with capsaicin administration. Previous experiments, however, reported no effect of *Capsicum* oleoresin on total tract digestibility of DM, OM, CP, NDF, and ADF in dairy cows (Tager and Krause, 2011; Oh et al., 2015). It is possible that in the current experiment the effect of *Capsicum* on nutrient digestibility in the lower gut was enhanced by its rumen protection, which resulted in greater delivery of capsaicinoids postruminally. The linear decrease in N excretion by RPC was consistent with the total tract digestibility data in the current experiment. The trends for quadratic decrease in UUN excretion and MUN concentration with RPC have resulted from reduction in ruminal proteolysis and deamination of dietary or microbial amino acids (Butler, 2005). Oh et al. (2015) reported that *Capsicum*

quadratically decreased the prevalence of *Prevotella* spp. in dairy cows. It is known that *Prevotella* spp. degrade proteins and are involved in the uptake and fermentation of peptides (Stewart et al., 1997).

Studies have reported that capsaicin supplementation reduced adipose tissue and increase blood free fatty acids in rats (Kawada et al., 1986; Yoshioka et al., 2000). According to Lee et al. (2013), capsaicin increased gene expression of lipoprotein lipase and adiponectin in mesenteric adipose tissues of capsaicin-treated rats, both of which are related to fat accumulation reduction and fatty acid combustion (Yamauchi et al., 2001; Koike et al., 2004). In the current experiment, however, RPC did not affect serum concentration of NEFA and BHBA. We found a quadratic increase in BHBA concentration with *Capsicum* oleoresin supplementation in Chapter 4 (Oh et al., 2015). Smaller amount of capsaicinoids delivered into the lower gut in the current experiment compared with Chapter 4 might be not enough to cause fat mobilization in dairy cows.

Effect of *Capsicum* oleoresin on responses to glucose tolerance test

While glucose concentration in blood remained similar between RPC and the control, RPC significantly decreased insulin concentration during GTT. These results suggest that cows treated with RPC were able to control and maintain glucose levels with lower levels of insulin secretion compared with the control cows, indicating an increase in insulin sensitivity in cows fed RPC during the glucose challenge. Van de Wall et al. (2005) demonstrated that capsaicin treatment increased insulin sensitivity in rats. These

authors found a lower insulin levels in rats neonatally treated with capsaicin during a glucose tolerance test. Increased insulin sensitivity in capsaicin-treated rats might be attributed to the inhibition of calcitonin-gene related peptide (CGRP) by capsaicin-induced desensitization of nerves. It is known that CGRP is released from sensory neurons and known to decrease insulin activity (Choi et al., 1991). According to Bley (2004), prolonged stimulation of capsaicin could functionally inactivate TRPV1, a receptor for capsaicinoids expressed mainly in the sensory neurons. In the current experiment, RPC may have caused desensitization of nerves in the intestine and exert the inhibitory effect on CGRP, which resulted in an increase of insulin sensitivity. However, data on glucose and insulin regulation by *Capsicum* have been conflicting. Dömötör et al. (2006) observed increases of glucose and glucagon concentration in blood serum after oral glucose challenge when 400 μg capsaicin was administrated to human subjects. In that study, serum insulin level was not affected by capsaicin. Chaiyasit et al. (2009) reported that a single dose of 5 g *Capsicum frutescens* (26.6 mg capsaicin) decreased plasma glucose concentration post oral glucose challenge and increased plasma insulin concentration in a human study. The quadratic increase in leptin concentration by RPC should be carefully interpreted because of a period × treatment interaction in the current experiment. It has been known that blood insulin concentration is correlated with blood leptin concentration (Havel et al., 1996; Rohner-Jeanrenaud and Jeanrenaud, 1997). Block et al. (2003) reported that hyperinsulinemia caused an increase in plasma leptin in dairy cows. Leury et al. (2003) also found an increase of plasma leptin concentration after an insulin infusion by hyperinsulinemic clamps. These data, however, were

opposite to the observed decrease in insulin and increase in leptin concentration in blood by RPC during the glucose tolerance test.

Effect of *Capsicum* oleoresin on responses to LPS challenge

Decreases in total white blood cells including neutrophils, lymphocytes, monocytes, and eosinophils and increases in rectal temperature, cortisol, and haptoglobin indicated that intravenous LPS infusion triggered acute phase responses in dairy cows in the current experiment. Leukocytes reduction by endotoxin administration has been found in other studies and known to be correlated to cortisol elevation in blood (Richardson et al., 1989; Wang et al., 2003). Drastic increase of serum cortisol by LPS infusion and return to the basal level at 8h were consistent with cattle studies (Waldron et al., 2003; Waggoner et al., 2009).

In previous experiments of this dissertation using dairy cows without immune challenges, *Capsicum* oleoresin decreased monocyte counts and increased lyphocyte proportion when it was post-ruminally infused at high (2 g/d) dose in dairy cows in Chapter 3 (Oh et al., 2013). Also, dietary supplementation of *Capsicum* oleoresin (250 to 1,000 mg/d) could linearly increase neutrophils and eosinophils in Chapter 4 (Oh et al., 2015). In the current experiment, however, RPC supplementation did not affect counts and proportion of pheripheral white blood cells before and after the immune challenge. The stimulatory effects of *Capsicum* on leukocytes might depend on dose amount delivered into the lower gut. Nevius et al. (2012) demonstrated in rats that capsaicin's

effects on T lymphocytes was different by dose amount. As previously decribed, capsaicinoids amount delivered into the lower gut in the current experiment was less than that of the previous experiments, Chapter 3 and 4.

No effect of LPS challenge on plasma TBARS, a byproduct from lipid oxidation, in the present experiment was also found in a poultry study (Star et al., 2008). Although a long-term stress such as heat could increase plasma TBARS, a short-term LPS administration did not affect the oxidative stress status in that experiment. The effect of RPC on TBARS in the current experiment is not consistent with Manjunatha and Srinivasan (2006), who found that capsaicin inclusion in diets could decreased serum TBARS induced by ion in rats. According to Bernabucci et al. (2005), higher TBARS could be found in cows with higher BHBA. In the current experiment, however, we did not analyze BHBA post LPS challenge.

Cortisol, a major stress hormone, is secreted from adrenal gland and its secretion is under control of the hypothalamic-pituitary-adrenal (HPA) axis. Choi et al. (2013) demonstrated that repeated oral capsaicin administration could affect the HPA axis and increased plasma cortisol in rats. In a study using pigs, subcutaneous injection of capsaicin has increased plasma corisol levels without immune challenges (Alving et al., 1991). It was suggested that stimulation of sensory nerve by capsaicin might release adrenocorticotrophic hormone to affect the HPA axis. However, we did not find differences in serum cortisol concentration by RPC treatment in the current experiment. Instead, RPC supplementation worked in a different way post LPS challenge in the

current experiment, which could alleviate LPS-induced stress in dairy cows by decreasing serum corisol concentration.

Haptoglobin is an acute phase protein, primarily produced in hepatocytes. Haptoglobin increases several fold during inflammation, tissue damage, and infection (Quaye, 2008). Haptoglobin has been known to play a role in inflammation by scavenging free haemoglobin that damages cellular constituents (Bicho et al., 2013). In addition, haptoglobin induces neutrophil recruitment to sites of inflammation (Quaye, 2008). In the present experiment, a decrease in serum haptoglobin by RPC post LPS administration suggests that *Capsicum* oleoresin supplementation might reduce acute phase response with LPS challenge in dairy cows. This result is consistent with Liu et al. (2014), who reported that *Capsicum* oleoresin supplementation could reduce gene expression profiles of ileal mucosa, which were associated with innate immunity in pigs challenged by *E. coli*.

Conclusion

Dietary supplementation of rumen-protected *Capsicum* tended to increase feed intake and milk production and increased feed efficiency in dairy cows. In addition, rumen-protected *Capsicum* appeared to improve insulin sensitivity during glucose tolerance test and have anti-inflammatory effect on the acute phase response after LPS challenge. Our data suggest that rumen-protected *Capsicum* fed at 100 to 200 mg/d may

increase productivity and insulin activity and alleviate acute phase responses via the neurological pathways in lactating dairy cows.

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Table 5-1. Ingredient and chemical composition of the basal diet fed during the experiment

Item	% of diet DM
Ingredients	
Corn silage[1]	43.5
Haylage[2]	12.0
Cottonseed, hulls	3.2
Corn grain, ground	6.0
Candy by-product meal[3]	6.5
Soybean seeds, whole heated[4]	8.65
Canola meal[5]	8.65
Molasses[6]	3.5
Vitamin and mineral premix[7]	3.0
SoyPLUS[8]	5.0
Composition,[9] % of DM (or as indicated)	
CP[9]	16.1
RDP[10]	9.8
RUP[10]	6.9
NDF[9]	30.9
ADF[9]	23.2
NE_L, Mcal/kg[9]	1.69
Ca[9]	1.02
P[9]	0.39
NFC[10]	45.2
Average NE_L balance,[11] Mcal/d	(4.5, 3.6, 3.2)
Average MP balance,[11] g/d	(359, 330, 304)

[1]Corn silage was 46.5% DM and contained (DM basis): 6.7% CP, 32.4% NDF and 42.7% Starch.
[2]Haylage was 90.7% DM and contained (DM basis): 21.0% CP and 43.7% NDF.
[3]Candy by-product meal (Graybill Processing, Elizabethtown, PA) contained (DM basis) 16.9% CP and 26.7% NDF.
[4]Soybean seeds contained (DM basis) 40.0% CP.
[5]Canola meal contained (DM basis) 40.9% CP.
[6]Molasses (Westway Feed Products, Tomball, TX) contained (DM basis) 3.9% CP and 66% total sugar.
[7]The premix (Cargill Animal Nutrition, Cargill Inc., Roaring Spring, PA) contained (%, as-is basis) trace mineral mix, 0.86; MgO (56% Mg), 8.0; NaCl, 6.4; vitamin ADE premix (Cargill Animal Nutrition, Cargill Inc.), 0.48; limestone, 37.2; selenium premix (Cargill Animal Nutrition, Cargill Inc.), 0.07; and dry corn distillers grains with solubles, 46.7. Ca, 14.1%; P, 0.39%; Mg, 4.59%; K, 0.44%; S, 0.39%; Se, 6.91 mg/kg; Cu, 362 mg/kg; Zn, 1,085 mg/kg; Fe, 186 mg/kg, vitamin A, 276,717 IU/kg; vitamin D, 75,000 IU/kg; and vitamin E, 1,983 IU/kg.
[8]SoyPLUS (West Central Cooperative, Ralston, IA) contained (DM basis) 47.2% CP.

[9]Values calculated using the chemical analysis (Cumberland Valley Analytical Services Inc., Maugansville, MD) of the ingredients of the diet.
[10]Estimated by NRC (2001).
[11]Estimated based on NRC (2001) using actual DMI, milk yield, milk composition, and BW of the cows throughout the experiment (Control, C100, and C200 respectively).

Table 5-2. Values of effective degradability (ED) of capsaicinoids[1] in CAP[2] and RPC[3] used in Chapter 4 and 5, respectively.

Parameters	CAP	RPC	SEM[4]	*P*-value
a[5], %	4.57	2.24	0.985	0.17
b[6], %	95.2	97.8	1.05	0.15
c[7]	0.30	0.19	0.021	0.03
ED				
(k[8] = 0.08)	79.7	70.6	2.47	0.07
(k = 0.10)	75.9	65.9	2.70	0.06
(k = 0.12)	72.5	61.8	2.85	0.06
(k = 0.15)	68.0	56.5	3.00	0.06

[1]Capsaicin and dihydrocapsaicin
[2]*Capsicum* oleoresin in unprotected granular form
[3]Rumen-protected *Capsicum* oleoresin
[4]Higher SEM shown; n = 15 for CAP and RPC (n represents number of observations used in the statistical analysis).
[5]Fraction of rapidly soluble capsaicinoids in the rumen
[6]Fraction of potentially degradable capsaicinoids in the rumen
[7]Fractional rate constant for the disappearance of fraction b
[8]Fractional passage rate

Table 5-3. Effect of rumen-protected *Capsicum* oleoresin on milk yield and composition in dairy cows

	Treatment[1]				*P*-value[2]		
Item	Control	C100	C200	SEM[3]	Con vs. T	L	Q
DM intake, kg/d	29.4	30.0	29.2	0.74	0.72	0.55	0.09
Milk yield, kg/d	42.8	44.7	43.9	1.27	0.06	0.22	0.08
Feed efficiency[4], kg/kg	1.48	1.52	1.57	0.056	0.03	<0.01	0.96
Milk fat, %	3.89	3.92	3.97	0.220	0.73	0.65	0.95
Yield, kg/d	1.64	1.72	1.73	0.102	0.34	0.38	0.66
ECM, kg/d	44.7	46.8	46.5	1.62	0.19	0.26	0.43
Milk true protein, %	3.12	3.11	3.10	0.088	0.69	0.68	0.91
Yield, kg/d	1.32	1.38	1.36	0.041	0.21	0.35	0.32
Milk lactose, %	4.72	4.75	4.71	0.052	0.88	0.77	0.46
Yield, kg/d	2.03	2.14	2.07	0.046	0.22	0.53	0.16
Total solid, %	12.7	12.6	12.7	0.39	0.89	0.99	0.77
MUN, mg/100ml	10.4	9.24	10.4	0.542	0.30	0.98	0.06
SCC, $\times 10^3$ cells/ml	36.5	33.8	73.2	24.41	0.50	0.21	0.42

[1]Control, 0 mg/d rumen-protected *Capsicum* oleoresin; C100, 100 mg/d rumen-protected *Capsicum* oleoresin; C200, 200 mg/d rumen-protected *Capsicum* oleoresin.

[2]Con vs. T, control vs. treatment; L, linear effect of rumen-protected *Capsicum* oleoresin; Q, quadratic effect of rumen-protected *Capsicum* oleoresin.

[3]Highest SEM shown; n = 215 for DMI, n = 202 for milk yield and feed efficiency, n = 24 for all other variables (n represents number of observations used in the statistical analysis).

[4]Milk yield÷DM intake

Table 5-4. Effect of rumen-protected *Capsicum* oleoresin on total tract digestibility of nutrients in dairy cows

	Treatment[1]				*P*-value[2]		
Item	Control	C100	C200	SEM[3]	Con vs. T	L	Q
Intake, kg/d[4]							
DM	29.6	29.7	29.0	1.13	0.73	0.46	0.56
OM	27.6	27.7	27.0	1..09	0.72	0.44	0.54
CP	4.71	4.74	4.61	0.186	0.70	0.42	0.51
NDF	9.45	9.49	9.28	0.375	0.74	0.50	0.60
ADF	7.25	7.30	7.11	0.287	0.72	0.44	0.54
Apparent digestibility, %							
DM	63.6	63.8	66.2	0.89	0.06	0.02	0.10
OM	64.8	64.9	67.4	0.85	0.06	0.02	0.10
CP	59.1	60.0	64.9	1.90	0.09	0.03	0.21
NDF	35.5	35.5	39.6	1.47	0.18	0.06	0.19
ADF	31.3	33.6	38.5	1.95	0.16	0.10	0.65

[1]Control, 0 mg/d rumen-protected *Capsicum* oleoresin; C100, 100 mg/d rumen-protected *Capsicum* oleoresin; C200, 200 mg/d rumen-protected *Capsicum* oleoresin.

[2]Con vs. T, control vs. treatment; L, linear effect of rumen-protected *Capsicum* oleoresin; Q, quadratic effect of rumen-protected *Capsicum* oleoresin.

[3]Highest SEM shown; n=18 for all variables. (n represents number of observations used in the statistical analysis).

[4]Data was collected on fecal sampling days in period 2 and 3.

Table 5-5. Effect of rumen-protected *Capsicum* oleoresin on nitrogen utilization in dairy cows

	Treatment[1]				*P*-value[2]		
Item	Control	C100	C200	SEM[3]	Con vs. T	L	Q
N intake, g/d[4]	754	759	738	29.1	0.70	0.41	0.51
N secretion and excretion, g/d							
Urine N	154	145	154	15.7	0.69	1.00	0.44
UUN, g/d	99.6	89.7	106	5.63	0.72	0.36	0.09
Fecal N	303	298	260	20.9	0.13	0.06	0.33
Total excreta N	466	443	414	15.8	0.09	0.07	0.89
As proportion of N intake, %							
Urine N	21.7	19.7	20.6	2.89	0.51	0.68	0.53
Fecal N	40.9	40.0	35.1	1.89	0.09	0.03	0.21
Total excreta N	61.8	59.9	56.0	1.65	0.16	0.09	0.65
Urine output, kg/d	23.2	20.1	20.7	1.06	0.08	0.14	0.22
Urinary PD excretion							
Allantoin, mmol/d	452	431	472	35.2	1.00	0.69	0.47
Uric acid, mmol/d	68.8	52.3	64.5	6.43	0.12	0.52	0.08
Total PD, mmol/d	511	494	528	38.8	0.99	0.77	0.57

[1]Control, 0 mg/d rumen-protected *Capsicum* oleoresin; C100, 100 mg/d rumen-protected *Capsicum* oleoresin; C200, 200 mg/d rumen-protected *Capsicum* oleoresin.

[2]Con vs. T, control vs. treatment; L, linear effect of rumen-protected *Capsicum* oleoresin; Q, quadratic effect of rumen-protected *Capsicum* oleoresin.

[3]Highest SEM shown; n=18 for all variables. (n represents number of observations used in the statistical analysis).

[4]Data was collected on fecal sampling days.

Table 5-6. Effect of rumen-protected *Capsicum* oleoresin on serum NEFA[1] and BHBA[2] in dairy cows

	Treatment[3]				*P*-value[4]		
Item	Control	C100	C200	SEM[5]	Con vs. T	L	Q
NEFA, μmol/L[6]	614	522	628	101.1	0.71	0.90	0.32
BHBA, μmol/L	310	403	391	41.9	0.15	0.21	0.31

[1]Non-esterified fatty acid
[2]Beta-hydroxybutyric acid
[3]Control, 0 mg/d rumen-protected *Capsicum* oleoresin; C100, 100 mg/d rumen-protected *Capsicum* oleoresin; C200, 200 mg/d rumen-protected *Capsicum* oleoresin.
[4]Con vs. T, control vs. treatment; L, linear effect of rumen-protected *Capsicum* oleoresin; Q, quadratic effect of rumen-protected *Capsicum* oleoresin.
[5]Highest SEM shown; n = 23 for NEFA and BHBA. (n represents number of observations used in the statistical analysis).
[6]Oleic acid equivalent.

Table 5-7. Effect of rumen-protected *Capsicum* oleoresin on serum glucose response to glucose tolerance test in dairy cows

	Treatment[1]				*P*-value[2]		
Item	Control	C100	C200	SEM[3]	Con vs. T	L	Q
Glucose $_{0\text{-}110\ min}$, mg/dL	133	126	127	7.5	0.17	0.24	0.32
Basal, mg/dL	72.0	69.1	67.8	3.39	0.36	0.32	0.80
Peak, mg/dL	366	330	335	56.4	0.59	0.64	0.71
CR[4], %/min	3.97	4.10	3.78	1.288	0.98	0.89	0.84
$T_{1/2}$[5], min	18.8	19.7	21.5	3.95	0.68	0.58	0.91
T_{basal}, min	181	202	202	29.9	0.65	0.68	0.82
AUC[6], mg/dL × min	4342	4078	4194	531.5	0.76	0.84	0.75
Time after glucose administration, min							
5	366	330	335	56.4	0.66	0.86	0.50
10	187	174	182	5.8	0.26	0.52	0.18
15	157	162	163	7.1	0.59	0.60	0.81
20	156	142	147	7.1	0.21	0.36	0.26
30	128	118	123	8.1	0.40	0.58	0.38
40	105	101	99.7	5.54	0.50	0.51	0.06
50	88.7	81.4	86.5	5.25	0.27	0.63	0.13
65	72.2	68.1	71.8	3.88	0.52	0.92	0.23
80	65.7	65.7	66.9	4.94	0.99	0.99	0.95
110	72.1	64.0	63.3	4.19	0.07	0.08	0.31

[1]Control, 0 mg/d rumen-protected *Capsicum* oleoresin; C100, 100 mg/d rumen-protected *Capsicum* oleoresin; C200, 200 mg/d rumen-protected *Capsicum* oleoresin.
[2]Con vs. T, control vs. treatment; L, linear effect of rumen-protected *Capsicum* oleoresin; Q, quadratic effect of rumen-protected *Capsicum* oleoresin.
[3]Highest SEM shown; n = 249 for glucose $_{0\text{-}110min}$, n = 23 for all other variables. (n represents number of observations used in the statistical analysis).
[4]Clearance rate.
[5]Half-life.
[6]Area under the curve.

Table 5-8. Effect of rumen-protected *Capsicum* oleoresin on serum insulin response to glucose tolerance test in dairy cows

	Treatment[1]				*P*-value[2]		
Item	Control	C100	C200	SEM[3]	Con vs. T	L	Q
Insulin $_{0\text{-}110\ min}$, μIU/ml	32.9	21.4	24.5	5.08	0.16	0.26	0.26
Insulin							
Basal, μIU/ml	8.75	5.95	5.81	1.81	0.52	0.50	0.84
Peak, μIU/ml	77.6	56.7	59.6	14.63	0.07	0.11	0.14
CR[4], %/min	10.2	18.3	14.1	3.32	0.25	0.52	0.19
$T_{1/2}$[5], min	8.36	5.43	7.43	1.581	0.38	0.70	0.24
T_{basal}, min	71.9	45.5	59.1	17.65	0.11	0.21	0.14
AUC[6], μIU/ml × min	1762	1249	1389	252.6	0.04	0.07	0.11
Time after glucose administration, min							
5	70.6	50.6	57.7	12.11	0.04	0.12	0.04
10	73.3	51.3	57.0	14.50	0.04	0.07	0.06
15	58.3	41.6	47.7	13.6	0.39	0.70	0.24
20	39.2	38.0	36.7	11.21	0.15	0.10	0.69
30	33.0	21.1	22.0	4.35	0.07	0.10	0.18
40	25.9	13.3	15.3	3.17	0.03	0.05	0.08
50	16.5	9.16	9.85	2.834	0.08	0.18	0.11
65	8.89	6.40	7.67	2.548	0.54	0.79	0.38
80	8.12	7.10	6.16	1.174	0.23	0.16	0.97
110	8.21	7.33	6.41	0.730	0.18	0.12	0.98

[1]Control, 0 mg/d rumen-protected *Capsicum* oleoresin; C100, 100 mg/d rumen-protected *Capsicum* oleoresin; C200, 200 mg/d rumen-protected *Capsicum* oleoresin.

[2]Con vs. T, control vs. treatment; L, linear effect of rumen-protected *Capsicum* oleoresin; Q, quadratic effect of rumen-protected *Capsicum* oleoresin.

[3]Highest SEM shown; n = 249 for insulin $_{0\text{-}110min}$, n = 23 for all other variables. (n represents number of observations used in the statistical analysis).

[4]Clearance rate.

[5]Half-life.

[6]Area under the curve.

Table 5-9. Effect of rumen-protected *Capsicum* oleoresin on serum leptin response to glucose tolerance test in dairy cows

	Treatment[1]				*P*-value[2]		
Item	Control	C100	C200	SEM[3]	Con vs. T	L	Q
Leptin $_{0\text{-}110\ min}$, ng/ml[4]	5.31	5.53	4.81	0.675	0.56	0.08	0.04
AUC[4], ng/ml × min	159	126	148	26.7	0.57	0.81	0.44
Time after glucose administration, min							
0	4.27	4.93	4.33	0.903	0.65	0.94	0.40
5	4.30	4.36	4.00	0.788	0.81	0.59	0.64
10	4.21	4.15	4.28	0.688	1.00	0.91	0.84
15	5.48	5.52	4.88	0.926	0.60	0.35	0.49
20	6.10	5.65	5.21	0.842	0.25	0.18	0.99
30	6.47	5.92	5.67	0.933	0.32	0.29	0.79
40	6.16	6.04	5.01	0.625	0.10	0.03	0.15
50	5.86	6.52	5.71	0.723	0.45	0.68	0.07
65	6.07	6.20	5.87	0.744	0.96	0.78	0.69
80	5.79	6.34	5.89	0.957	0.63	0.89	0.42
110	5.23	4.90	4.45	0.870	0.63	0.54	0.95

[1]Control, 0 mg/d rumen-protected *Capsicum* oleoresin; C100, 100 mg/d rumen-protected *Capsicum* oleoresin; C200, 200 mg/d rumen-protected *Capsicum* oleoresin.

[2]Con vs. T, control vs. treatment; L, linear effect of rumen-protected *Capsicum* oleoresin; Q, quadratic effect of rumen-protected *Capsicum* oleoresin.

[3]Highest SEM shown; n = 216 for leptin $_{0\text{-}110min}$, n = 20 for all other variables. (n represents number of observations used in the statistical analysis).

[4]Period × Treatment, $P < 0.01$

[5]Area under the curve.

Table 5-10. Effect of rumen-protected *Capsicum* oleoresin on DMI[1], milk production[1], white blood cells and TBARS[2] after lipopolysacchride challenge in dairy cows[3]

	Treatment[4]				*P*-value[5]		
Item	Control	C100	C200	SEM[6]	Con vs. T	L	Q
DM intake, kg/d	25.3	26.2	25.5	1.73	0.77	0.92	0.70
Milk yield, kg/d	34.0	37.4	35.8	2.44	0.39	0.60	0.40
Feed efficiency[7], kg/kg	1.44	1.47	1.56	0.127	0.60	0.45	0.79
White blood cells, $10^3/\mu L$	5.68	5.55	5.23	0.503	0.61	0.48	0.85
Neutrophils	3.32	3.10	2.83	0.463	0.50	0.41	0.95
Lymphocytes	1.93	2.19	2.14	0.309	0.21	0.31	0.38
Monocytes	0.14	0.12	0.12	0.036	0.72	0.71	0.93
Eosinophils	0.29	0.23	0.21	0.060	0.20	0.18	0.75
Neutrophils:lymphocytes	1.68	1.54	1.35	0.380	0.46	0.37	0.95
TBARS, μM	5.96	7.21	6.79	0.527	0.07	0.19	0.13

[1]Data were collected for 5 days after lipopolysacchride challenge
[2]Thiobarbituric acid reactive substances
[3]Analyzed as repeated measure
[4]Control, 0 mg/d rumen-protected *Capsicum* oleoresin; C100, 100 mg/d rumen-protected *Capsicum* oleoresin; C200, 200 mg/d rumen-protected *Capsicum* oleoresin.
[5]Con vs. T, control vs. treatment; L, linear effect of rumen-protected *Capsicum* oleoresin; Q, quadratic effect of rumen-protected *Capsicum* oleoresin.
[6]Highest SEM shown; n = 114 for DMI, n = 109 for milk yield and TBARS, n = 108 for feed efficiency and white blood cells. (n represents number of observations used in the statistical analysis).
[7]Milk yield÷DM intake

Table 5-11. Effect of rumen-protected *Capsicum* oleoresin on serum cortisol and haptoglobin response to lipopolysacchride challenge in dairy cows

	Treatment[1]				*P*-value[2]		
Item	Control	C100	C200	SEM[3]	Con vs. T	L	Q
Cortisol $_{0\text{-}24\ h}$, μg/dL	1.50	1.03	1.27	0.255	0.22	0.48	0.19
Cortisol $_{2\text{-}4\ h}$, μg/dL	4.23	2.98	3.49	0.520	0.03	0.13	0.04
Haptoglobin $_{0\text{-}24\ h}$, mg/mL	0.57	0.29	0.25	0.118	0.04	0.06	0.40

[1]Control, 0 mg/d rumen-protected *Capsicum* oleoresin; C100, 100 mg/d rumen-protected *Capsicum* oleoresin; C200, 200 mg/d rumen-protected *Capsicum* oleoresin.

[2]Con vs. T, control vs. treatment; L, linear effect of rumen-protected *Capsicum* oleoresin; Q, quadratic effect of rumen-protected *Capsicum* oleoresin.

[3]Highest SEM shown; n=120 for Cortisol $_{0\text{-}24\ h}$, n=46 for Cortisol $_{2\text{-}4\ h}$, and n=114 for Haptoglobin $_{0\text{-}24\ h}$. (n represents number of observations used in the statistical analysis).

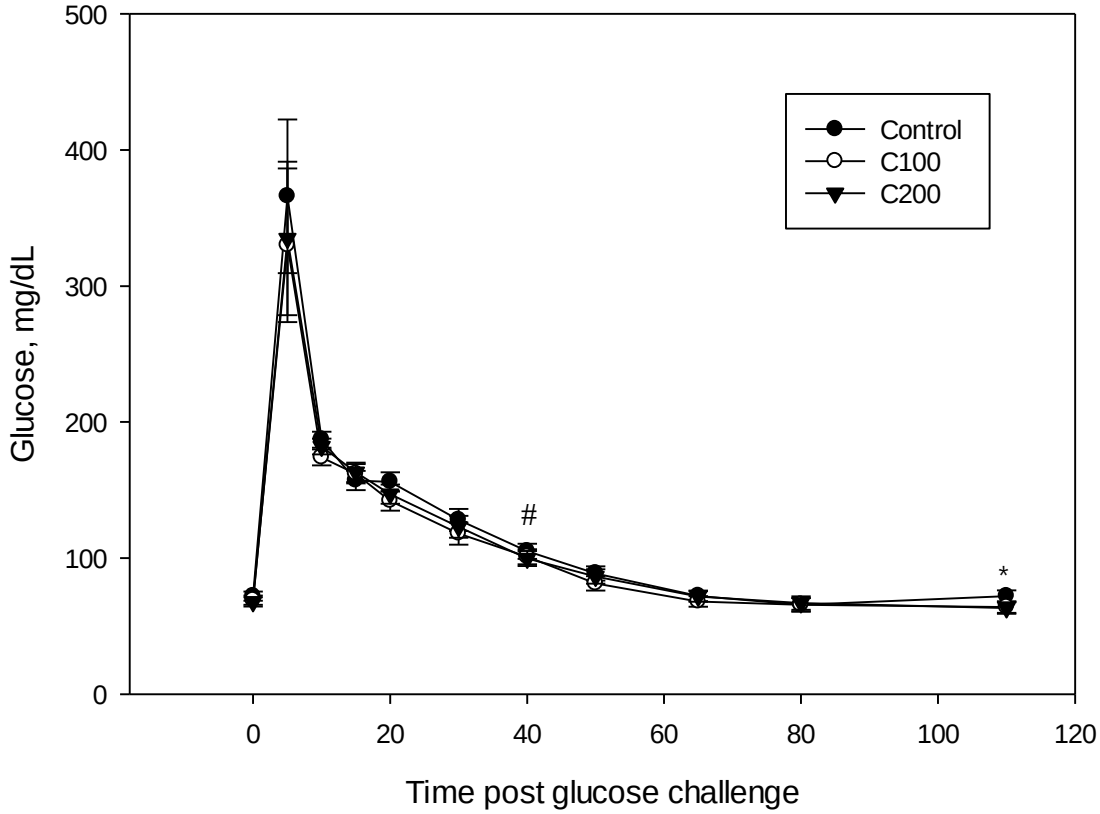

Figure 5-1. Effect of rumen-protected *Capsicum* oleoresin (RPC) on serum glucose concentration at 0, 5, 10, 15, 20, 30, 40, 50, 65, 80, and 110 after intravenous administration of glucose. Control, 0 mg/d RPC; C100, 100 mg/d RPC; C200, 200 mg/d RPC. Orthogonal contrast between control and treatments (n = 23; n represents number of observations used in the statistical analysis), ** $P \leq 0.05$, * $0.05 < P \leq 0.10$ in control vs treatment; †† $P \leq 0.05$, † $0.05 < P \leq 0.10$ linear effect; and ## $P \leq 0.05$, # $0.05 < P \leq 0.10$ quadratic effect.

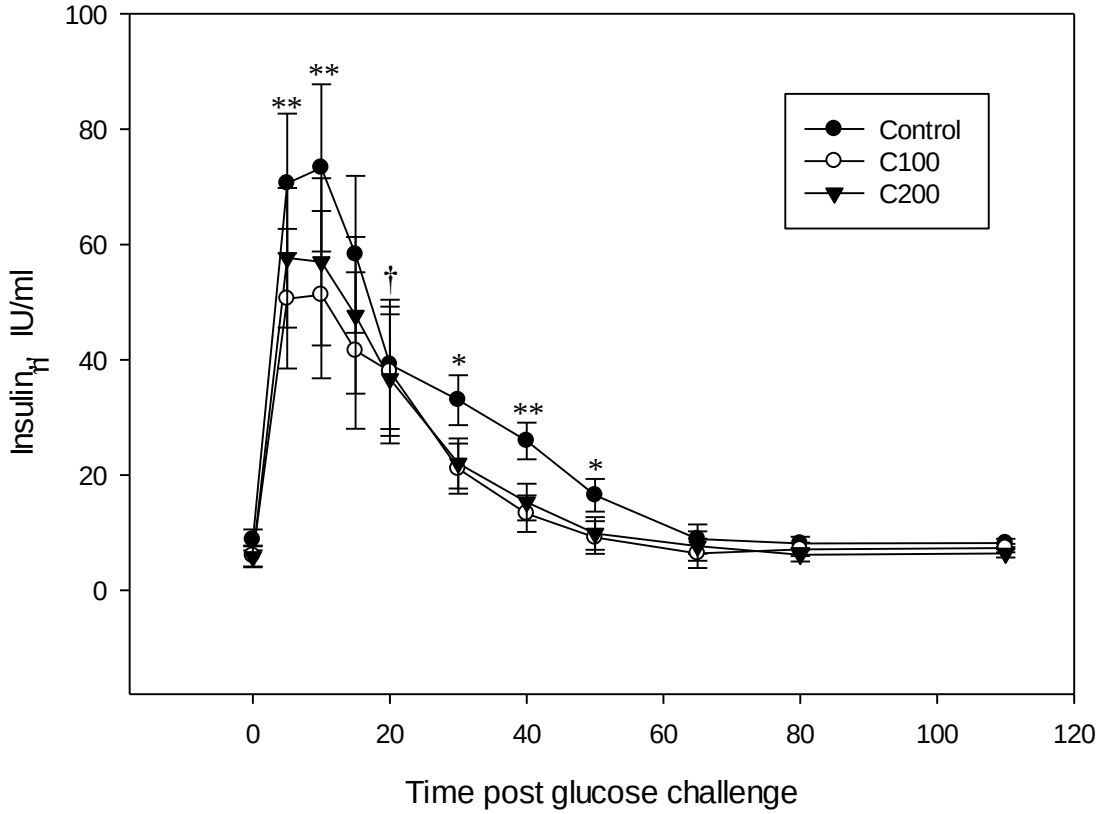

Figure 5-2. Effect of rumen-protected *Capsicum* oleoresin (RPC) on serum insulin concentration at 0, 5, 10, 15, 20, 30, 40, 50, 65, 80, and 110 after intravenous administration of glucose. Control, 0 mg/d RPC; C100, 100 mg/d RPC; C200, 200 mg/d RPC. Orthogonal contrast between control and treatments (n = 23; n represents number of observations used in the statistical analysis), ** $P \leq 0.05$, * $0.05 < P \leq 0.10$ in control vs treatment; †† $P \leq 0.05$, † $0.05 < P \leq 0.10$ linear effect; and ## $P \leq 0.05$, # $0.05 < P \leq 0.10$ quadratic effect.

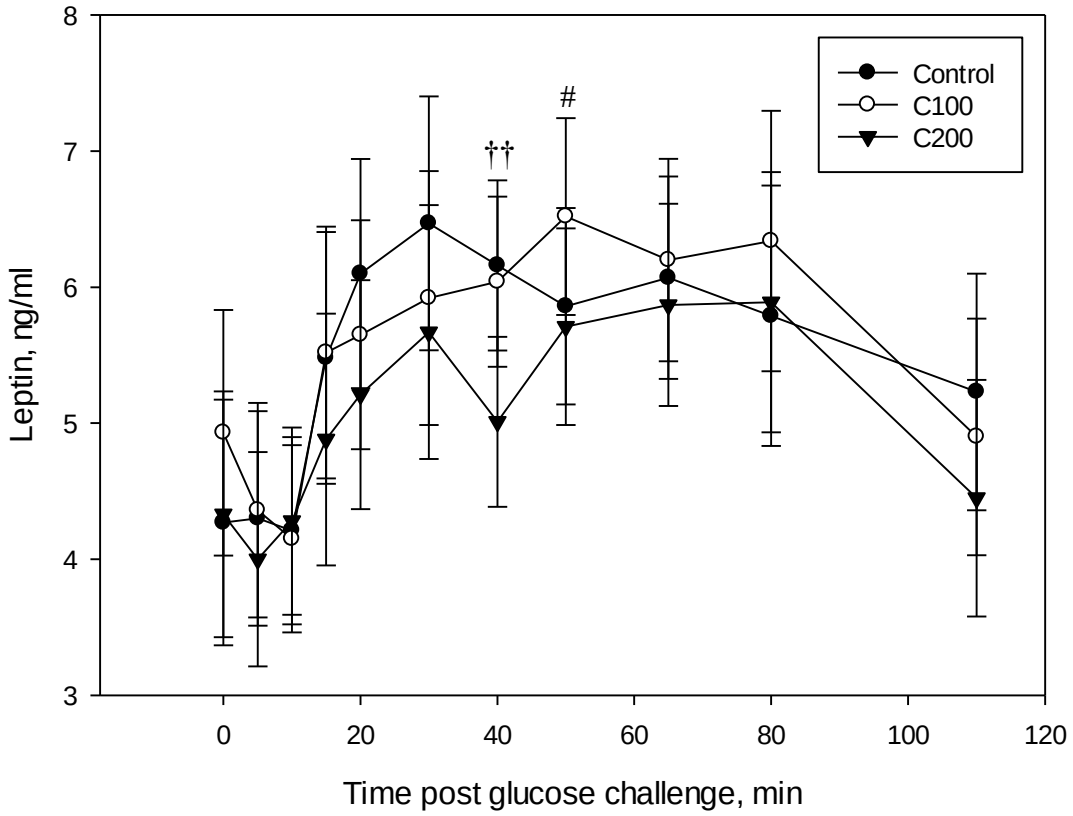

Figure 5-3. Effect of rumen-protected *Capsicum* oleoresin (RPC) on serum leptin concentration at 0, 5, 10, 15, 20, 30, 40, 50, 65, 80, and 110 after intravenous administration of glucose. Control, 0 mg/d RPC; C100, 100 mg/d RPC; C200, 200 mg/d RPC. Orthogonal contrast between control and treatments (n = 20; n represents number of observations used in the statistical analysis), ** $P \leq 0.05$, * $0.05 < P \leq 0.10$ in control vs treatment; †† $P \leq 0.05$, † $0.05 < P \leq 0.10$ linear effect; and ## $P \leq 0.05$, # $0.05 < P \leq 0.10$ quadratic effect.

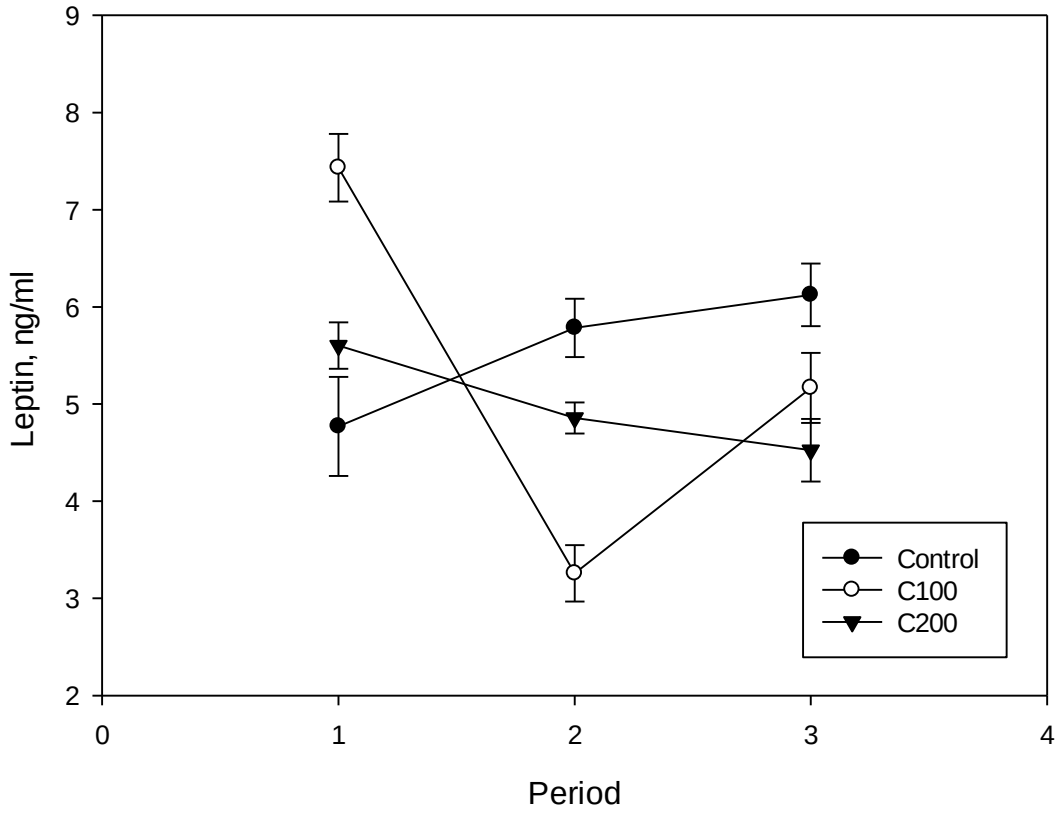

Figure 5-4. Average plasma leptin concentration by periods. A period × treatment interaction ($P < 0.01$) was observed in the leptin data.

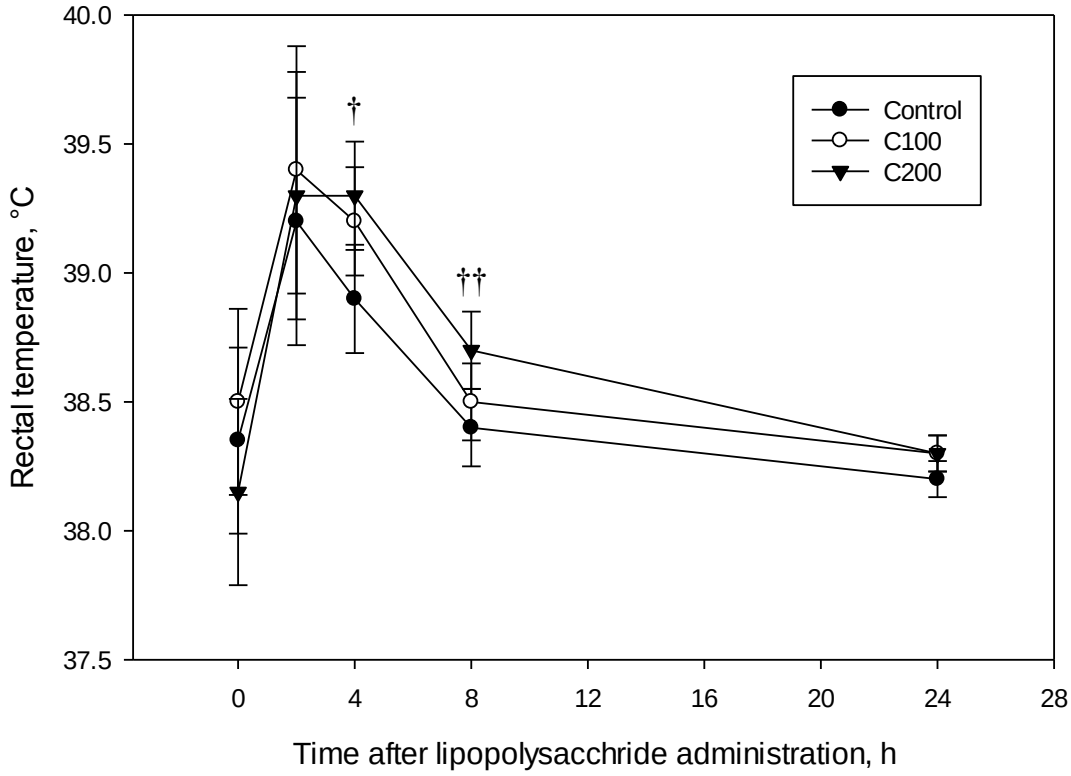

Figure 5-5. Effect of rumen-protected *Capsicum* oleoresin (RPC) on rectal temperature at 0, 2, 4, 8, and 24 h after intravenous administration of lipopolysaccharide. Control, 0 mg/d RPC; C100, 100 mg/d RPC; C200, 200 mg/d RPC. Orthogonal contrast between control and treatments (n = 20; n represents number of observations used in the statistical analysis), ** $P \leq 0.05$, * $0.05 < P \leq 0.10$ in control vs treatment; †† $P \leq 0.05$, † $0.05 < P \leq 0.10$ linear effect; and ## $P \leq 0.05$, # $0.05 < P \leq 0.10$ quadratic effect.

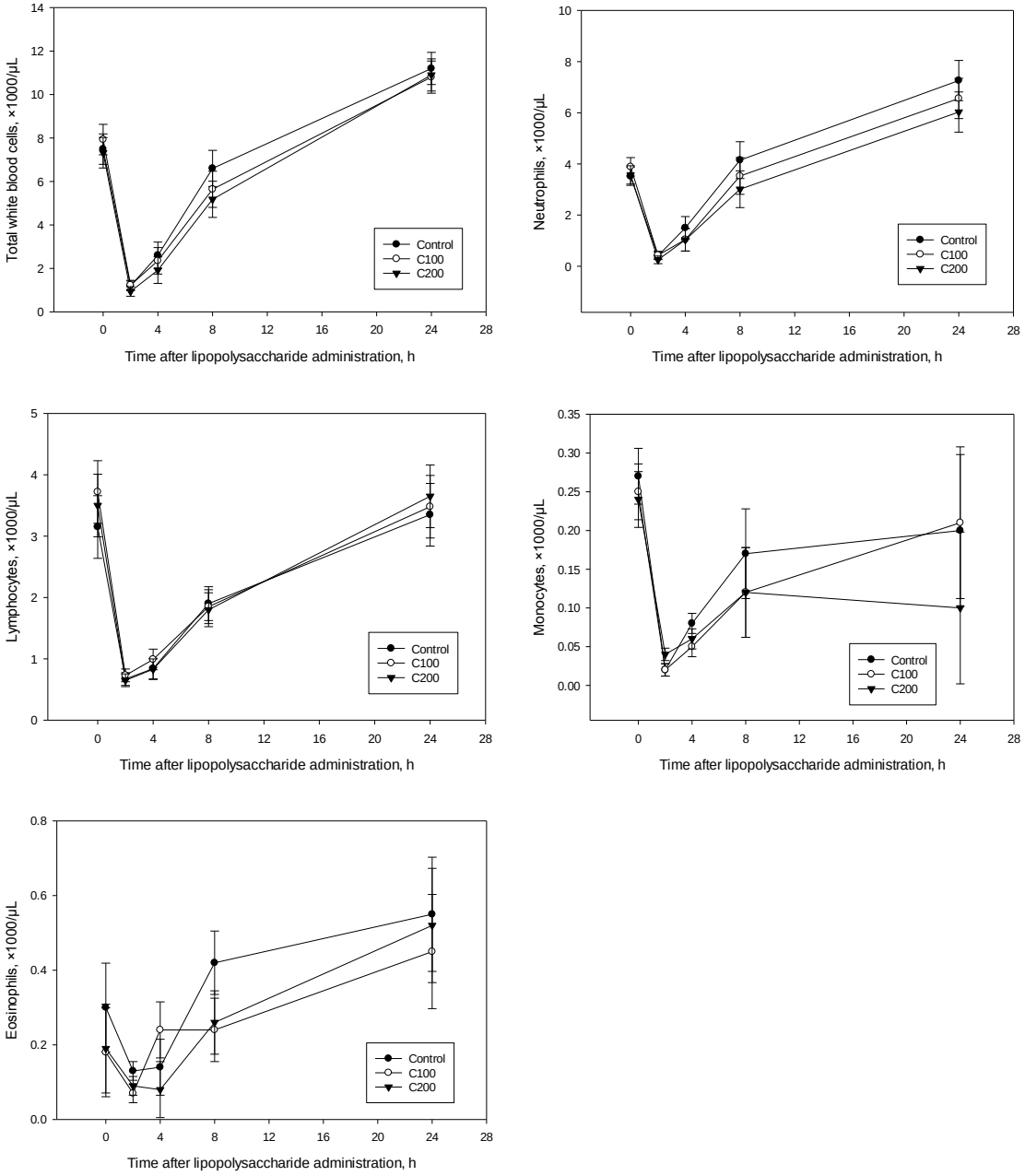

Figure 5-6. Effect of rumen-protected *Capsicum* oleoresin (RPC) on white blood cells at 0, 2, 4, 8, and 24 h after intravenous administration of lipopolysaccharide. Control, 0 mg/d RPC; C100, 100 mg/d RPC; C200, 200 mg/d RPC. A, total white blood cells; B, neutrophils; C, lymphocytes; D, monocytes; E, Eosinophils. Orthogonal contrast between control and treatments (n = 20; n represents number of observations used in the statistical analysis), ** $P \leq 0.05$, * $0.05 < P \leq 0.10$ in control vs treatment; †† $P \leq 0.05$, † $0.05 < P \leq 0.10$ linear effect; and ## $P \leq 0.05$, # $0.05 < P \leq 0.10$ quadratic effect.

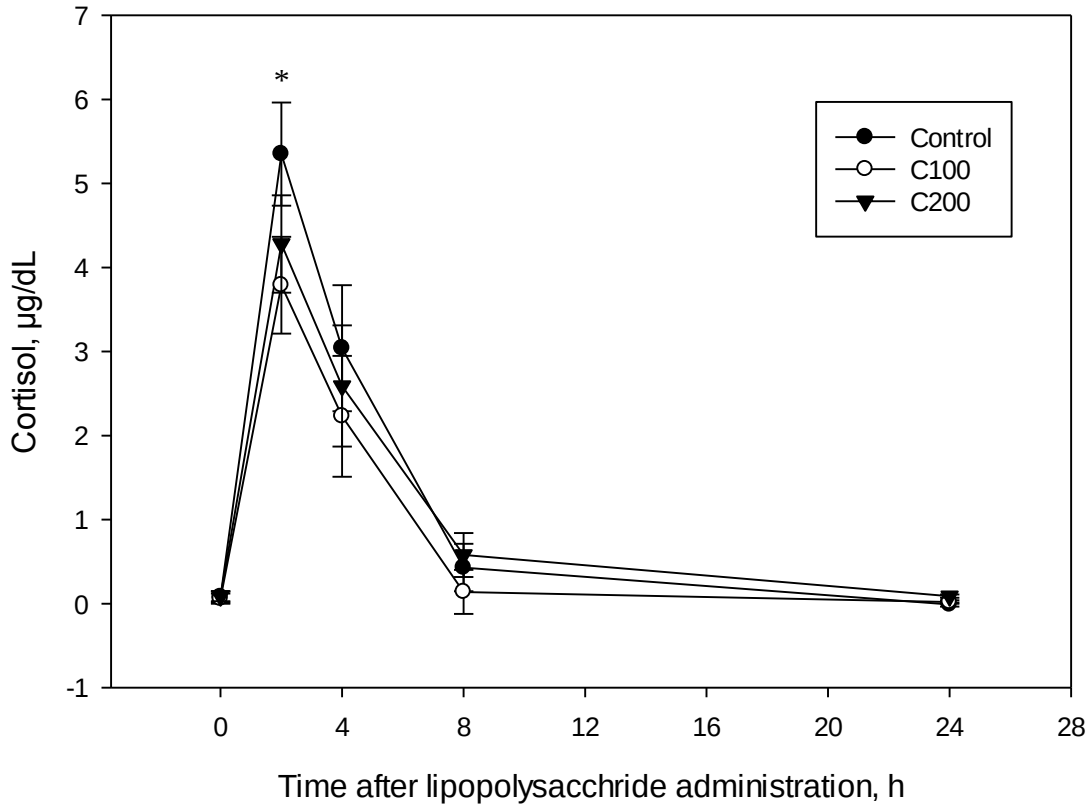

Figure 5-7. Effect of rumen-protected Capsicum oleoresin (RPC) on serum cortisol concentration at 0, 2, 4, 8, and 24 h after intravenous administration of lipopolysaccharide. Control, 0 mg/d RPC; C100, 100 mg/d RPC; C200, 200 mg/d RPC. Orthogonal contrast between control and treatments (n = 20; n represents number of observations used in the statistical analysis), ** $P \leq 0.05$, * $0.05 < P \leq 0.10$ in control vs treatment; †† $P \leq 0.05$, † $0.05 < P \leq 0.10$ linear effect; and ## $P \leq 0.05$, # $0.05 < P \leq 0.10$ quadratic effect.

Chapter 6

Conclusions and Future study

Effects of *Capsicum* oleroresin in lactating dairy cows were investigated on feed intake, milk production, immune responses, blood metabolites, and hormones in a series of experiments. *Capsicum* oleoresin treatment did not affect nutrient digestibility, fecal bacterial diversity, and antioxidant status when administerted postruminally in the first experiment with lactating dairy cows. Milk yield was also decreased during the treatment period. We concluded that a high dose of *Capsicum* oleoresin (2 g/d) may have negative effects on productivity of dairy cows. Data from this experiment, however, suggested that *Capsicum* oleoresin might have an immunostimulatory effect by activating and inducing the expansion of $CD4^+$ cells. In a follow-up experiment, dietary supplementation of unprotected *Capsicum* oleresin (at 250 to 1,000 mg/d) was tested to investigate both ruminal and post-ruminal effects of *Capsicum* in dairy cows. Treatments increased energy-corrected milk yield, beta-hydroxybutyrate, and neutrophil numbers and activity. In that experiment, *Capsicum* had a modulatory effect on certain genera of bacteria, although it did not affect feed intake, rumen fermentation, digestibility, and the antioxidant status of the cows. The conclusion from this experiment was that dietary supplementation of unprotected *Capsicum* oleresin had a positive effect, perhaps through enhanced mobilization of body fat reserves, on the energy balance of the cows, resulting in increased energy-corrected milk yield. *Capsicum* oleoresin also facilitated neutrophil

activity and immune cells related to the acute phase response. In a third experiment, we explored the possibility of delivering *Capsicum* post-ruminally thus triggering physiological, rather than ruminal effects. In addition, we immunologically challenged dairy cows to investigate the effect of *Capsicum* on acute phase responses. In that experiment, rumen-protected *Capsicum* tended to increase feed intake and milk production and increased feed efficiency and total tract digestibility of nutrients of the cows without any challenge. Furthermore, insulin sensitivity during a glucose tolerance test was increased by rumen-protected *Capsicum* supplementations. After an immune challenge, serum cortisol and haptoglobin levels were decreased by the treatments. Data suggested that *Capsicum* might increase productivity and insulin activity and alleviate acute phase responses. Collectively, our data in this dissertation indicate that *Capsicum* oleoresin may have a positive effect on milk yield in dairy cows. In addition, we observed positive, but subtle, effects of *Capsicum* on feed intake, rumen microbial species distribution, and immune responses with or without an immune challenge. Interestingly, insulin sensitivity was increased by *Capsicum* treatment during a glucose challenge.

High producing dairy cows are susceptible to metabolic diseases during the periparturient period. Increased energy requirements to support the onset of copious milk production postpartum causes a negative energy balance, leading to metabolic disorders such as immunosuppression and ketosis. It is also known that insulin resistance is observed in transition cows. The stimulatory effects of *Capsicum* on feed intake, milk production, immune responses, and insulin sensitivity observed in this dissertation

should be tested in the periparturient cows. Average days in milk (DIM) of cows used in this dissertation was 175 ± 19.8, 50 ± 9.6, 100 ± 9.1 d for Chapter 3, 4, and 5, respectively, and there was no immunosuppression in the cows unless they were challenged by an immune stimulus. An experimental design is suggested to be a randomized block design with cows from -30 to 30 DIM in which cows commonly experience immnosuppression. Rumen protected *Capsicum* oleoresin can be used in a range from 100 to 500 mg, estimated from previous experiments. The effects of *Capsicum* are investigated on feed intake, glucose, insulin, BHBA, NEFA, milk production after calving, immune parameters including leukocytes and acute phase proteins.

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PEER REVIEWED PUBLICATIONS

Hristov, A. N., **J. Oh**, F. Giallongo, T. Frederick, M. Harper, H. Weeks, A. Branco, P. Moate, M. Deighton, R. Williams, M. Kindermann, and S. Duval. 2015. A novel inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. PNAS. (in press)

Hristov, A. N., **J. Oh**, F. Giallongo, T. Frederick, H. Weeks, P. R. Zimmerman, R. A. Hristova, S. R. Zimmerman, and A. F. Branco. 2015. The use of an automated system to monitor enteric methane and carbon dioxide emissions from ruminant animals. J. Visualized Experiments (in press).

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