PHARMACOLOGICAL AND DIETARY METHODS TO INFLUENCE THE INFLAMMATORY STATE OF DAIRY CATTLE

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ABSTRACT

The replacement rate of dairy cattle is, on average, 35%, while cattle will leave the herd for reasons other than health events; this rate is not sustainable. Replacement rate can be defined as the percentage of lactating cows that leave the herd and are replaced by new primiparous cows. Societal pressure has led to the implementation of a veterinary feed directive (VFD), established in 2017 to restrict supplementing antibiotics in feed or water to livestock without written consent from a veterinarian. Then in 2023 further legislation was introduced requiring veterinarian consent to administer any prescription drugs. While this had little impact on the dairy industry when first implemented, the aim of the VFD is to lessen the likelihood of further microbial antibiotic resistance in livestock production. To help improve the resilience of cows to pathogens, research has been done to investigate naturally occurring compounds that may help decrease the need for antibiotic use. β-Caryophyllene (BCP) has been demonstrated to possess anti-inflammatory properties. In this experiment, 20 mid or late lactation dairy cows, enrolled using a randomized complete block design, received two lipopolysaccharide (LPS) intramammary challenges 28 days apart and received 1 of 2 treatments for 42 days: 1) CON (basal TMR); 2) BCP, consisting of 5 mg/kg of body weight, as liquid BCP delivered as a top dress treatment and mixed in with the top 20% of the ration. No changes in dry matter intake, body weight, milk yield or components were observed between treatment groups. BCP supplementation resulted in a lower haptoglobin concentration during the first LPS challenge and a lower somatic cell count (SCC) prior to the second LPS challenge. Plasma haptoglobin concentrations were lower in the second LPS challenge and a higher peak in SCC was observed in BCP cows. I conclude that supplementation of BCP during a LPS intramammary challenge lessens the systemic inflammatory response experienced by late lactation dairy cows.

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CHAPTER 1:

REVIEW OF THE LITERATURE

Introduction

In the U.S. dairy industry, there is an average lactating cow replacement rate of 35% (Overton and Dhuyvetter, 2020). Replacement rate can be defined as the percentage of lactating cows that leave the herd and are replaced by new primiparous cows. Often cows are replaced for reasons other than health events, but regardless of the cause, this replacement rate is far from optimal in terms of economic, social, or environmental sustainability (Murat Tatar et al., 2017). Mastitis and metritis are common infections experienced by dairy cattle and can often require antibiotic treatment (Cheng and Han, 2020). Due to societal pressure, a veterinary feed directive (VFD) was established in 2017 to restrict supplementing antibiotics in feed or water to livestock without written consent from a veterinarian, then further legislation was passed in 2023 to require a veterinarian prescription for any antibiotics. While this had little impact on the dairy industry when first implemented, the aim of the VFD is to lessen the likelihood of further microbial antibiotic resistance in livestock production. To help improve the resilience of cows to pathogens, research has been done to investigate naturally occurring compounds that may help decrease the need for antibiotic use. This review of the literature will examine the mechanisms of inflammation and common pharmaceutical and dietary methods to modulate the inflammatory state of the dairy cow.

Inflammation during an immunological challenge

Inflammation is part of the innate immune system that is commonly associated with localized heat, swelling, pain, and a reduction in appetite (Vane and Botting, 1987). Inflammation consists of three phases: alarm, mobilization, and resolution. The acute inflammatory response is activated when pattern recognition receptors (PRR) detect either pathogen-associated molecular patterns (PAMP) or damage-associated molecular patterns

(DAMP). PAMP consist of microbial nucleic acids, lipoproteins, and carbohydrates (Mogensen, 2009). PRR are found on both tissue-resident immune cells like macrophages, mast cells, and dendritic cells, as well as on leukocytes and many non-immune cells. Upon PRR activation, there is an increase in expression of pro-inflammatory mediators, including cytokines and chemokines, aiding in the recruitment of leukocytes and production of complement proteins, adhesion molecules, histidine, and eicosanoids (Newton and Dixit, 2012). Transcription factors such as nuclear factor kappa B (NFκB) and activator protein-1 (AP-1) are found in the cytoplasm of cells and are activated thorough extracellular signaling (Adcock and Caramori, 2009). These transcription factors coordinate the activation of many genes, including proinflammatory cytokines (Schottelius et al., 1999; Hoffmann and Baltimore, 2006). Depending on the pathogen load, NFκB and/or AP-1 will trigger positive or negative feedback, thus allowing the body to mount an appropriate response to the pathogen load (Adcock and Caramori, 2009; Sung et al., 2014). Inflammatory cytokines include tumor necrosis factor α (TNFα), interleukin (IL)-1 and IL-6 (Takeuchi and Akira, 2010). Histidine and adhesion molecules work together with the increased blood flow to allow for neutrophils to enter the site of irritation. This passage of neutrophils across the vascular epithelium is the cause for heat, swelling, and pain associated with inflammation (Sordillo, 2018). If the acute response is adequate, the body switches from producing prostaglandins (pro-inflammatory mediators) to lipoxins that aid in the recruitment of macrophages and begin tissue repair (Medzhitov, 2008). If the initial inflammatory response is unable to resolve the irritation, the neutrophil cascade is accompanied by macrophages and T-cells (Medzhitov, 2008). Once the body has successfully eliminated the pathogen, it will switch from pro-inflammatory to resolving signaling by producing cytokines such as IL-4, IL-10, IL-11 and IL-13. Interleukins IL-4 and IL-10 aid in the attenuation of inflammatory transcription

factors (Schottelius et al., 1999; Iribarren et al., 2003). If the body cannot eliminate the pathogen, it is possible that granulomas and tertiary lymphoid tissues can form (Medzhitov, 2008). The inflammatory response is the first step in a immune response and may either be isolated and resolved or may require other branches of the immune system to intervene to help clear the pathogen.

Oxidative status

Oxidants consist of substances able to oxidize other molecules (Abuelo et al., 2015). Free radicals are atoms or molecules that have one or more unpaired electrons in their outer electron shell, and are oxidizing agents (Sordillo and Aitken, 2009). Reactive oxygen species (ROS) are a class of free radicals that are produced from superoxide anions as a byproduct of cellular metabolism, and produced by activated neutrophils and macrophages (Sordillo and Aitken, 2009; Abuelo et al., 2015). These superoxide anions can interact with other molecules to generate various ROS (Sordillo and Aitken, 2009). ROS aid in the host immune response by promoting the expression of cytokines and eicosanoids, as well as through the destruction of phagocyted pathogens (Abuelo et al., 2015). Antioxidants may be synthesized within the host, or may be consumed in the diet, and are generally described as any substance that prevents, delays, or removes oxidative damage (Sordillo and Aitken, 2009). Examples of antioxidants include vitamin E and selenium (Miller et al., 1993; Finch and Turner, 1996). Antioxidants and ROS work together to maintain the oxidative status of the host. However, when abundant ROS are produced and there are insufficient antioxidants present, the condition called oxidative stress develops (Abuelo et al., 2015). Oxidative stress can cause tissue damage and deteriorate cell membrane integrity and may even result in cell death (Abuelo et al., 2015; Bradford et al., 2015). Oxidants in themselves are harmless, but if the oxidative status shifts towards a high

concentration of ROS damage can occur to the host. In order to balance the oxidative status of cattle, it is important to limit ROS production, and or provide sufficient antioxidants to offset ROS production.

Inflammation during the transition period

The transition period poses an interesting challenge to both dairy producers and nutritionists, leading to it being studied extensively. During the last week preceding parturition, the dairy cow begins to back off feed intake while initiating colostrogenesis. Parturition is associated with tissue damage, and an immediate increase in energy demands due to lactogenesis (Bradford et al., 2015). The combination of already expressing feed adverse behavior and new tissue damage following parturition provides the perfect storm for other insults to the immune system. This could explain why this period often accounts for most illness events on a dairy farm, especially for mastitis and metritis (Barragan et al., 2021). This predicament has led to the search for methods to influence the inflammatory state during this period so that the cow may return to consuming adequate feed more quickly than a cow with no intervention. Studies have shown mixed results when attempting to alter the inflammatory state during the transition period; some show an increase in milk yield (Bertoni et al., 2004; Barragan et al., 2021). Others observed an increase in the rate of dystocia when flunixin was administered pre-calving and an increase in retained placentas when administered post-calving (Newby et al., 2017). While inflammation during the transition period is responsible for most illness events on a dairy, influencing the inflammatory state is more complex than just reducing inflammation around calving.

Mastitis

Mastitis, which consists of inflammation of the mammary gland, results in roughly 2 billion dollars lost annually in the U.S dairy industry (Cha et al., 2011). Mastitis is either contagious or environmental (coliform) but both result in lower fluid milk production, milk component concentrations and an increase of milk immunoglobulins due to increased permeability of the blood-milk barrier, and may cause tissue damage (Ogala et al., 2007). Mastitis cases can be clinical or sub-clinical. A clinical case of mastitis results in the clinical signs of inflammation in the udder accompanied by watery milk with visible flakes and clots, whereas sub-clinical mastitis demonstrates no visible signs of udder inflammation, but a decrease in milk production and increase in the somatic cell count (SCC) (Cheng and Han, 2020). Symptoms experienced during clinical mastitis may be local, with only heat and redness in the infected quarter, or may be more severe, causing systemic fever and a reduction in feed intake (Cheng and Han, 2020).

Coliform bacteria are able to survive in near anaerobic conditions and are able to utilize lactose as a fuel source, allowing them to proliferate within the mammary gland (Hogan and Smith, 2003). The low oxygen concentration in the mammary gland can inhibit production of ROS like hydrogen peroxide, used by neutrophils to help kill/breakdown pathogens, thus compromising the host's immune defense (Sordillo and Streicher, 2002). Contagious bacteria may originate from the bedding area or on milk machines as a result of poor milking hygiene (Smith et al., 1985). Once a cow is infected with contagious bacteria, the udder becomes the major reservoir and the bacteria is primarily spread between cattle while milking; these pathogens tend to result in chronic sub-clinical infections with occasional flares of clinical cases (Abebe et al., 2016). Factors such as age, breed, and udder structure can increase a cow's

susceptibility to mastitis and paired with reduced immune function due to low oxygen concentrations, the mammary gland may be unable to clear the infection on its own, thus requiring antibiotic intervention (Cheng and Han, 2020). Mastitis, regardless of type, results in profit loss to the producer through lowered milk production and sometimes treatment costs. Thus, management practices to limit the spread of environmental infections or other anti-infection interventions should be used to help limit milk withdrawal times to help minimize loss of profit by producers.

Methods to modulate the inflammatory state in dairy cattle

Inflammation is commonly associated with illness, but it is not inherently bad for the host. Often, inflammation is necessary, like during parturition or when fighting off infection (Medzhitov, 2008; Bradford et al., 2015). By inhibiting specific enzymes or pathways using anti-inflammatory drugs, not only are the clinical signs of inflammation lessened, but the response to pathogens can also be delayed. It has been demonstrated that inhibiting key functions of inflammation pre-partum can result in increased stillbirth rate as well as an increased odds of clinical signs of inflammation, metritis, retained placenta, and a decrease in milk production (Newby et al., 2017). As different methods to modulate inflammation are discussed in this chapter, it is important to keep in mind what the potential adverse effects of blindly blocking pathways may be.

Trained immunity. One method for immunomodulation is innate memory, where innate immune cells demonstrate either an enhanced or suppressed response to a repeated immune challenge (Byrne et al., 2020). Innate immunity can be thought of as the instant and non-specific response arm of the immune system, which has traditionally been considered to be incapable of developing an immunological memory. Adaptive immunity, in contrast, is able develop a

memory response to pathogens, allowing for a more targeted and heightened immune response. This greater immune response is achieved through the production of memory B and T cells that allow for long-term protection against the same pathogen (Chaplin, 2010). Trained immunity works as a protection against reinfection independent of memory B and T cells. Instead, trained immunity works through other cells, such as natural killer cells, and is achieved through epigenetics (altering gene expression without DNA sequence modulation) and metabolic programming (Byrne et al., 2020). Adaptive immunity can be distinguished from trained immunity by the pathogen specificity and duration of immunity. Adaptive immunity is highly specific to the pathogen and provides long-term immunity (years-lifetime); conversely, trained immunity is often non-specific and provides short term altered immunity (Netea, 2013). LPS is one of the best-known innate priming agonists able to alter the immune system, and has been demonstrated to alter the immune system response to repeated challenges in a dose-dependent manner (Byrne et al., 2020). When LPS is administered in low doses, tolerance is observed (Yoza and McCall, 2011); however, administration of even lower concentrations results in heightened immune responses (Ifrim et al., 2014). In a bovine mastitis model, repeated LPS challenges with an interval of 14 days resulted in a lower concentration of immune indicators in the plasma as well as milder local signs of inflammation (Suojala et al., 2008). In another study, cattle received an intramammary infusion of LPS and were later challenged with *Escherichia coli*. Cows who had received the LPS infusion demonstrated lesser signs of systemic and local inflammation (Petzl et al., 2012). Other forms of innate priming agonists include β-glucans and bacilli Calmette-Guérin (BCG), a live attenuated vaccine for tuberculosis (Byrne et al., 2020). One study demonstrated that monocytes isolated from calves who received the BCG vaccine produced more pro-inflammatory cytokines when stimulated with LPS and Pam3CSK4,

compared to cells isolated from non-vaccinated calves (Guerra-Maupome et al., 2019). Trained immunity poses as a short-term method to lessen the inflammatory response to non-specific inflammatory markers such as LPS.

Selective and non-selective cyclooxygenase inhibitors. One class of anti-inflammatory drugs are the cyclooxygenase (COX) inhibitors, also commonly known as nonsteroidal anti-inflammatory drugs (NSAID). COX inhibitors are commonly used in the dairy industry and are readily available as over-the-counter (OTC) drugs. The COX enzymes (COX 1 & COX 2) catalyze the rate-limiting step in biosynthesis of many eicosanoids, including prostaglandins (Chandrasekharan and Simmons, 2004). COX-1 is expressed at a relatively constant rate and plays a role in homeostasis, whereas COX-2 expression is associated with inflammation and is stimulated by LPS, IL-1, IL-2, and TNFα (Vane et al., 1998). In the gastrointestinal tract, prostaglandins from COX-1 aid in the production of the mucosal lining. The available COX inhibiting drugs are either selective to COX-1 or COX-2 or nonselective, inhibiting both enzymes. Examples of selective COX inhibitors include carprofen, meloxicam, and tolfenamic acid; non-selective COX inhibitor examples include flunixin and ketoprofen (Breen, 2017). COX-2 inhibition during an immune challenge reduces the fever response, along with mitigation of negative effects on dry matter intake (DMI) during an immune challenge, dependent upon the half-life of the drug administered (Yeiser et al., 2012).

COX inhibitors have been studied as potential tools for resolution of acute coliform mastitis cases. Orally administered non-selective inhibitors such as aspirin, ketoprofen, and flunixin meglumine should be used with caution, as excessive COX-1 inhibition around parturition may increase the risk of retained placenta, culling, or metritis (Trimboli et al., 2020).

COX inhibitors have been shown to lower the SCC in cattle experiencing mastitis (Anderson and Hunt, 1989), reduce body temperature (Morkoç et al., 1993), and improve the recovery from clinical mastitis cases (Shpigel et al., 1994). When considering usage of COX inhibitors, there is a meat and milk withhold time of 24 hours or more (Smith et al., 2008). COX inhibitors may blunt the inflammatory response and its effects on DMI and production. When administering COX inhibitors, selective COX-2 inhibitors should be considered as excessive COX-1 inhibition may have deleterious effects around parturition.

Glucocorticoids and prostaglandins. Glucocorticoids (GC) are a class of anti-inflammatory mediators synthesized in the adrenal cortex. Adrenal GC production is regulated by the hypothalamic-pituitary-adrenal axis (Timmermans et al., 2019). During an immunological challenge, cytokines stimulate the hypothalamus, causing the release of corticotropin-releasing hormone; in turn, corticotropin is released from the anterior pituitary. Corticotropin then acts on the adrenal cortex to trigger synthesis and secretion of cortisol, which is then converted to cortisone (Rhen and Cidlowski, 2005). GC secretion inhibits inflammation via the downregulation of chemokine and cytokine activity, inhibition of eicosanoid production from macrophages, and inhibition of endothelial production of intercellular and vascular adhesion enzymes (Cain and Cidlowski, 2017). GC activate glucocorticoid receptors and can inhibit prostaglandin production through the inhibition of annexin I, MAPK phosphatase 1, and COX-2 (Rhen and Cidlowski, 2005). Inhibition of these pathways ultimately results in anti-inflammatory and analgesic effects.

There are many synthetic GC that are widely available, such as prednisone, prednisolone, and dexamethasone. When treating acute cases of mastitis, administration of GC such as dexamethasone can result in lower body temperatures as well as less milk production loss than

an untreated cow with mastitis (Lohuis et al., 1988). Prednisone may decrease the production of pro-inflammatory cytokines and decrease SCC, depending on the type of infection (Wall et al., 2016). Because treatment with GC may lessen immune responses, it may impair the immune system's ability to resolve the infection due to impaired immune cell migration (Roth, 1982). GC supplementation during an immune challenge may reduce the inflammatory response, but should be used with caution as the reduction in inflammatory response may also lessen the host's ability to clear the pathogen thus prolonging the infection.

Omega-3 fatty acids. During an immune challenge, immune cells can utilize FA, glutamine, or glucose as fuel sources (Sordillo, 2016). Immune cells utilize these energy sources at varying rates depending on severity of immune challenge and DMI (Calder, 2013). Oxylipids are synthesized from substrates including omega-6 linoleic and arachidonic acids and omega-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA; Sordillo and Raphael, 2013). Commonly, omega-6 FAs activate NFκB and enhance inflammatory responses, whereas omega-3 FAs tend to inhibit NFκB and promote resolution of inflammation (Serhan, 2009; Calder, 2012). DHA and EPA demonstrate anti-inflammatory functions by inhibiting NFκB activation indirectly by altering the concentrations of inflammation mediators such as prostaglandin $F_{2\alpha}$ (Badinga and Caldari-Torres; Lee et al., 2010; Calder, 2012). It has been demonstrated that increasing the omega-3 FA content of endothelial cells can lessen their pro-inflammatory response (Contreras et al., 2012). By altering the omega-6 to omega-3 ratio of FA supplemented to dairy cattle, it may be possible to lessen the inflammatory state during times such as the periparturient period or during mastitis (Bradford, 2020). When feeding diets differing in the ratio of omega-6 and omega-3 acids, increasing omega-3 supply led to an increase in DMI and milk component yields as well as a decrease in the acute phase response to an intramammary

LPS challenge. However, increasing the amount of omega-3 in the diet did not attenuate the decrease in DMI during an immune challenge (Greco et al., 2015). By altering the FA supplied in the diet to a higher concentration of omega-3 acids, the body may produce less pro-inflammatory mediators during an infection. Feeding omega-3 acids serves as a preventative measure to lessen the inflammatory state, but during an active infection, other interventions may be required to help clear up the cause of infection.

Phytogenics. As demonstrated by the VFD of 2017, the use of feed antibiotics in livestock has become a greater consumer concern, leading to the interest in naturally occurring compounds that may support immune system function to reduce the need for antibiotic treatment of disease and limit the risk of antibiotic resistance in livestock. Phytogenics refers to a broad array of secondary metabolites produced by plants (Swartz and Bradford, 2023). Phytogenics have been studied as a dietary method to decrease methanogenesis (Sinz et al., 2019). Ku-vera et al. (2020) demonstrated that some tannins, saponins, free phenolics, and flavonoid compounds inhibit methane production from ruminal fermentation (Ku-Veraet al., 2020).This review is going to discuss primarily one class of these naturally occurring compounds, polyphenols, which exert antioxidative and immunostimulatory effects. Polyphenols are a group of over 8,000 compounds, all containing phenol rings. Polyphenols can be further divided into flavonoids and other phenolic acids. It is worth noting that the concentration of plant polyphenols is highly dependent on the maturity and part of the plant that is fed (Miranda et al., 2014). Because plant secondary metabolites are more difficult to patent than synthetic compounds, there has been limited private sector investment in these compounds. The limited literature, in turn, means that for most polyphenols, there is no standard dose recommendation, and publications vary widely in doses administered (Soltan et al., 2023). Phytogenics pose as a natural solution to combat

inflammation while avoiding the adverse effects of animal product withhold times due to treatment.

Flavonoids. Flavonoids are a class of polyphenols known to have anti-inflammatory and antioxidative functions. Flavonoids are made up of a three-ring core and are divided into five subgroups: flavanols, anthocyanidins, flavones, flavanones, and chalcones (Middleton et al., 2000). Even though there are thousands of known flavonoids, they almost all promote broad anti-inflammatory effects on different cell types (Gonzálezet al., 2011). Classically, flavonoids have been studied in ruminants as a tool to decrease methane production (Sinz et al., 2019; Ku-Vera et al., 2020). While research is limited, groups have set out to analyze flavonoid supplementation during the transition period and mastitis challenges (Olagaray and Bradford, 2019).

Morin is a flavonoid isolated from the mulberry/fig family of plants (Moraceae) and has been demonstrated to help lessen the inflammatory response to bovine mammary epithelial cells during LPS-induced mastitis in a cell culture model. Morin acts through inhibition of the NF-κB pathway (Wang et al., 2016). Supplementing silymarin (extracted from milk thistle) pre- and post-partum tended to lower the SCC in cows during the first 21 DIM as well as lead to increased milk production and no noticeable change in milk composition (Tedesco et al., 2004; Garavaglia et al., 2015). Another group looking at supplementing a cocktail of flavonoids to lactating cattle with moderate or high SCC found that supplementation lowered SCC in cows with high SCC but had no effects on moderate SCC. An increase in DMI, fluid milk, and feed efficiency was also observed (Hashemzadeh-Cigari et al., 2014). Flavanoid supplementation may help reduce the inflammatory state, but further research needs to be done looking at the effects during an active infection.

Other phenolic acids. Another group of phytogenic compounds are other phenolic acids, including free phenolics, essential oils (EO), lignans, saponins, terpenes, quinones, and alkaloids (Swartz and Bradford, 2023; Worku and Ismail, 2020). Essential oils are a liquid and volatile form of secondary metabolites. Supplementation of non-flavonoid polyphenols in diabetic murine and human models decreased the concentration of pro-inflammatory molecules (Miranda et al., 2014). EO found in ginger have been used as folk medicine for the treatment of pain and inflammation (Worku and Ismail, 2020). Eugenol is an EO found in clove oil, nutmeg, cinnamon, and basil which has demonstrated anti-inflammatory effects (Fujisawa et al., 2002; Carrasco et al., 2009). Phenolic compounds found in plants may be polymerized into larger molecules such as proanthocyanidins (condensed tannins) and lignins (Waghorn and McNabb, 2003). Feeding birdsfoot trefoil (a forage with high concentrations of tannins) to lactating dairy cows improved feed efficiency, increased milk yield and milk protein yield, but also decreased milk fat concentration (Woodward et al., 1999; Harris et al., 1998). Hydroxycinnamic acids are free phenolic acids with the most common ones including caffeic acid, ferulic acid, p-coumaric acid, sinapic acid, and chlorogenic acid (Chen and Ho, 1997). Using inflammatory bowel disease as a model of inflammation, Zielińska et al. (2021) observed that caffeic acid exerts anti-inflammatory properties by targeting COX-2 and PGE_2 (Zielińska et al., 2021). Supplementation of phenolic acids may serve as a preventative measure to inflammatory responses, but much like omega-3 supplementation, other methods may be required to clear a pathogen in the event of an active immune challenge.

Endocannabinoid system. Endocannabinoids, closely related to oxylipids, are a class of lipid mediators (Walker et al., 2022). Endocannabinoids are derived from arachidonic acid and synthesized in the body, while phytocannabinoids are synthesized in plants and are only active in

mammals after ingestion (Chayasirisobhon, 2021). Phytocannabinoids have properties similar to cannabinoids and/or interact with the cannabinoid receptors (Gertsch et al., 2010). Examples of endocannabinoids include anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), and examples of phytocannabinoids include β-caryophyllene (BCP), cannabidiol (CBD) and Δ^9-tetrahydrocannabivarin (THCV) (Bolognini et al., 2010; Gertsch et al., 2010). The endocannabinoid system (ECS) is activated by two receptors - CB1 and CB2 - and is involved in metabolism, energy homeostasis, and inflammation (Zachut et al., 2018). The CB1 receptor is associated with the central nervous system and is responsible for the psychoactive effects such as catalepsy and memory/cognitive impairment reported with some cannabinoids (Pertwee and Ross, 2002a; Aly et al., 2020). In contrast, CB2 activation is associated with peripheral and immune cell responses (Pertwee and Ross, 2002). Both the CB1 and CB2 receptors can be found in immune cells (macrophages, dendritic cells and B cells), but the CB2 receptors are 10-100 times more abundant than CB1 in these cell types (Ziring et al., 2006; Chiurchiù et al., 2015; Rahaman and Ganguly, 2021).

CB2 activation has been demonstrated to exert anti-inflammatory effects in vitro and vivo (Gertsch et al., 2008). During infection, endocannabinoid production is increased, paired with a downregulation of the AEA-degrading enzymes (Klein, 2005). Depending on both the concentration and timing, endocannabinoids may serve as pro- or anti-inflammatory signals (Basu and Dittel, 2011; Walker et al., 2022b). CB2 aids in reducing the inflammatory state by inhibiting the release of pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α (Correa et al., 2009; Capozzi et al., 2022). During LPS activation of the immune system, CB2 agonists inhibit the secretion of TNF-α and IL-1β (Gertsch et al., 2008; Jürg Gertsch and Gertsch, 2017). Endocannabinoids function as lipid mediators, thus increasing the dietary supply of

endocannabinoids may act as a preventative method to the inflammatory response, but once again, other interventions may be required in the event of an immune challenge.

β-caryophyllene. BCP is a sesquiterpene phytochemical that is relatively abundant in plants such as black pepper, oregano, and cinnamon (Gertsch et al., 2008). BCP selectively activates the CB2 receptor and has been shown to have analgesic, anti-inflammatory (lower production of TNF-α, IL-1β, IL-6 and NF-κB), and other immunomodulatory effects (Klauke et al., 2014; Francomano et al., 2019; Scandiffio et al., 2020). BCP stimulation of the CB2 receptor has been demonstrated in cell culture models stimulated with LPS, and when cells were treated with a CB2 antagonist the positive effects of BCP were reverted (Picciolo et al., 2020). In another study, researchers found BCP treatment exerted anti-inflammatory effects and decreased the oxidative status of cells exposed to a conditioned medium from activated monocytes (Mattiuzzo et al., 2021). In murine models, oral supplementation of BCP demonstrated a 70% reduction of carrageenan-induced paw edema (Gertsch et al., 2008). In another murine model, oral BCP supplementation suppressed the expression pro-inflammatory cytokines in the context of antiretroviral drug-induced neuropathic pain (Aly et al., 2020). BCP, a dietary cannabinoid, may help blunt the response to an immune challenge. Before BCP can be recommended for commercial use, further studies need to be conducted to define an effective dose, as well as confirm that there are no residuals found in meat or milk products.

Conclusions

Inflammation is an essential part of homeostasis, but too much inflammation can impede lactation performance (Bradford et al., 2015). Although compounds have been identified which can influence immune responses, there are few tools that are proven effective for enhancing resilience to disease challenges. Supplementation of phytogenic compounds such as BCP may

potentially decrease pro-inflammatory signals (Klauke et al., 2014) so that a healthy degree of inflammation is still allowed while not completely eliminating its effects. BCP selective activation of the CB2 receptor is of primary interest, as this avoids potential psychotropic effects while providing all of the anti-inflammatory effects (Scandiffio et al., 2020). While BCP has been shown effective in murine and cell culture models (Gertsch et al., 2008; Mattiuzzo et al., 2021), the effect of BCP supplementation on dairy cattle in an LPS mastitis model is pertinent to further understanding its effects on inflammation.

CHAPTER 2:

EFFECTS OF β-CARYOPHYLLENE SUPPLEMENTATION ON DRY MATTER INTAKE AND PRODUCTIVITY OF LATE-LACTATION DAIRY COWS THROUGH REPEATER LIPOPOLYSACCHARIDE CHALLENGES

Abstract

Inflammation greatly impacts the health and performance of dairy cows during lactation. The objectives of this study were to evaluate the effect of β-caryophyllene (BCP) supplementation on somatic cell score (SCS) and inflammatory blood markers in late lactation dairy cattle experiencing an acute mastitis challenge, as well as carryover effects of a second mastitis challenge. Twenty late lactation Holstein cows (240 ± 60 DIM; 33 ± 6.9 kg/d of milk) were enrolled in a randomized complete block design experiment lasting 70 d. Cows were assigned treatments of no top-dress (CON, n = 10) or BCP (n = 10) consisting of 5 mg/kg BW liquid BCP added as a top-dress as a top dress treatment and mixed in with the top 20% of the ration. All cows received the same diet. Cows were challenged in the right-rear quarter (RR) with 10 µg of lipopolysaccharide (LPS) on d 28 and d 56. Quarter-level milk (RR) was collected at 0, 8, 16, 24, 48, 72, 96, 120, 144, and 168 h after each LPS challenge. Treatment effects were determined using linear repeated mixed models in SAS, including fixed effect of treatment, time, parity, and their interactions and random effects of block and cow. Before LPS, BCP did not affect DMI, milk yield, milk composition, SCS, or plasma glucose concentration (all $P > 0.10$). After each LPS challenge, BCP did not affect DMI, milk yield or composition, but reduced composite milk SCS ($P < 0.01$) A treatment × time interaction ($P < 0.001$) for SCS revealed a tendency for more complete resolution in BCP-treated cows at d 52 ($P = 0.06$). Conversely, SCS in the RR quarter was greater for BCP vs. CON ($P = 0.02$) at 16 h after challenge 2 (treatment × time: $P < 0.001$). Plasma haptoglobin was significantly reduced by BCP in the 5 d following challenge 1 ($P = 0.02$) but not challenge 2 ($P > 0.10$). Overall, results suggest that BCP supplementation may reduce the inflammatory response observed from acute mastitis. Further work is required to understand the underlying mechanisms of the changes observed in this study.

Introduction

Nutritional methods to influence the inflammatory state of dairy cattle have been a strong area of interest in the research community. Improving the understanding and delivery of nutraceuticals has the ability to further dairy production while working towards economic and environmental sustainability. The use of nutraceuticals may reduce the need for other pharmaceuticals, thus lowering the quantity of dairy products that are unusable due to drug withdrawal times from products.

Phytocannabinoids, such as β-caryophyllene (BCP), are secondary plant metabolites that have similar properties to endocannabinoids and/or interact with the cannabinoid receptors (Gertsch et al., 2010). BCP has been proposed as a nutrient potentially capable of reducing inflammation through its selective activation of the CB2 receptor (Francomano et al., 2019). BCP is also known to have antioxidant, neuroprotective, anxiolytic, anti-cancer, and analgesic effects (Aly et al., 2020). The CB2 receptor is widely found among immune cells, modulating cytokine release and immune cell migration (Pertwee, 2006). Unlike the CB1 receptor, it provides these results without psychoactive effects. Activation of the CB2 receptor through agonists can reduce inflammation by inhibiting the release of inflammatory cytokines such as IL-6, IL-8, IL-1β and TNF-α in LPS-stimulated monocytes (Capozzi et al., 2022; Correa et al., 2009; Gertsch et al., 2008; Jürg Gertsch & Gertsch, 2017). BCP supplementation, therefore, may help lessen the inflammatory state experienced by a dairy cow during an immunological challenge through its relationship with the CB2 receptor.

While not previously studied in dairy cattle, BCP supplementation has been studied in multiple murine models. Oral supplementation in mice models has demonstrated suppression of pro-inflammatory cytokines (Aly et al., 2020) and a decrease in paw edema (Gertsch et al., 2008)

in different inflammatory models. Poulopoulou & Hadjigeorgiou (2021) suggest that the rumen microbiome takes 1 week to adjust to BCP supplementation; after adaptation, the mean ruminal degradation rate was just 4.3% per h (compared to 0.1% in PBS solution) using an ovine model. In comparison to expected ruminal liquid outflow rates in dairy cattle of ~19% per h (Taylor et al., 2005), these findings point to a strong potential for BCP delivery to the small intestine.

Trained immunity is the process in which innate immune cells undergo epigenetic changes resulting in either an enhanced or suppressed response to a repeated immune challenge (Byrne et al., 2020). Unlike adaptive immunity, trained immunity provides short term, often non-specific alteration of immune function (Netea, 2013). The concept of trained immunity raises a question: if innate immunity can be trained from a primary challenge, can what is consumed during a primary infection impact the body's trained response to a repeated challenge?

The objectives of this experiment were to evaluate if BCP supplementation reduces the SCS and inflammatory blood markers in late lactation dairy cattle experiencing an acute mastitis challenge. Additionally, we investigated carryover effects of BCP supplementation on SCS and inflammatory blood markers during a second acute mastitis challenge once BCP supplementation ceased, as an initial assessment of potential BCP impacts on immune training. We hypothesized that BCP supplementation would lower the SCS, inflammatory blood markers, and improve the resolution of mastitis.

Materials and Methods

Cows, treatments, and experimental design. All procedures were approved by the Michigan State University Institutional Animal Care and Use Committee (protocol no. PROTO202200231). The experiment was conducted at the Michigan State University Dairy Cattle Teaching and Research Center (Lansing, MI) between August 2022 and November 2022. Twenty mid or late-lactation Holstein dairy cows (240 ± 60 days in milk [DIM]; 6 primiparous, 14 multiparous), averaging 33 ± 6.9 kg/d of milk yield and free from clinical mastitis, SCS < 4, were enrolled in a randomized complete block experiment. Using data from a previous intramammary LPS challenge study (Swartz et al., 2023), we conducted a power analysis based on body temperature. In the previous study the peak mean body temperature occurred at 5 h post-challenge, with a standard deviation of 0.34 degrees C. Based on these data, 10 cows per treatment are sufficient to detect a mean treatment effect of 0.44 °C in body temperature at 5 h post-challenge with 80% power at $\alpha = 0.05$. This is sufficiently powerful to detect a meaningful strategy to reduce LPS-induced inflammation.

The cows were enrolled in two cohorts of 10 cows each, two weeks apart. Cows were blocked, in blocks of two, by parity, bovine leukemia virus (BLV) status, and DIM, in that order, at start of challenge, within each cohort. BLV status was used as a blocking factor as it has been demonstrated that cows which are BLV positive have impaired immune response to stimuli (Erskine et al., 2011). Treatments were randomly assigned within each block. The experiment consisted of two treatment groups; 10 cows were assigned to receive the control (CON; no treatment) and 10 cows were assigned to receive 5 mg/kg of bodyweight (BW) per d of BCP (#W225207 Sigma-Aldrich). This dose was selected in part due to anti-inflammatory effects of this dose in mice (Gertsch et al., 2008) and because this is the recommended greatest dose in

livestock feed under a European Union approval (https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32017R0065).

Cows were housed in a tie-stall barn, milked three times daily (0500, 1300, and 2100 h) in a milking parlor, and fed a total mixed ration (TMR) once per day (1000 h) for a 10% refusal rate. Cows had *ad libitum* access to water. Upon morning feeding (1000 h), BCP treatments were administered as a top-dress from d 0 until d 42; cows were monitored until d 70. Prior to the first mastitis challenge, quarter-level milk samples from the right rear (RR) quarter were taken to screen for an active mastitis case in that quarter. The 5 cows within each treatment group in each cohort (out of 6) with the lowest quarter-level somatic cell count (SCC) were selected for mastitis challenge (total $n = 10$ per treatment). Both CON and BCP groups included 5 BLV-positive and 5 BLV-negative cows.

Data collection and sample analysis. TMR samples were collected (prior to BCP administration) every week throughout the experiment and composited at two-week intervals prior to analysis (Table 1). TMR samples were evaluated for dry matter (DM), crude protein (CP), starch, ethanol-soluble carbohydrates, neutral detergent fiber (NDF), lignin, crude fat, ash, and minerals using NIR spectroscopy (Cumberland Valley Analytical Services).

Bodyweight was measured once per week and body condition score (BCS) was assessed once per week by 3 trained investigators on a 1-5 scale (Wildman et al., 1982). Milk yield and milk conductivity were measured at each milking in the milking parlor with an electronic monitoring system (AFIMILK, Kibbutz Afikim, Israel). Composite milk samples (~50 mL) were collected on d 14, 23, 24, 30, 37, 38, 45, and 52 in a sealed tube with a preservative (bronopol) and stored at 4°C for milk component (milkfat, protein, lactose) and SCC analyses (Bentely FTM/FCS, Bentely Instruments Inc.; CentralStar DHI). Energy-corrected milk (ECM) yield was

calculated using the formula: ECM = (0.327 × milk kg) + (12.95 × fat kg) + (7.65 × protein kg). Somatic cell score (SCS) was calculated using the formula: $SCS = \log_2 (SCC/100,000) + 3$ (Ali and Shook, 1980). Quarter-level milk samples were collected from the RR quarter at the evening milking (2100 h) on d 24 and 0, 8, 16, or 24 h after each challenge. Samples during the first 24 h after challenge were diluted 1:10 in PBS for flow cytometric analysis. Quarter level samples were then taken once daily for the next 6 d following each LPS challenge; these samples were not diluted in PBS. All quarter-level milk samples were analyzed for SCC as described above.

Body temperature was collected every 10 min from 24 h prior to each LPS challenge until 72 h after each LPS challenge. The body temperature was collected using a thermometer (iButton DS1921H; Embedded Data Systems) which was placed on a blank controlled internal drug release device (CIDR; Zoetis). Prior to insertion of the CIDR, the vulva of the cow was cleaned with water and disinfected with betadine solution. Then, a lubricant was applied to the CIDR and vulva then the CIDR containing the iButton inserted into the vagina of each cow.

Blood samples were collected on d 14, 24, 28-34 and d 56-62 after the 2100 h milking (~2200 h). Blood samples were also collected 8 h and 16 h after each LPS challenge. Blood was collected by coccygeal venipuncture with tubes containing K_2EDTA; samples were immediately placed on ice and transported to the lab where they were centrifuged for 15 min at 1,500 × *g* to collect the plasma. Two aliquots of plasma were kept at -20°C for later analysis. Plasma was analyzed for glucose by enzymatic methods (kit #997-03001; Fujifilm, Osaka, Japan), insulin using a bovine specific ELISA (no. 10–1201–01; Mercodia AB, Uppsala, Sweden), haptoglobin using a bovine specific ELISA (Hp; kit # HAPT-11; Life Diagnostics, West Chester, PA), LPS binding protein by ELISA (LBP; cat # CKH113; Cell Scienes, Newburyport, MA), and β-hydroxybutyrate by enzymatic assay (BHB; kit no. H7587-58; Pointe Scientific Inc., Canton,

MI). The inter-assay coefficients of variation (CV) were 5.81, 6.48, 7.22, 5.26, and 5.23 for glucose, insulin, Hp, LBP and BHB, respectively. Intra-assay CV were 5.17, 5.11, 6.55, 5.02, and 5.37 5.81, 6.48, 7.22, 5.26, and 5.23 for glucose, insulin, Hp, LBP, and BHB, respectively.

The Mass Spectrometry and Metabolomics Core at Michigan State University conducted the analysis of BCP in feed and milk using gas chromatography-mass spectrometry. For quantification in the TMR, approximately 3.0 – 4.5 g of feed (frozen and thawed, not dried) was transferred into 40 mL amber vials capped with Teflon septa. Samples were loaded onto a sampling tray set at 40°C and equilibrated for 30 min before headspace was sampled with an autosampler equipped with a solid phase microextraction (SPME) syringe and fiber (30 mm divinylbenzene/Carboxen 50 µm polydimethylsiloxane, Milipore-Sigma). The headspace extraction time was 10 min and volatiles were desorbed at 250°C with splitless mode on an Agilent 7890B GC with a 7010b triple quadrupole mass spectrometer. Separation was achieved on an Agilent J&W VF5ms (30 m × 0.25 mm × 0.25 mm; Agilent) using the following temperature profile: 40°C for 4 min; 20°C min^{-1} to 250°C; 250°C for 2 min. Ionization employed 70 eV electron ionization and the mass spectrometer was operated in selected ion mode for m/z 93 and 204 for BCP.

Milk samples were prepared by pipetting 500 µL of milk sample, 100 µL 0.05 ng/µL of tetradecane, and 9.4 mL of Milli-Q water to 40 mL amber vials capped with Teflon septa. Samples were loaded onto a purge and trap unit (Atomx XYZ). The sample cup was held at 50°C and volatiles were purged in the sample vials for 11 min with 40 mL/min of nitrogen gas onto a #9 trap held at room temperature. The trap was heated to 250°C for 2 min and volatiles were desorbed with a 1:5 pulsed split mode on an 7890A GC with a 5975 single quadrupole mass spectrometer (Agilent). Separation was achieved on an Agilent J&W VF5ms (30 m × 0.25 mm ×

0.25 mm; Agilent) using the following temperature profile: 40°C for 4 min; 20°C min^{-1} to 250°C; 250°C for 2 min. Ionization employed 70 eV electron ionization and the mass spectrometer was operated in selected ion mode for m/z 93 and 204 for BCP and m/z 198 for tetradecane.

Lipopolysaccharide challenges. Lipopolysaccharide (LPS; E. coli O11:B4, Millipore Sigma, St. Louis, MO) challenges were conducted at the final milking (2100 h) on d 27 and d 55 of the experiment. The challenges were all conducted in the RR quarter of each cow. To conduct the challenge, cows were milked according to farm protocols. After milking was completed, the teat end was scrubbed with an alcohol swab to remove bacteria. Then, 5 mL of PBS containing 100 µg of LPS (20 µg/mL) were infused into the RR quarter. The solution was then massaged upward into the mammary gland. The post-milking procedure was then completed according to farm protocols.

Statistical analysis. All data were analyzed using linear mixed models (GLIMMIX; SAS 9.4, Cary NC). Dry matter intake (DMI), milk yield, milk composition, and milk component yields were analyzed using repeated measures mixed linear models for pre-challenge, post-challenge 1, and post-challenge 2 data. Change in BW and BCS (ΔBW, ΔBCS) were evaluated as repeated measures and included fixed effects of treatment, time, their interaction, BLV status, and parity. Random effects included cohort and cow. For ΔBW, ΔBCS, SCS, and body temperature, the spatial power covariance structure was used due to unequal spacing of measurements. Normality of residuals was visually appraised. Observations with a Studentized residual ≥ 4 or ≤ -4 were removed from the data set for each variable and the models were refit if observations were removed.

Quarter-level somatic cell score (SCS; [$\log_2$(somatic cell count/100,000) + 3]) was analyzed as a repeated measure using a compound symmetry covariance structure due to equal spacing of measurements. The model included the fixed effects of treatment, time post challenge, and challenge (1st vs. 2nd). All two and three-way interactions were included in the initial model and removed if $P > 0.10$. For plasma markers, the model used was the same except for the use of autoregressive covariance structure. Cow was included as a random effect in each model. Model assumptions were evaluated as described previously.

Body temperature and blood analytes were analyzed as an area under the curve (AUC; ΔC° × min) using the trapezoidal rule. Baseline body temperatures were calculated by averaging the temperature of each cow for 12 h prior to LPS infusion. The model included treatment, challenge (challenge 1 vs. challenge 2) and their interaction. AUC was evaluated as a repeated measure using the compound symmetry covariance structure. Cow was included as a random effect in the model.

Statistical significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$ for each variable and model that were run.

Results and Discussion

Baseline period & BCP analysis. Prior to LPS challenge, no treatment effects on DMI were observed (Table 2), similar to results reported by Oliveira (2018) using BCP as an oral supplement in mice models. Treatment did not significantly affect milk yield or energy-corrected milk (ECM; Table 2). Furthermore, the ΔBW and ΔBCS were not affected by treatment (Table 5), consistent with both the lack of DMI differences and findings in mice (Oliveira et al., 2018). BCP was detected in purified samples used for treatment (95% BCP according to the LC-MS analysis), consistent with specifications from the supplier, but we found no evidence of BCP in

milk or any basal presence in the TMR. Milk and TMR samples were stored in a -20°C freezer for 6 months, then thawed prior to analysis; storage and handling of the milk and TMR samples may have affected our ability to detect BCP due to its volatile nature (Gertsch et al., 2008). The volatile nature of BCP also raises the possibility of all of it evaporating prior to ingestion thus explaining our inability to detect it in the milk and TMR.

Prior to LPS challenges, our findings suggest that BCP supplementation had no apparent adverse or beneficial effects on late-lactation dairy cattle. This could possibly be due to rumen degradation of BCP. Poulopoulou and Hadjigeorgiou (2021) evaluated terpene degradation in rumen fluid using an ovine model and found that BCP degradation rate was greatest after 1 week of oral supplementation (approximately 4.3% per hour, dosed at 1 g). These baseline period results, paired with our inability to detect BCP in milk collected from treated cows, suggests no risks of meaningful quantities of BCP getting into the food chain, thus setting the stage for further research on BCP supplementation in dairy cattle.

One potential explanation for the inability to detect BCP in milk samples during this study is BCP's low oral bioavailability and high sensitivity to oxidation when exposed to oxygen and light (Liu et al., 2013; Di Sotto et al., 2018). Bioavailability of a molecule depends on its chemical and physical properties, and typically reflects water solubility and membrane permeability (Di Sotto et al., 2018). It is important to note that BCP has high lipophilicity, which subsequently limits its bioavailability and absorption. Assuming the BCP is not degraded in the rumen, it would be taken up in the small intestine (Di Sotto et al., 2018). In a human study by Mödinger et al. (2022) using similar dosage of 100 mg per subject, using a self-emulsifying drug delivery system to orally supplement BCP significantly increased the BCP $AUC_{0\text{-}12h}$ and $AUC_{0\text{-}24h}$ concentrations by 2.2-fold and 2.0-fold, respectively. They also found a 3.6-fold increase in

maximum plasma concentration and time to maximum blood concentration was 1.43 vs. 3.04 h for BCP delivered as a neat oil. Therefore, the form of supplementation in this experiment may have affected its uptake. Little information is available on rumen degradation of BCP other than results from Poulopoulou and Hadjigeorgiou (2021) using a sheep model, who found that rumen degradation of BCP was approximately 13%/h on average for the first week of supplementation and then dropped to approximately 4.3%/h in the following week. Further work should be done to evaluate the degradation of BCP using a bovine model, as well as determining the efficacy of formulating BCP to self-emulsify through oral and abomasal supplementation.

LPS challenge 1. During an intramammary LPS challenge, dairy cattle experience a drop in DMI and milk production while also having an increase in body temperature and somatic cell count in milk (Johnzon et al., 2018). Temporal patterns in this study were similar to those reported previously, with a noticeable rise in body temperature beginning roughly 2 h post-challenge, peaking (~41°C) at 6 h post-challenge, and returning to baseline 12 h post-challenge (Figure 1). There was no detectable impact of treatment on these body temperature patterns. After challenge, DMI and milk production were not significantly affected by BCP supplementation (Table 2). On the other hand, BCP supplementation significantly reduced SCS (Figure 3) on d 52 of the study (10 days after BCP supplementation ended), potentially pointing to more complete resolution of challenge-induced inflammation in the mammary gland. Treatment did not affect plasma glucose, insulin, BHB or lipopolysaccharide binding protein (LBP) concentrations in the 16 h after the first LPS challenge (Table 3). However, BCP supplementation significantly lowered haptoglobin concentrations (Table 3) over the 16 h post-challenge.

The reduction in plasma haptoglobin and the greater drop in SCS 10 d after challenge may be in part due to BCP's ability to decrease the expression of pro-inflammatory signals, allowing for a more complete resolution (Aly et al., 2020; Johnzon et al., 2018). When looking at the results following the first of the LPS challenge, we did not observe sufficient evidence to support our hypothesis that BCP would lessen the inflammatory response. A recent study by Jermann et al. (2023) varying the dietary supply of macronutrients found no difference in the systemic inflammatory response to an intramammary LPS injection based on macronutrient profile, but they did observe a significant increase in acute phase proteins (APPs) in milk before APP changes were detectable in plasma. This discovery raises the question of whether inflammatory mediators from the challenged quarter cause the systemic reactions observed, or if LPS itself is entering the bloodstream. Haptoglobin is a positive APP synthesized by the liver; it is found in low concentrations in healthy animals but is quickly released into the bloodstream during an infection. It is a protein with antioxidant, antimicrobial, and anti-inflammatory properties through its ability to scavenge free hemoglobin and bind to neutrophil integrins (Gulhar et al., 2023). It results in the release of other anti-inflammatory mediators such as IL-10 (Quaye, 2008; Ceciliani et al., 2012). While we didn't detect any improvements in feed intake or milk yield in this study, lower concentrations of plasma haptoglobin suggests that cows experienced less inflammation and hepatic stress as a result of BCP supplementation. The lower SCS observed at 24 d post-challenge in cows previously supplemented with BCP could also be explained by the anti-inflammatory effects of BCP potentially blunting the severity of the APP response in the affected quarter. The decrease in SCS and haptoglobin that was observed provides evidence that further research needs to be conducted looking at supplementing BCP to dairy cattle in a challenge setting. However, without any significant changes in any other acute

phase proteins, body temperature, or milk production, it is unclear if inflammation was mitigated during this challenge as a result of BCP supplementation.

LPS Challenge 2. Repeated exposure to the same pathogen or pathogen-associated molecule can alter the immune system through innate training, resulting in the host having either a heighted or a suppressed innate immune response (Byrne et al., 2020). BCP treatment administration ended 2 weeks after the initial LPS challenge and 2 weeks prior to the second LPS challenge, to evaluate whether BCP supplementation influenced trained immunity. The 2 weeks prior to the second LPS challenge was to allow for BCP to leave the guy/body, however since we didn't collect adipose samples there is a possibility that BCP may be stored in adipocytes.

In accordance with our hypothesis, BCP treatment appeared to have an effect on response to repeated LPS challenge. BCP supplementation resulted in a significantly greater quarter-level SCS score 16 hours after the second LPS challenge (Figure 4); there was no significant effect at any other time point. This could be due to increased sensitivity after the first LPS challenge (Byrne et al., 2020), but more work needs to be conducted into nutritional impacts on innate training to explain this response. After the second LPS challenge no treatment effects were observed for plasma glucose, insulin, haptoglobin, BHB, or LBP (Table 3). BCP supplementation has not been previously studied in cattle, so while the lack of difference in DMI and BW changes are supported by murine models, further research needs to be conducted to elucidate BCP effects on performance responses in dairy cattle.

We expected to see a lesser acute phase response when comparing challenge 2 to challenge 1, as demonstrated in other studies looking at innate training (Ifrim et al., 2014; Sullivan et al., 2023). For haptoglobin, there was indeed a significant reduction in plasma AUC for the second challenge compared to the first (Table 4). However, the body temperature

response for the second LPS challenge was similar to that of the first challenge (Figures 1 & 2). When comparing the AUC for other plasma metabolites between challenges, we expected to see a lower AUC value for the second challenge but instead glucose, insulin, BHB, and LBP were not significantly different. Our findings when comparing between challenges was unexpected as other groups that have looked at repeated LPS challenges observed a lesser fever response due to innate training (Petzl et al., 2012).

In contrast to other plasma analytes, we observed a lower concentration of plasma haptoglobin after challenge 2 compared to challenge 1. This lesser concentration in the second challenge points to the possibility of tolerance to LPS following the first challenge, but this idea conflicts with the increased SCS response 16 hours after challenge, pointing to increased sensitivity as opposed to the tolerance observed with the haptoglobin AUC. This increased sensitivity in the mammary gland may be due to the anti-inflammatory effects of BCP that were present during the first LPS challenge. It is possible that BCP limited the local inflammatory tone of cows during the first challenge, "training" the intramammary immune cells in BCP-treated cows compared to CON cows and thus leading to, increased immune cell migration and a higher SCS in the BCP cows during the second challenge. This raises a question of whether an observable difference in the inflammatory response would be expected, potentially impacting body temperature, feed intake, and milk responses, if this altered immune training was monitored while BCP supplementation was maintained. To test this, future studies could be conducted looking at repeated LPS challenges with BCP being supplied through both LPS challenges.

While these findings are interesting, without other evidence of altered systemic responses – such as a lower body temperature or SCS – we are unable to clearly state that we successfully trained the innate immune system through repeated LPS challenges.

Conclusions

Supplementation of BCP to late lactation dairy cattle did not cause any detrimental effects to production or homeostasis during a basal period. When looking at BCP supplementation during the first LPS challenge, a decrease in SCS and a reduction in haptoglobin concentrations suggest that BCP could help mitigate the inflammatory responses to an intramammary LPS challenge. During the second LPS challenge, results suggest that BCP supplementation did not have an impact on innate training except for when looking at plasma haptoglobin concentrations compared to challenge 1. One measurement that was not collected in this study but would be advisable for future research is to confirm BCP supplementation was effective by testing its plasma concentration. The few significant results observed could be due to BCP's low bioavailability and uptake, which we are unable to determine with this experiment. We conclude that BCP supplementation appears to be safe at a rate of 5 mg/kg BW without disrupting the function of late lactation dairy cattle, however, we are unable to conclude that it is a useful treatment for inflammation. Although BCP treatment significantly decreased SCC and haptoglobin concentrations at particular timepoints after intramammary LPS challenge, future research needs to be conducted to fully elucidate the function of BCP as an inflammatory modulator in lactating dairy cattle.

CHAPTER 3:

IMPLICATIONS AND CONCLUSIONS

Implications and Conclusions

The average lactating cow replacement rate in the U.S. dairy industry is 35%, while cows are often replaced due to health events, this replacement rate is far from optimal in terms of economic, social, or environmental sustainability (Murat Tatar et al., 2017; Overton and Dhuyvetter, 2020). Due to societal pressure, a veterinary feed directive (VFD) was established in 2017 to reduce the use of antibiotics in livestock production. To help improve the resilience of cows to pathogens, further research needs to be done to investigate naturally occurring compounds that may help decrease the need for antibiotic use. By studying compounds such as BCP, we may better understand ways in which nutrition can improve the immune function of dairy cattle.

The goal of this work is to access how BCP supplementation changes the inflammatory response in late lactation dairy cattle experiencing repeated intramammary LPS challenges. The study evaluated the effect of BCP supplementation on the inflammatory responses from late lactation Holstein cows as well as any carryover effects of BCP supplementation on a subsequent LPS challenge. Parameters such as BHB, glucose, haptoglobin, insulin, LBP, and body temperature were evaluated to measure the inflammatory response. BCP supplementation significantly reduced haptoglobin during the first LPS challenge and led to a significant decrease in SCC 10 days after supplementation ended, but had no effect on DMI, milk or component yields, or any of the other analytes measured. BCP supplementation resulted in a higher peak for SCC at 16 h after the second challenge, but similarly had no impact on DMI, milk or component yields, or any of the other analytes measured. When comparing the first challenge to the second challenge there was a tendency for cows to have lower levels of haptoglobin during the second challenge. Considering the anti-inflammatory effects of BCP it may have lowered the haptoglobin levels following the first challenge, while trained immunity may be the reason for

the lower levels of haptoglobin when comparing the two challenges, as well as the higher peak for SCC observed in the second LPS challenge.

This study concludes that further work should be done to determine if BCP supplementation would alter the inflammatory response upon repeated LPS challenges. Work investigating BCP supplementation for both of the LPS challenges is warranted. Evaluation of BCP supplementation on the inflammatory response will potentially help the current problem of susceptibility to some infectious diseases further lessening the need of antibiotics. With this study, we conclude that BCP affects haptoglobin and SCC of dairy cows experiencing an intramammary LPS challenge.

BIBLIOGRAPHY

Abebe, R., H. Hatiya, M. Abera, B. Megersa, and K. Asmare. 2016. Bovine mastitis: Prevalence, risk factors and isolation of Staphylococcus aureus in dairy herds at Hawassa milk shed, South Ethiopia. BMC Vet Res 12. Doi:10.1186/s12917-016-0905-3.

Abuelo, A., J. Hernández, J.L. Benedito, and C. Castillo. 2015. The importance of the oxidative status of dairy cattle in the periparturient period: Revisiting antioxidant supplementation. J Anim Physiol Anim Nutr (Berl) 99:1003–1016. Doi:10.1111/jpn.12273.

Adcock, I.M., and G. Caramori. 2009. Transcription Factors. Asthma and COPD: Basic Mechanisms and Clinical Management 373–380. Doi:10.1016/B978-0-12-374001-4.00031-6.

Ali, A.K.A., and G.E. Shook. 1980. An Optimum Transformation for Somatic Cell Concentration in Milk. J Dairy Sci 63:487–490. Doi:10.3168/jds.S0022-0302(80)82959-6.

Aly, E., M.A. Khajah, and W. Masocha. 2020. B-caryophyllene, a CB2-receptor-selective phytocannabinoid, suppresses mechanical allodynia in a mouse model of antiretroviral-induced neuropathic pain. Molecules 25. Doi:10.3390/molecules25010106.

Anderson KL, Hunt E. Anti-inflammatory therapy in acute endotoxin-induced bovine mastitis. Vet Res Commun. 1989;13(1):17-26. doi: 10.1007/BF00366848. PMID: 2672555.Badinga, L., and C. Caldari-Torres. Omega-3 Fatty Acids Control Immune Stress in Dairy Cattle 1.

Barragan AA, Bas S, Hovingh E, Byler L. Effects of postpartum acetylsalicylic acid on uterine diseases and reproductive performance in dairy cattle. JDS Commun. 2021 Jan 22;2(2):67-72. doi: 10.3168/jdsc.2020-0047. PMID: 36338778; PMCID: PMC9623653.Basu S, Dittel BN.

Unraveling the complexities of cannabinoid receptor 2 (CB2) immune regulation in health and disease. Immunol Res. 2011 Oct;51(1):26-38. doi: 10.1007/s12026-011-8210-5. PMID: 21626285; PMCID: PMC4624216.Bertoni, G., E. Trevisi, and F. Piccioli-Cappelli. 2004.

Effects of acetyl-salicylate used in post-calving of dairy cows. Vet Res Commun 28:217–219. Doi:10.1023/B:VERC.0000045410.86004.03.

Bolognini, D., B. Costa, S. Maione, F. Comelli, P. Marini, V. Di Marzo, D. Parolaro, R.A. Ross, L.A. Gauson, M.G. Cascio, and R.G. Pertwee. 2010. The plant cannabinoid Δ 9-tetrahydrocannabivarin can decrease signs of inflammation and inflammatory pain in mice. Br J Pharmacol 160:677–687. Doi:10.1111/j.1476-5381.2010.00756.x.

Bradford, B. 2020. Transition Cows-How Fatty Acids Affect Immunity, Production and Health. Gainesville.

Bradford, B.J., K. Yuan, J.K. Farney, L.K. Mamedova, and A.J. Carpenter. 2015. Invited review: Inflammation during the transition to lactation: New adventures with an old flame. J Dairy Sci 98:6631–6650. Doi:10.3168/jds.2015-9683.

Breen, J. 2017. The importance of non-steroidal anti-inflammatory drugs (NSAIDs) in mastitis therapeutics. Livestock 22:182–185. Doi:10.12968/live.2017.22.4.182.

Byrne, K.A., C.L. Loving, and J.L. McGill. 2020. Innate Immunomodulation in Food Animals: Evidence for Trained Immunity?. Front Immunol 11. Doi:10.3389/fimmu.2020.01099.

Cain, D.W., and J.A. Cidlowski. 2017. Immune regulation by glucocorticoids. Nat Rev Immunol 17:233–247. Doi:10.1038/nri.2017.1.

Calder PC. Long-chain fatty acids and inflammation. Proc Nutr Soc. 2012 May;71(2):284-9. doi: 10.1017/S0029665112000067. Epub 2012 Feb 28. PMID: 22369781.

Calder PC. Feeding the immune system. Proc Nutr Soc. 2013 Aug;72(3):299-309. doi: 10.1017/S0029665113001286. Epub 2013 May 21. PMID: 23688939.

Capozzi, A., D. Caissutti, V. Mattei, F. Gado, S. Martellucci, A. Longo, S. Recalchi, V. Manganelli, G. Riitano, T. Garofalo, M. Sorice, C. Manera, and R. Misasi. 2022. Anti-inflammatory activity of a cb2 selective cannabinoid receptor agonist: Signaling and cytokines release in blood mononuclear cells. Molecules 27. Doi:10.3390/molecules27010064.

Carrasco, F.R., G. Schmidt, A.L. Romero, J.L. Sartoretto, S.M. Caparroz-Assef, C.A. Bersani-Amado, and R.K.N. Cuman. 2009. Immunomodulatory activity of <I>Zingiber officinale</I> Roscoe, <I>Salvia officinalis</I> L. and <I>Syzygium aromaticum</I> L. essential oils: evidence for humor- and cell-mediated responses. Journal of Pharmacy and Pharmacology 61:961–967. Doi:10.1211/jpp/61.07.0017.

Ceciliani, F., J.J. Ceron, P.D. Eckersall, and H. Sauerwein. 2012. Acute phase proteins in ruminants. J Proteomics 75:4207–4231. Doi:10.1016/j.jprot.2012.04.004.

Cha, E., D. Bar, J.A. Hertl, L.W. Tauer, G. Bennett, R.N. González, Y.H. Schukken, F.L. Welcome, and Y.T. Gröhn. 2011. The cost and management of different types of clinical mastitis in dairy cows estimated by dynamic programming. J Dairy Sci 94:4476–4487. Doi:10.3168/jds.2010-4123.

Chandrasekharan, N., Simmons, D.L. The cyclooxygenases. Genome Biol 5, 241 (2004). https://doi.org/10.1186/gb-2004-5-9-241Chaplin, D.D. 2010. Overview of the immune response. Journal of Allergy and Clinical Immunology 125. Doi:10.1016/j.jaci.2009.12.980.

Chayasirisobhon, S. 2021. Mechanisms of Action and Pharmacokinetics of Cannabis. Permanente Journal 25. Doi:10.7812/TPP/19.200.

Chen, J.H., and C.-T. Ho. 1997. Antioxidant Activities of Caffeic Acid and Its Related Hydroxycinnamic Acid Compounds.

Cheng, W.N., and S.G. Han. 2020. Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments — A review. Asian-Australas J Anim Sci 33:1699–1713. Doi:10.5713/ajas.20.0156.

Chiurchiù, V., M. Lanuti, M. De Bardi, L. Battistini, and M. Maccarrone. 2015. The differential characterization of GPR55 receptor in human peripheral blood reveals a distinctive expression in monocytes and NK cells and a proinflammatory role in these innate cells. Int Immunol 27:153–160. Doi:10.1093/intimm/dxu097.

Contreras, G.A., S.A. Mattmiller, W. Raphael, J.C. Gandy, and L.M. Sordillo. 2012. Enhanced n-3 phospholipid content reduces inflammatory responses in bovine endothelial cells. J Dairy Sci 95:7137–7150. Doi:10.3168/jds.2012-5729.

Correa, F., F. Docagne, L. Mestre, D. Clemente, M. Hernangómez, F. Loría, and C. Guaza. 2009. A role for CB2 receptors in anandamide signalling pathways involved in the regulation of IL-12 and IL-23 in microglial cells. Biochem Pharmacol 77:86–100. Doi:10.1016/j.bcp.2008.09.014.

Erskine, R.J., P.C. Bartlett, K.M. Sabo, and L.M. Sordillo. 2011. Bovine leukemia virus infection in dairy cattle: Effect on serological response to immunization against J5 Escherichia coli Bacterin. Vet Med Int 2011. Doi:10.4061/2011/915747.

Finch, J.M., and R.J. Turner. 1996. Effects of selenium and vitamin E on the immune responses of domestic animals.

Francomano, F., A. Caruso, A. Barbarossa, A. Fazio, C. La Torre, J. Ceramella, R. Mallamaci, C. Saturnino, D. Iacopetta, and M.S. Sinicropi. 2019. B-caryophyllene: A sesquiterpene with countless biological properties. Applied Sciences (Switzerland) 9. Doi:10.3390/app9245420.

Fujisawa, S., T. Atsumi, Y. Kadoma, and H. Sakagami. 2002. Antioxidant and prooxidant action of eugenol-related compounds and their cytotoxicity.

Garavaglia, L., S. Galletti, and D. Tedesco. 2015. Silymarin and lycopene administration in periparturient dairy cows: effects on milk production and oxidative status. N Z Vet J 63:313–318. Doi:10.1080/00480169.2015.1047911.

Gertsch, J., Leonti, M., Raduner, S., Racz, I., Chen, J.-Z., Xie, X.-Q., Altmann, K.-H., Karsak, M., & Zimmer, A. (2008). Beta-caryophyllene is a dietary cannabinoid. Proc Natl Acad Sci 105:9099– 9104.

Gertsch, J., R.G. Pertwee, and V. Di Marzo. 2010. Phytocannabinoids beyond the Cannabis plant - Do they exist? Br J Pharmacol 160:523–529. doi:10.1111/j.1476-5381.2010.00745.x.

González, R., I. Ballester, R. López-Posadas, M.D. Suárez, A. Zarzuelo, O. Martínez-Augustin, and F. Sánchez de Medina. 2011. Effects of flavonoids and other polyphenols on inflammation. Crit Rev Food Sci Nutr 51:331–362. doi:10.1080/10408390903584094.

Greco, L.F., J.T.N. Neto, A. Pedrico, R.A. Ferrazza, F.S. Lima, R.S. Bisinotto, N. Martinez, M. Garcia, E.S. Ribeiro, G.C. Gomes, J.H. Shin, M.A. Ballou, W.W. Thatcher, C.R. Staples, and J.E.P. Santos. 2015. Effects of altering the ratio of dietary n-6 to n-3 fatty acids on performance and inflammatory responses to a lipopolysaccharide challenge in lactating Holstein cows. J Dairy Sci 98:602–617. doi:10.3168/jds.2014-8805.

Guerra-Maupome, M., D.X. Vang, and J.L. McGill. 2019. Aerosol vaccination with Bacille Calmette-Guerin induces a trained innate immune phenotype in calves. PLoS One 14. doi:10.1371/journal.pone.0212751.

Gulhar, R., M.A. Ashraf, and I. Jialal. 2023. Physiology, Acute Phase Reactants.

Harris, S.L., D.A. Clark, and P.J. Laboyrie. 1998. Birdsfoot trefoil-an alternative legume for New Zealand dairy pastures.

Hashemzadeh-Cigari, F., M. Khorvash, G.R. Ghorbani, M. Kadivar, A. Riasi, and Q. Zebeli. 2014. Effects of supplementation with a phytobiotics-rich herbal mixture on performance, udder health, and metabolic status of Holstein cows with various levels of milk somatic cell counts. J Dairy Sci 97:7487–7497. doi:10.3168/jds.2014-7989.

Hoffmann, A., and D. Baltimore. 2006. Circuitry of nuclear factor κB signaling. Immunol Rev 210:171–186. doi:10.1111/j.0105-2896.2006.00375.x.

Hogan, J., and K.L. Smith. 2003. Coliform mastitis. Vet Res 34:507–519. doi:10.1051/vetres:2003022.

Ifrim, D.C., J. Quintin, L.A.B. Joosten, C. Jacobs, T. Jansen, L. Jacobs, N.A.R. Gow, D.L. Williams, J.W.M. Van Der Meer, and M.G. Netea. 2014. Trained immunity or tolerance: Opposing functional programs induced in human monocytes after engagement of various pattern recognition receptors. Clinical and Vaccine Immunology 21:534–545. doi:10.1128/CVI.00688-13.

Iribarren, P., H. Cui, Y. Le, G. Ying, X. Zhang, W. Gong, and J.M. Wang. 2003. IL-4 Down-Regulates Lipopolysaccharide-Induced Formyl Peptide Receptor 2 in Murine Microglial Cells by Inhibiting the Activation of Mitogen-Activated Protein Kinases 1,2.

Jermann, P.M., L.A. Wagner, D. Fritsche, J.J. Gross, O. Wellnitz, and R.M. Bruckmaier. 2023. Acute phase reaction to LPS induced mastitis in early lactation dairy cows fed nitrogenic, glucogenic or lipogenic diets. J Dairy Sci. doi:10.3168/jds.2023-23582.

Johnzon, C.F., J. Dahlberg, A.M. Gustafson, I. Waern, A.A. Moazzami, K. Östensson, and G. Pejler. 2018. The effect of lipopolysaccharide-induced experimental bovine mastitis on clinical parameters, inflammatory markers, and the metabolome: A kinetic approach. Front Immunol 9. doi:10.3389/fimmu.2018.01487.

Jürg Gertsch, C., and J. Gertsch. 2017. Cannabimimetic phytochemicals in the diet-an evolutionary link to food selection and metabolic stress adaptation? * LINKED ARTICLES. Br J Pharmacol 174:1464–1483. doi:10.1111/bph.v174.11/issuetoc.

Klauke, A.L., I. Racz, B. Pradier, A. Markert, A.M. Zimmer, J. Gertsch, and A. Zimmer. 2014. The cannabinoid CB2 receptor-selective phytocannabinoid beta-caryophyllene exerts analgesic effects in mouse models of inflammatory and neuropathic pain. European Neuropsychopharmacology 24:608–620. doi:10.1016/j.euroneuro.2013.10.008.

Klein, T.W. 2005. Cannabinoid-based drugs as anti-inflammatory therapeutics. Nat Rev Immunol 5:400–411. doi:10.1038/nri1602.

Ku-Vera, J.C., R. Jiménez-Ocampo, S.S. Valencia-Salazar, M.D. Montoya-Flores, I.C. Molina-Botero, J. Arango, C.A. Gómez-Bravo, C.F. Aguilar-Pérez, and F.J. Solorio-Sánchez. 2020. Role of Secondary Plant Metabolites on Enteric Methane Mitigation in Ruminants. Front Vet Sci 7. doi:10.3389/fvets.2020.00584.

Lee, J.Y., L. Zhao, and D.H. Hwang. 2010. Modulation of pattern recognition receptor-mediated inflammation and risk of chronic diseases by dietary fatty acids. Nutr Rev 68:38–61. doi:10.1111/j.1753-4887.2009.00259.x.

Liu, H., G. Yang, Y. Tang, D. Cao, T. Qi, Y. Qi, and G. Fan. 2013. Physicochemical characterization and pharmacokinetics evaluation of β-caryophyllene/β-cyclodextrin inclusion complex. Int J Pharm 450:304–310. doi:10.1016/j.ijpharm.2013.04.013.

Lohuis, J.A.C.M., W. Van Leeuwen, J.H.M. Verheijden, A.S.J.P.A.M. Van Miert, and A. Brand. 1988. Effect of Dexamethasone on Experimental Escherichia coli Mastitis in the Cow. J Dairy Sci 71:2782–2789. doi:10.3168/jds.S0022-0302(88)79872-0.

Mattiuzzo, E., A. Faggian, R. Venerando, A. Benetti, E. Belluzzi, G. Abatangelo, P. Ruggieri, and P. Brun. 2021. In vitro effects of low doses of β-Caryophyllene, ascorbic acid and D-Glucosamine on human chondrocyte viability and inflammation. Pharmaceuticals 14. doi:10.3390/ph14030286.

Medzhitov, R. 2008. Origin and physiological roles of inflammation. Nature 454:428–435. doi:10.1038/nature07201.

Middleton, E., C. Kandaswami, and T.C. Theoharides. 2000. The Effects of Plant Flavonoids on Mammalian Cells: Implications for Inflammation, Heart Disease, and Cancer.

Miller, J.K., E. Brzezinska-Slebodzinska, and F.C. Madsen. 1993. Oxidative Stress, Antioxidants, and Animal Function. J Dairy Sci 76:2812–2823. doi:10.3168/jds.S0022-0302(93)77620-1.

Miranda, J., A. Lasa, L. Aguirre, A. Fernandez-Quintela, I. Milton, and M.P. Portillo. 2014. Potential Application of Non-flavonoid Phenolics in Diabetes: Antiinflammatory Effects. Curr Med Chem 22:112–131. doi:10.2174/09298673216661408151235O7.

Mödinger, Y., K. Knaub, T. Dharsono, R. Wacker, R. Meyrat, M.H. Land, A.L. Petraglia, and C. Schön. 2022. Enhanced Oral Bioavailability of β-Caryophyllene in Healthy Subjects Using the VESIsorb® Formulation Technology, a Novel Self-Emulsifying Drug Delivery System (SEDDS). Molecules 27. doi:10.3390/molecules27092860.

Mogensen, T.H. 2009. Pathogen recognition and inflammatory signaling in innate immune defenses. Clin Microbiol Rev 22:240–273. doi:10.1128/CMR.00046-08.

Morkoç, A.C., W.L. Hurley, H.L. Whitmore, and B.K. Gustafsson. 1993. Bovine Acute Mastitis: Effects of Intravenous Sodium Salicylate on Endotoxin-Induced Intramammary Inflammation. J Dairy Sci 76:2579–2588. doi:10.3168/jds.S0022-0302(93)77592-X.

Murat Tatar, A., D. Şireli, and M. Tutkun. 2017. REASONS FOR CULLING AND REPLACEMENT RATE IN DAIRY CATTLE Animal Mechanization View project Structural Analysis of Dairy enterprises in Diyarbakır Province/Turkey View project.

Netea MG. Training innate immunity: the changing concept of immunological memory in innate host defence. Eur J Clin Invest. 2013 Aug;43(8):881-4. doi: 10.1111/eci.12132. PMID: 23869409.

Newby, N.C., K.E. Leslie, H.D.P. Dingwell, D.F. Kelton, D.M. Weary, L. Neuder, S.T. Millman, and T.F. Duffield. 2017. The effects of periparturient administration of flunixin meglumine on the health and production of dairy cattle. J Dairy Sci 100:582–587. doi:10.3168/jds.2016-11747.

Newton, K., and V.M. Dixit. 2012. Signaling in innate immunity and inflammation. Cold Spring Harb Perspect Biol 4. doi:10.1101/cshperspect.a006049.

Ogala, H., A. Shitandi, and J. Nanua. 2007. Effect of mastitis on raw milk compositional quality. Journal of Veterinary Science 8:237–242.

Olagaray, K.E., and B.J. Bradford. 2019. Plant flavonoids to improve productivity of ruminants – A review. Anim Feed Sci Technol 251:21–36. doi:10.1016/j.anifeedsci.2019.02.004.

Oliveira, G.L. da S., K.C. Machado, K.C. Machado, A.P. dos S.C.L. da Silva, C.M. Feitosa, and F.R. de Castro Almeida. 2018. Non-clinical toxicity of β-caryophyllene, a dietary cannabinoid: Absence of adverse effects in female Swiss mice. Regulatory Toxicology and Pharmacology 92:338–346. doi:10.1016/j.yrtph.2017.12.013.

Overton, M.W., and K.C. Dhuyvetter. 2020. Symposium review: An abundance of replacement heifers: What is the economic impact of raising more than are needed?. J Dairy Sci 103:3828–3837. doi:10.3168/jds.2019-17143.

Pertwee, R.G. 2006. Cannabinoid pharmacology: The first 66 years. Br J Pharmacol 147. doi:10.1038/sj.bjp.0706406.

Pertwee, R.G., and R.A. Ross. 2002a. Cannabinoid receptors and their ligands.

Pertwee, R.G., and R.A. Ross. 2002b. Cannabinoid receptors and their ligands.

Petzl, W., J. Günther, T. Pfister, C. Sauter-Louis, L. Goetze, S. Von Aulock, A. Hafner-Marx, H.J. Schuberth, H.M. Seyfert, and H. Zerbe. 2012. Lipopolysaccharide pretreatment of the udder protects against experimental Escherichia coli mastitis. Innate Immun 18:467–477. doi:10.1177/1753425911422407.

Picciolo, G., G. Pallio, D. Altavilla, M. Vaccaro, G. Oteri, N. Irrera, and F. Squadrito. 2020. β-Caryophyllene reduces the inflammatory phenotype of periodontal cells by targeting CB2 receptors. Biomedicines 8. doi:10.3390/BIOMEDICINES8060164.

Poulopoulou, I., and I. Hadjigeorgiou. 2021. Evaluation of Terpenes' Degradation Rates by Rumen Fluid of Adapted and Non-adapted Animals. Nat Prod Bioprospect 11:307–313. doi:10.1007/s13659-020-00289-3.

Quaye, I.K. 2008. Haptoglobin, inflammation and disease. Trans R Soc Trop Med Hyg 102:735–742. doi:10.1016/j.trstmh.2008.04.010.

Rahaman, O., and D. Ganguly. 2021. Endocannabinoids in immune regulation and immunopathologies. Immunology 164:242–252. doi:10.1111/imm.13378.

Rhen, T., and J.A. Cidlowski. 2005. Antiinflammatory Action of Glucocorticoids-New Mechanisms for Old Drugs.

Roth, J.A. 1982. Effect of glucocorticoids on the bovine immune system Pathogenesis of Mycoplasma hyopneumoniae View project.

Scandiffio, R., F. Geddo, E. Cottone, G. Querio, S. Antoniotti, M. Pia Gallo, M.E. Maffei, and P. Bovolin. 2020. Protective effects of (E)-β-caryophyllene (bcp) in chronic inflammation. Nutrients 12:1–24. doi:10.3390/nu12113273.

Schottelius, A.J.G., M.W. Mayo, R.B. Sartor, and A.S. Baldwin. 1999. Interleukin-10 Signaling Blocks Inhibitor of B Kinase Activity and Nuclear Factor B DNA Binding*.

Serhan, C.N. 2009. Systems approach to inflammation resolution: Identification of novel anti-inflammatory and pro-resolving mediators. Journal of Thrombosis and Haemostasis 7:44–48. doi:10.1111/j.1538-7836.2009.03396.x.

Shpigel, N.Y., M. Wlnkler, A. Saran, and G. Ziv. 1994. Anti-inflammatory ketoprofen in the treatment of field cases of bovine mastitis.

Sinz, S., S. Marquardt, C.R. Soliva, U. Braun, A. Liesegang, and M. Kreuzer. 2019. Phenolic plant extracts are additive in their effects against in vitro ruminal methane and ammonia formation. Asian-Australas J Anim Sci 32:966–976. doi:10.5713/ajas.18.0665.

Smith, G., J. Davis, L. Tell, A. Webb, and J. Riviere. 2008. Extralabel use of nonsteroidal anti-inflammatory drugs in cattle. American Veterinary Medical Association 232:697–701.

Smith, K.L., D.A. Todhunter, and P.S. Schoenberger. 1985. Environmental Mastitis: Cause, Prevalence, Prevention. J Dairy Sci 68:1531–1553. doi:10.3168/jds.S0022-0302(85)80993-0.

Soltan, M.A. 2010. Effect of dietary chromium supplementation on productive and reproductive performance of early lactating dairy cows under heat stress. J Anim Physiol Anim Nutr (Berl) 94:264–272. doi:10.1111/j.1439-0396.2008.00913.x.

Soltan, Y.A., P.E. Simitzis, N. Zhang, M. Besharati, and A. Lara-Bueno. 2023. Meta-analysis of flavonoids use into beef and dairy cattle diet: Performance, antioxidant status, ruminal fermentation, meat quality, and milk composition.

Sordillo, L.M. 2016. Nutritional strategies to optimize dairy cattle immunity. J Dairy Sci 99:4967–4982. doi:10.3168/jds.2015-10354.

Sordillo, L.M. 2018. Symposium review: Oxylipids and the regulation of bovine mammary inflammatory responses1. J Dairy Sci 101:5629–5641. doi:10.3168/jds.2017-13855.

Sordillo, L.M., and S.L. Aitken. 2009. Impact of oxidative stress on the health and immune function of dairy cattle. Vet Immunol Immunopathol 128:104–109. doi:10.1016/j.vetimm.2008.10.305.

Sordillo, L.M., and W. Raphael. 2013. Significance of metabolic stress, lipid mobilization, and inflammation on transition cow disorders. Veterinary Clinics of North America - Food Animal Practice 29:267–278. doi:10.1016/j.cvfa.2013.03.002.

Sordillo LM, Streicher KL. Mammary gland immunity and mastitis susceptibility. J Mammary Gland Biol Neoplasia. 2002 Apr;7(2):135-46. doi: 10.1023/a:1020347818725. PMID: 12463736.

Swartz, T. H., B. J. Bradford, L. K. Mamedova, and K. A. Estes. 2023. Effects of dietary rumen-protected choline supplementation to periparturient dairy cattle on inflammation, metabolism, and performance during an intramammary lipopolysaccharide challenge. J Dairy Sci. 106:8561–82. https://doi.org/10.3168/jds.2023-23259.

Di Sotto, A., P. Paolicelli, M. Nardoni, L. Abete, S. Garzoli, S. Di Giacomo, G. Mazzanti, M.A. Casadei, and S. Petralito. 2018. SPC liposomes as possible delivery systems for improving bioavailability of the natural sesquiterpene β-caryophyllene: Lamellarity and drug-loading as key features for a rational drug delivery design. Pharmaceutics 10. doi:10.3390/pharmaceutics10040274.

Sullivan, T., A. Sharma, K. Lamers, B.A. Mallard, A. Cánovas, and N.A. Karrow. 2023. Immunotolerance of dairy heifers in response to repeated exposure to bacterial lipopolysaccharide endotoxin. Vet Immunol Immunopathol 259. doi:10.1016/j.vetimm.2023.110579.

Sung, M.H., N. Li, Q. Lao, R.A. Gottschalk, G.L. Hager, and I.D.C. Fraser. 2014. Switching of the relative dominance between feedback mechanisms in lipopolysaccharide-induced NF-κB signaling. Sci Signal 7. doi:10.1126/scisignal.2004764.

Suojala, L., T. Orro, H. Järvinen, J. Saatsi, and S. Pyörälä. 2008. Acute phase response in two consecutive experimentally induced E. coli intramammary infections in dairy cows. Acta Vet Scand 50. doi:10.1186/1751-0147-50-18.

Swartz, T.H., and B.J. Bradford. Phytogenics and potential uses for enhancing resilience of ruminant livestock.

Takeuchi, O., and S. Akira. 2010. Pattern Recognition Receptors and Inflammation. Cell 140:805–820. doi:10.1016/j.cell.2010.01.022.

Taylor, C.C., M.S. Allen, and M.S. Allen. 2005. Corn Grain Endosperm Type and Brown Midrib 3 Corn Silage: Site of Digestion and Ruminal Digestion Kinetics in Lactating Cows. American Dairy Science Association.

Tedesco, D., A. Tava, S. Galletti, M. Tameni, G. Varisco, A. Costa, and S. Steidler. 2004. Effects of silymarin, a natural hepatoprotector, in periparturient dairy cows. J Dairy Sci 87:2239–2247. doi:10.3168/jds.S0022-0302(04)70044-2.

Timmermans, S., J. Souffriau, and C. Libert. 2019. A general introduction to glucocorticoid biology. Front Immunol 10. doi:10.3389/fimmu.2019.01545.

Trimboli, F., M. Ragusa, C. Piras, V. Lopreiato, and D. Britti. 2020. Outcomes from experimental testing of nonsteroidal anti-inflammatory drug (Nsaid) administration during the transition period of dairy cows. Animals 10:1–13. doi:10.3390/ani10101832.

Vane, J., and R. Botting. 1987. Inflammation and the mechanism of action of anti-inflammatory drugs. The FASEB Journal 1:89–96. doi:10.1096/fasebj.1.2.3111928.

Vane, J.R., Y.S. Bakhle, and R.M. Botting. 1998. CYCLOOXYGENASES 1 AND 2.

Waghorn, G.C., and W.C. McNabb. 2003. Consequences of plant phenolic compounds for productivity and health of ruminants. Proceedings of the Nutrition Society 62:383–392. doi:10.1079/pns2003245.

Walker, C.C.F., L.M. Sordillo, and G.A. Contreras. 2022a. Anandamide Alters Barrier Integrity of Bovine Vascular Endothelial Cells during Endotoxin Challenge. Antioxidants 11. doi:10.3390/antiox11081461.

Walker, C.C.F., L.M. Sordillo, and G.A. Contreras. 2022b. Anandamide Alters Barrier Integrity of Bovine Vascular Endothelial Cells during Endotoxin Challenge. Antioxidants 11:1461. doi:10.3390/antiox11081461.

Wall, S.K., L.E. Hernández-Castellano, A. Ahmadpour, R.M. Bruckmaier, and O. Wellnitz. 2016. Differential glucocorticoid-induced closure of the blood-milk barrier during lipopolysaccharide- and lipoteichoic acid-induced mastitis in dairy cows. J Dairy Sci 99:7544–7553. doi:10.3168/jds.2016-11093.

Wang, J., C. Guo, Z. Wei, X. He, J. Kou, E. Zhou, Z. Yang, and Y. Fu. 2016. Morin suppresses inflammatory cytokine expression by downregulation of nuclear factor-κB and mitogen-activated protein kinase (MAPK) signaling pathways in lipopolysaccharide-stimulated primary bovine mammary epithelial cells. J Dairy Sci 99:3016–3022. doi:10.3168/jds.2015-10330.

Wildman, E.E., G.M. Jones, P.E. Wagner, R.L. Boman, H.F. Troutt, and T.N. Lesch. 1982. A Dairy Cow Body Condition Scoring System and Its Relationship to Selected Production Characteristics. J Dairy Sci 65:495–501. doi:10.3168/jds.S0022-0302(82)82223-6.

Woodward, S., M. Auldist, P. Laboyrie, and E. Jansen. 1999. Effect of Lotus corniculatus and condensed tannins on milk yield and milk composition of dairy cows.

Worku, M., and H. Ismail. 2020. A Review of the Effect of Plant-derived Bioactive Substances on the Inflammatory Response of Ruminants (Sheep, Cattle, and Goats) Biopsia Project View project Mutation detection View project. doi:10.31021/ijvam.20203130.

Yeiser, E.E., K.E. Leslie, M.L. McGilliard, and C.S. Petersson-Wolfe. 2012. The effects of experimentally induced Escherichia coli mastitis and flunixin meglumine administration on activity measures, feed intake, and milk parameters. J Dairy Sci 95:4939–4949. doi:10.3168/jds.2011-5064.

Yoza, B.K., and C.E. McCall. 2011. Facultative heterochromatin formation at the IL-1 beta promoter in LPS tolerance and sepsis. Cytokine 53:145–152. doi:10.1016/j.cyto.2010.10.007.

Zachut, M., G. Kra, U. Moallem, L. Livshitz, Y. Levin, S. Udi, A. Nemirovski, and J. Tam. 2018. Characterization of the endocannabinoid system in subcutaneous adipose tissue in periparturient dairy cows and its association to metabolic profiles. PLoS One 13. doi:10.1371/journal.pone.0205996.

Zielińska, D., H. Zieliński, J.M. Laparra-Llopis, D. Szawara-Nowak, J. Honke, and J.A. Giménez-Bastida. 2021. Caffeic acid modulates processes associated with intestinal inflammation. Nutrients 13:1–15. doi:10.3390/nu13020554.

Ziring, D., B. Wei, P. Velazquez, M. Schrage, N.E. Buckley, and J. Braun. 2006. Formation of B and T cell subsets require the cannabinoid receptor CB2. Immunogenetics 58:714–725. doi:10.1007/s00251-006-0138-x.

APPENDIX

TABLES AND FIGURES

Table 1. Nutrient composition of the total mixed ration fed to lactating dairy cows throughout the experiment[1].

Item, %DM	Mean	SD
DM	50.70	2.455
CP	15.76	0.802
Starch	25.50	1.298
ESC[2]	4.64	1.369
NDF	32.92	2.061
Lignin	3.63	0.347
Crude Fat	3.79	0.256
Ash	6.45	0.483
Ca	0.81	0.219
P	0.38	0.109
Mg	0.34	0.095
K	1.23	0.275
S	0.19	0.011
Na	0.35	0.083
Cl	0.28	0.025

[1]n = 5 composite samples analyzed

[2]ESC = ethanol soluble carbohydrates

Table 2. Effect of β-Caryophyllene (BCP) on feed intake, milk production, and milk composition prior to, during, and after intramammary challenges with lipopolysaccharide (LPS).

Item	Treatment		SEM	*P* -values			
	BCP	CON		Trt	Day	Trt × Day	Lactgrp2
Pre-challenge							
DMI, kg/d	24.40	24.90	1.10	0.68	<0.01	0.26	0.02
Milk, kg/d	35.30	37.00	2.22	0.50	<0.01	0.76	0.06
ECM, kg/d	34.97	34.67	0.60	0.68	<0.01	0.89	0.28
Fat, kg/d	1.39	1.43	0.25	0.67	0.01	0.81	0.10
Protein, kg/d	1.14	1.22	0.07	0.39	0.75	0.72	0.02
SCS[1]	0.52	1.07	0.35	0.14	0.17	0.71	0.96
Post -challenge 1							
DMI, kg/d	24.84	25.74	0.20	0.51	<0.01	<0.01	0.03
Milk, kg/d	30.51	33.31	3.03	0.41	<0.01	0.84	0.46
ECM, kg/d	35.40	35.30	0.80	0.90	<0.01	0.72	0.96
Fat, kg/d	1.39	1.46	0.10	0.55	0.09	0.92	0.73
Protein, kg/d	1.11	1.17	0.11	0.54	<0.01	0.33	0.28
SCS[1]	2.12	2.85	0.36	0.06	<0.01	<0.01	0.63
Post -challenge 2							
Milk, kg/d	26.86	30.00	2.71	0.38	<0.01	0.81	0.77
ECM, kg/d	35.73	35.12	0.95	0.53	<0.01	<0.01	0.44
SCS[1]	8.23	7.82	0.45	0.29		<.0001	0.63

[1]Somatic cell score (SCS) = $\log_2$ (SCC/100,000)+3

[2]Lactgrp = lactation group, primiparous vs. multiparous

Table 3. Effect of β-caryophyllene (BCP) on acute phase proteins after intramammary challenges with lipopolysaccharide (LPS).

Item	Treatment		SEM	*P* -values				
	BCP	CON		Trt	Time	Trt × Time	Lactgrp[1]	BLV
LPS Challenge 1								
BHB, mmol/L	0.59	0.52	0.05	0.16	<0.01	<.04	0.92	0.26
Glucose, mg/dL	72.98	75.06	3.02	0.50	<0.01	0.96	0.56	0.13
Haptoglobin, mg/L	79.87	117.88	12.52	<0.01	<0.01	0.22	0.01	0.63
Insulin, ng/mL	1.48	1.33	0.21	0.50	<0.01	0.78	0.76	0.42
LBP, mg/L	6.41	6.49	1.06	0.95	<0.01	0.11	0.49	0.87
LPS Challenge 2								
BHB, mmol/L	0.51	0.49	0.03	0.42	<0.01	0.79	0.60	0.28
Glucose, mg/dL	77.29	76.58	4.40	0.87	0.39	0.39	0.53	0.55
Haptoglobin, mg/L	52.38	68.84	14.19	0.26	<0.01	0.38	0.80	0.82
Insulin, ng/mL	1.38	1.28	0.19	0.47	<0.01	0.13	0.29	0.51
LBP, mg/L	6.28	7.45	0.80	0.16	<0.01	0.67	0.03	0.66

[1]Lactgrp = lactation group, primiparous vs. multiparous

Table 4. Effect of β-caryophyllene (BCP) on the area under the curve of acute phase proteins and body temperature after intramammary challenges with lipopolysaccharide (LPS). All reported values are relative to the basal period.

Item	LPS Challenge		SEM	*P* -values			
	First	Second		Trt	Lactgrp	BLV	Challenge
BHB	-3.17	-1.42	-1.75	0.84	0.41	0.3	0.37
Glucose	-28.61	112.00	-140.61	0.64	0.02	0.71	0.57
Haptglobin	543291	353952	45739	0.06	<.01	0.64	<.01
Insulin	18.29	19.22	5.06	0.95	0.78	0.39	0.86
LBP	496314	444139	28955	0.75	0.35	0.96	0.75
Body Temp	538.88	552.52	50.65	0.29	0.11	0.9	0.29

[1]Lactgrp = lactation group, primiparous vs. multiparous

Table 5. Effect of β-caryophyllene (BCP) on bodyweight and body condition score change in late lactation cows challenged with intramammary lipopolysaccharide (LPS).

Item	Treatment		SEM	*P* -values				
	BCP	CON		Trt	Week	Trt × Week	Lactgrp[2]	BLV
BW, kg/d	692.81	718.64	28.33	0.37	<0.01	0.75	<0.01	0.48
BCS, units/wk[1]	3.29	3.33	0.04	0.81	0.18	0.46	0.8	0.32

[1]Measured on a 1-5 scale (Wildman et al., 1982)
[2]Lactgrp = lactation group, primiparous vs. multiparous

Figure 1. Effect of β-caryophyllene (BCP) on intravaginal body temperature after the first lipopolysaccharide challenge. The solid vertical line indicates the time of the lipopolysaccharide challenge. Supplementing BCP did not affect body temperature after the first lipopolysaccharide challenge (*P* = .83)**.**

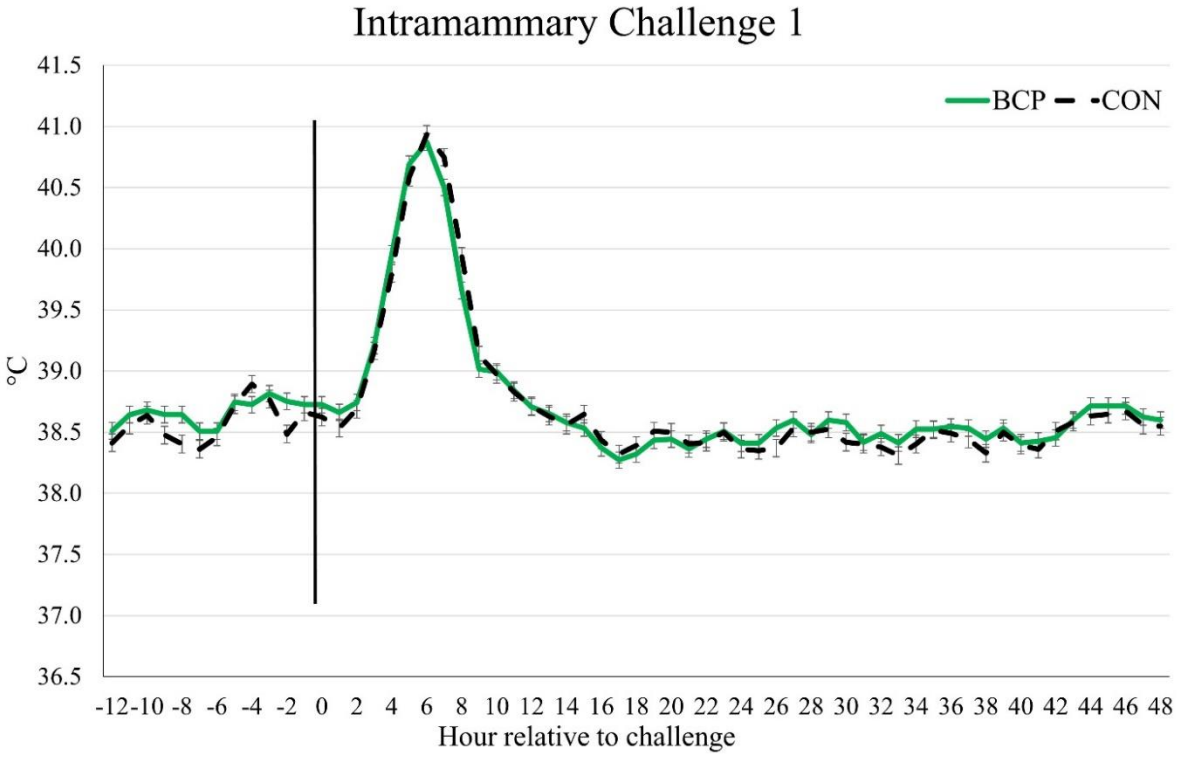

Figure 2. Effect of β-Caryophyllene (BCP) on body temperature after the second lipopolysaccharide challenge. BCP was not supplemented during the second lipopolysaccharide challenge. the solid vertical line indicates the time of the lipopolysaccharide challenge. Supplementing BCP did not affect body temperature after the second lipopolysaccharide challenge (*P* = .81).

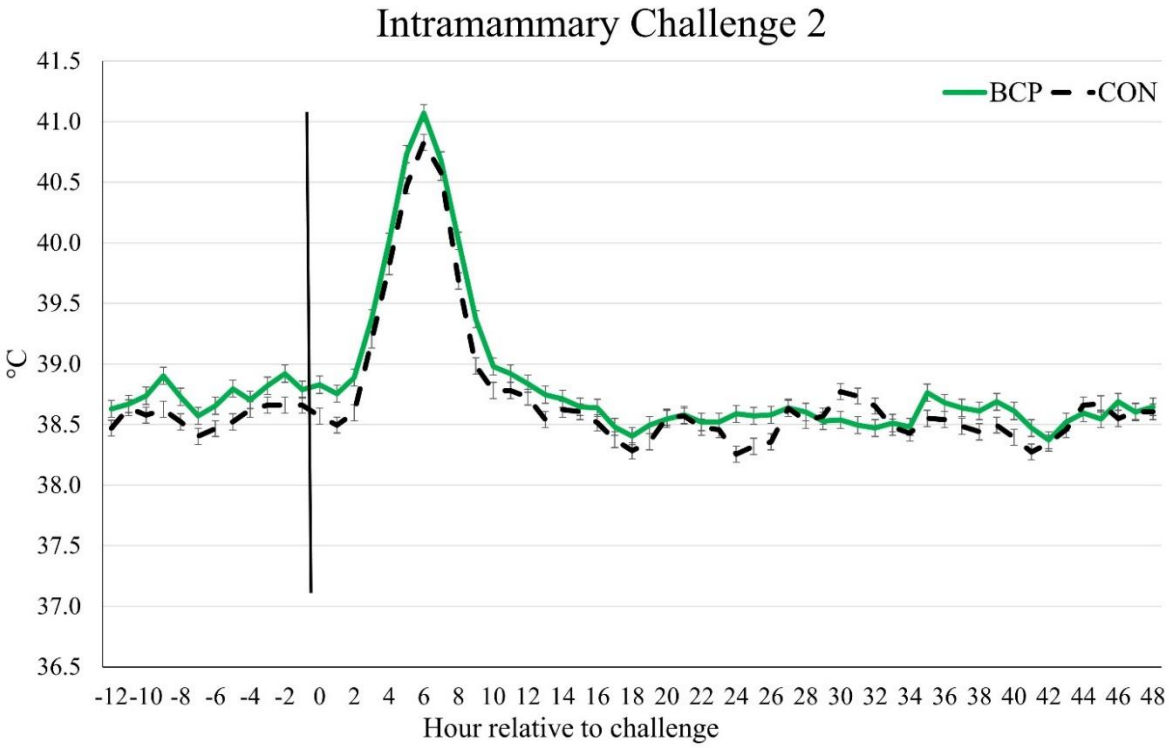

Figure 3. Composite somatic cell score (SCS = $\log_2$ [SCC / 100,000] + 3) of dairy cows fed β-caryophyllene (BCP) or a no-treatment control (CON). Cows were challenged with lipopolysaccharide in their right rear mammary gland, and results are shown for the challenged quarter. Treatment tended to affect SCS ($P = 0.06$), and there was a significant treatment × time interaction ($P < 0.01$), reflecting a tendency for BCP to reduce SCS relative to CON on d 24 post-challenge ($P = 0.06$).

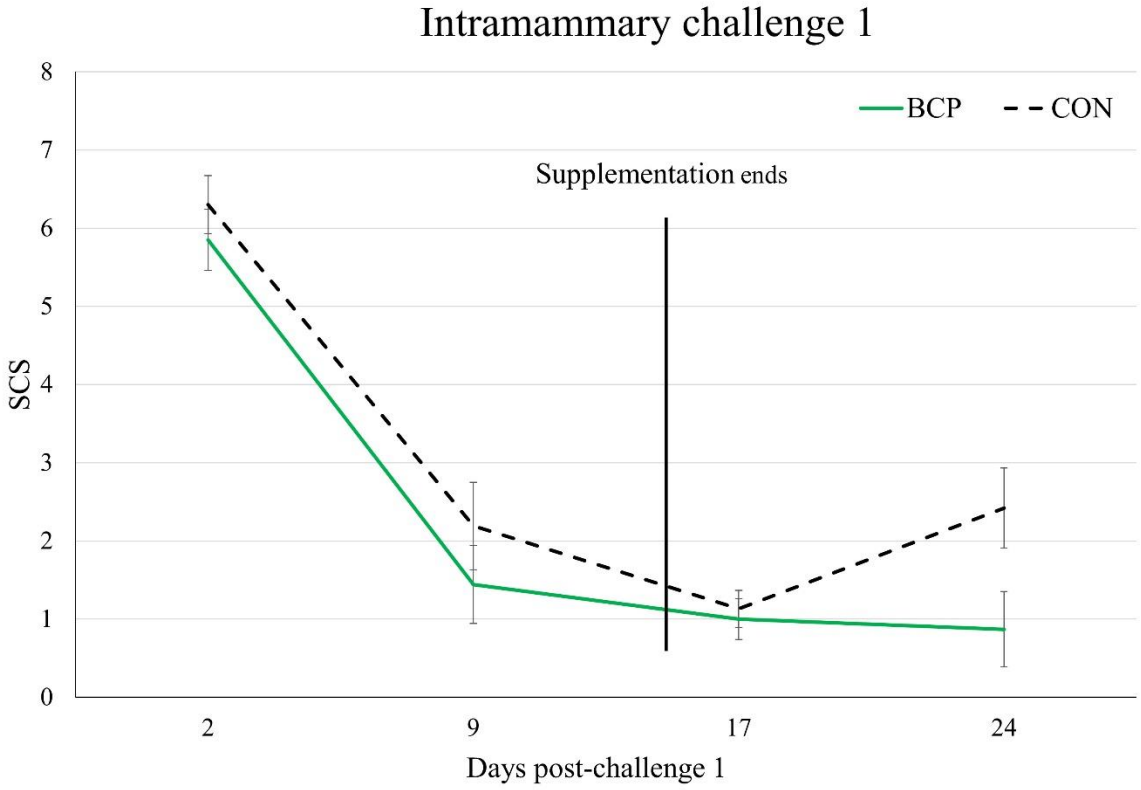

Figure 4. Quarter level somatic cell score somatic cell score (SCS = $\log_2$ [SCC / 100,000] + 3) of dairy cows fed β-caryophyllene (BCP) or a no-treatment control (CON). Cows were challenged a second time with lipopolysaccharide in their right rear quarter. Treatment did not affect SCS ($P = 0.43$). There was a significant effect of BCP supplementation ($P < 0.01$) at 16 hours post challenge ($P < 0.01$).