USING CHITOSAN MICROPARTICLES TO TREAT METRITIS IN LACTATING DAIRY COWS

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

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To my wife Tania and my dad Ribamar for all the support and encouragement given to me all my life.
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<tr>
<td>AI</td>
<td>Artificial Insemination</td>
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<td>APP</td>
<td>Acute phase proteins</td>
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<td>BCS</td>
<td>Body condition score</td>
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<td>BHB</td>
<td>Beta-hydroxybutyrate</td>
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<td>CON</td>
<td>Control</td>
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<td>CM</td>
<td>Chitosan microparticle</td>
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<tr>
<td>D</td>
<td>Day</td>
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<td>DIM</td>
<td>Days in milk</td>
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<td>DMI</td>
<td>Dry mater intake</td>
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<td>DPP</td>
<td>Days postpartum</td>
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<tr>
<td>EXD</td>
<td>Excede®</td>
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<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
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<tr>
<td>GH</td>
<td>Growth hormone</td>
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<td>GHR</td>
<td>Growth hormone receptor</td>
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<tr>
<td>GRAS</td>
<td>Generally Recognized as Safe</td>
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<td>Hb</td>
<td>Hemoglobin</td>
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<td>Hp</td>
<td>Haptoglobin</td>
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<td>HTH</td>
<td>Healthy</td>
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<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>i.m.</td>
<td>Intramuscular</td>
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<td>IUPEC</td>
<td>Intra-uterine pathogenic <em>E. coli</em></td>
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<td>Kg</td>
<td>Kilogram</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>L</td>
<td>Liter</td>
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<tr>
<td>LAP</td>
<td>Lingual antimicrobial peptide</td>
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<td>LPS</td>
<td>Lipopolysaccharides</td>
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<td>mmol</td>
<td>Millimole</td>
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<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
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<td>NLRS</td>
<td>Nod-like receptors</td>
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<tr>
<td>PAMPS</td>
<td>Pathogen-associated molecular patterns</td>
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<td>PMNs</td>
<td>Polymorphonuclear neutrophils</td>
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<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PU</td>
<td>Unit point</td>
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<tr>
<td>TAP</td>
<td>Tracheal antimicrobial peptide</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factors</td>
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<tr>
<td>VLS</td>
<td>Vaginal-vulvar laceration</td>
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A Thesis Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Master of Science

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By

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The objective was to evaluate the efficacy of intrauterine administration of chitosan microparticles (CM) for cure of metritis in dairy cows. Secondary objectives were to evaluate the effects of CM treatment on milk yield, reproductive performance, and survival of cows previously diagnosed with metritis. Holstein cows (n = 826) with metritis from three dairies located in northern FL were blocked by parity (primiparous or multiparous) and, within each block, randomly assigned to one of three treatments: CM (n = 276): intrauterine infusion of 24 g of CM dissolved in 40 mL of sterile distilled water at the time of metritis diagnosis (D0) and two (D2) and four (D4) days later; Excede® (EXD; n = 275): subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) in the base of the ear at D0 and D3; Control (CON; n = 275): no intrauterine or subcutaneous treatment. A group of healthy (HTH) cows was used for comparison. Data were analyzed by generalized linear mixed models. Proportion of cows cured on D12 was greater for EXD than for CM and CON (79.6 vs. 61.2 vs. 64.7%, respectively). The proportion of cows cured did not differ between CM and CON treatments. Culling in the first 60 days in milk (DIM) was lesser for EXD and CON than CM (8.4 vs. 10.6 vs. 19.2%), which were all greater than HTH (4.4%). Culling in the first
60 DIM did not differ between EXD and CON treatments. Treatment did not affect plasma NEFA (CM = 0.49 ± 0.03, CON = 0.46 ± 0.03, EXD = 0.51 ± 0.03, HTH = 0.54 ± 0.02 mmol/L), BHB (CM = 0.60 ± 0.03, CON = 0.56 ± 0.03, EXD = 0.60 ± 0.03, HTH = 0.63 ± 0.03 mmol/L), and haptoglobin (CM = 649.07 ± 54.49, CON = 490.38 ± 55.70, EXD = 556.11 ± 58.24, HTH = 541.08 ± 53.05 μg/mL) concentrations. Milk yield in the first 60 DIM was lowest for CM (37.12 ± 0.19 kg/d), followed by CON (38.22 ± 0.19 kg/d) and EXD (39.52 ± 0.19 kg/d), and greatest for HTH (42.26 ± 0.06 kg/day). In addition, milk yield in the first 10 months was lowest for CM (35.52 ± 0.44 kg/d), followed by CON (35.79 ± 0.43 kg/d) and EXD (36.46 ± 0.43 kg/d), and greatest for HTH (37.43 ± 0.25 kg/day). First service pregnancy per breeding did not differ among treatments, but hazard of pregnancy up to 300 DIM was less for CM or CON than EXD, which were all less than HTH. Median time to pregnancy was 149, 137, 131, and 113 DIM for CM, CON, EXD, and HTH, respectively. In summary, CM did not improve cure of metritis or hazard of pregnancy, and was detrimental to milk yield and culling compared with CON. Treatment of cows with EXD increased cure of metritis, milk yield and hazard of pregnancy compared with CON.
CHAPTER 1
LITERATURE REVIEW

Transition Period

The transition period is well described in the literature consisting in the 3 weeks before parturition and the following 3 weeks post parturition (Drackley, 1999; Grummer et al., 1995). The Latin origin of the word transition means, “go across” and it is defined as the process or period of changing from one state or condition to another. During this time, dairy cows experience several physiological and biochemical changes going from a pregnant non-lactating to a lactating non-pregnant state, which is accompanied with exerting stress and thus making them more susceptible to various metabolic and infectious diseases (Goff and Horst, 1991). There is also an increased demand for nutrients after parturition because of lactogenesis and galactopoiesis. The decrease in feed intake that occurs around calving results in an insufficient dietary intake of nutrients during the first 4 to 6 weeks of lactation (Hayirli et al., 2002; Grummer et al., 2004; Butler et al., 2006). The glucose, amino acids and fatty acids required by dairy cows between 21 days before parturition and 4 days post parturition (DPP) increased by four-fold for glucose, two-fold for amino acids and nearly a five-fold increase for fatty acids (Bell et al., 1995).

The nutritional requirements shift at parturition, such as milk production rapidly increases and cows enter a period of negative nutrient balance (Butler, 2000). Under the increased glucose requirements and the 20 to 35% decrease in dry matter intake (DMI) (Urton et al., 2005; Bell et al., 1995) during the last week of gestation, cows undergo an extensive period of body fat mobilization. The severity and duration of the negative energy balance experienced by post-partum cows is primarily related to DMI
and correlated with the cow’s body condition at calving (Butler, 2000). The extensive period of body fat mobilization results in an elevated concentration of blood plasma non-esterified fatty acid (NEFA) and beta-hydroxybutyrate (BHB) (NRC, 2001, Bell, 1995). As a consequence of the reduced DMI and the increased demand of energy after calving, NEFA is mobilized from body fat deposits as a source of energy (Bauman and Currie, 1980). Non-esterified fatty acids release from adipose tissue is also related to norepinephrine and epinephrine secretion. Epinephrine is released during stress; this means that a cow going through the stress of the transition period can have NEFA production increased via epinephrine secretion (Herdt, 2000).

Two characteristics of the lipolytic state are the decline in plasma insulin concentration and the reduced insulin sensitivity in adipose tissue and muscle (De Koster et al., 2013). The negative energy balance in early lactation resulting from continued increase in milk yield with insufficient nutrient intake results in uncoupling of the somatotropic axis, characterized by decreased growth hormone receptor (GHR) expression in the liver and consequently reduced IGF-1 synthesis by the liver, and reduced negative feedback of IGF-1 on GH secretion (Lucy et al., 2009). Genetic selection for high milk production has created Holstein cows with exacerbated insulin resistance (Matsuzaki et al., 1997). Efficient gluconeogenesis is most important in high-producing cows because it is essential for maintaining adequate glucose supply for the mammary gland and other tissues. The glucose-derived lactose output in milk exceeds the animal’s glucose demand for maintenance several times during peak lactation. Reduced insulin responsiveness by the adipose tissue (McNamara et al., 1986; Vernon et al., 1988.) and muscle (Saremi et al., 2014), allows glucose to be spared and shifted
towards the insulin independent mammary gland for lactose synthesis. Lactose draws water into the mammary epithelial cells, into the Golgi apparatus, and ultimately becoming part of milk; therefore, increased glucose availability leads to more lactose synthesis, which leads to increased milk production. Reduced insulin responsiveness also enhances lipolysis and muscle breakdown (De Koster et al., 2013), such that a high-producing Holstein cow loses ~1.5 kg/day of body weight during the first weeks postpartum (Bell et al., 1995, Koltès et al., 2011). This catabolic state increases the availability of glucogenic substrates such as amino acids and glycerol to support hepatic synthesis of glucose. Finally, increased hepatic resistance to insulin stimulates gluconeogenesis by increasing the uptake of glucogenic substrates (Brockman, 1985).

**The Puerperium**

The puerperium is a period that cover after parturition until the resumption of reproductive function (Senger, 2003). The involution of the uterus in healthy cows typically completes within 40 to 47 days postpartum (Buch et al., 1955; Gier et al., 1968). However, the greatest change in uterus size occurs within 3 to 5 days post-partum (Gier et al., 1968). The period of uterine involution comprises important events such the reduction in uterine size and loss of tissue and epithelial repair these events are important and necessary before another pregnancy can be sustained. In the involution process, the endometrium undergoes a massive tissue remodeling, myometrium contractions, shrinkage of the uterus, sloughing of the caruncles and endometrial regeneration (LeBlanc et al., 2011). One critical point during the involution process is that the presence of epithelial tissue debris and fluid on this period makes the uterine environment favorable for bacterial growth, and impairment of the immune
status during this period likely makes uterine infection more prevalent (Borsberry et al., 1989; Sheldon et al., 2002b).

The cervix, a physical barrier that prevents contamination of the uterus, remains open for several days after calving, allowing the entrance of bacteria into the uterus (Sheldon et al., 2004b). If all physiological processes such as uterine involution and epithelial repair take place under normal circumstances, most cows can eliminate bacteria within 5 weeks (Borsberry et al., 1989). Factors such as uterine involution, regeneration of endometrium and the immune status leads to bacterial clearance in the uterine lumen (Sheldon et al., 2006). Within the first 2 to 3 weeks post-partum, 100% of dairy cows have bacterial contamination of the uterus (Jeon et al., 2015), which often leads to uterine infection and disease. The incidence of uterine disease remains under 50% (Markusfeld, 1987; Bell et al., 2007; Sheldon et al., 2008; Galvão et al., 2009), which reinforces the idea that bacterial contamination is not the same as bacterial infection. Uterine infection involves adherence of pathogenic organisms to the mucosa, colonization or penetration of the epithelium, and release of bacterial toxins that lead to the establishment of uterine disease (Sheldon et al., 2006).

**Uterine Diseases**

Dairy cows can be affected by various uterine diseases post-partum, but the most commonly reported are clinical metritis, puerperal metritis, clinical endometritis, subclinical endometritis, and pyometra. During several years, many definitions have been applied to these diseases, with differences in diagnostic method and time at which the diagnosis was made (Dohoo et al., 1983; Kelton et al., 1998). Sheldon et al., (2006) tried to standardize definitions for uterine diseases, giving a better understanding of the differences that exist among them.
Puerperal metritis and clinical metritis: Puerperal metritis is defined as an animal with an abnormally enlarged uterus and a fetid watery red-brown uterine discharge, within 21 days after parturition, associated with signs of systemic illness and pyrexia >39.5° C. This condition also can course with a reduction in intake or anorexia, decrease milk yield, elevated heart rate, apparent dehydration and affect animal welfare (Stojkov et al., 2015). The incidence of puerperal metritis is 20 to 30% (Giuliodori et al., 2013; Martinez et al., 2012). Animals that are not systemically ill but have an abnormally enlarged uterus and a fetid watery red-brown uterine discharge, within 21 days after parturition, may be classified as having clinical metritis. Clinical metritis and puerperal metritis are characterized histologically by a deep inflammation that affects all layers of the uterus such as endometrium, submucosa, muscularis, and serosa (Bondurant., 1999). The incidence of clinical metritis ranges from eight to 40 % (Chapinal et al., 2011; Hammon et al., 2006; Galvão et al., 2009; Curtis et al., 1985; Giuliodori et al., 2013; Markusfeld 1987). The peak of the disease is at 6 to 7 DIM, and around 80% of the metritis in dairy cows happens in the first 10 DIM and 95% in the first 14 DIM (Galvão et al., 2011).

Clinical endometritis: histologically, it is characterized by the inflammation of the endometrium not deeper than the stratum spongiosum, disruption of the endometrial epithelium, infiltration of inflammatory cells and accumulations of lymphocytes, vascular congestion, and stromal edema (Bondurant, 1999; Bonnett et al., 1991). Clinically, it is characterized by the presence of > 50% of pus 21 days after parturition or mucopurulent discharge detectable in the vagina (approximately 50% pus and 50% mucus) after 26 days post-partum without systemic signs (LeBlanc et al., 2002). The incidence of clinical
endometritis ranges from 3 to 20% (Curtis et al., 1985; LeBlanc et al., 2002; Galvão et al., 2009; McDougall et al., 2007).

Subclinical endometritis: It is defined as an endometrial inflammation of the uterus that can be determined by cytology examining the presence of neutrophils within the uterine lumen (Sheldon et al., 2006). Two variables are considered to define subclinical endometritis: the number of polymorphonuclear neutrophils (PMNs) divided by PMNs and endometrial cells visualized in the smear and days post-partum, according to Sheldon et al., (2006). Based on the work of Kasimanickam et al. (2004), subclinical endometritis is defined by > 18% neutrophils in uterine cytology samples collected 21 to 33 DPP, or >10% neutrophils at 34 to 47 DPP, in the absence of clinical endometritis. The incidence of subclinical endometritis ranges from 1 to 50% (Hammon et al., 2006; Kasimanickam et al., 2004; Ribeiro et al., 2013; Gilbert et al., 2005; Barański et al., 2012), and cows diagnosed with RP or metritis have twice the odds of developing subclinical endometritis after 30 DPP (Rutigliano et al., 2008).

Pyometra: is defined as the accumulation of purulent material within the uterine lumen, usually the cervix is constricted enough to prevent the exudate from draining freely generating a uterine distention (Lewis, 1997; Sheldon et al., 2006), in the presence of a persistent corpus luteum (Sheldon et al., 2006).

**Risk Factors for Metritis**

Risk factors for the development of metritis can be separated into calving problems (dystocia, retained placenta), nutritional status (body condition score, negative energy balance), immunosuppression and environmental causes (season, housing).
**Calving Problems**

In cows with risk factors such as dystocia, delivery of twin calves, retained placenta or stillbirth the incidence of metritis is greater than in health cows. Primiparous cows have an increased risk for metritis compared with multiparous cows because the former require more calving assistance (Bruun et al., 2002; Bell et al., 2007). Besides that, Huzzey et al., (2007) showed that cows with calving assistance had 15.8 times the odds to develop puerperal metritis that unassisted calving. In addition, several authors have identified RP as a risk factor to develop metritis (Kaneene et al., 1995; Dohoo et al., 1983). Several studies have found a strong association between RP and metritis, in which cows with RP had 4 to 6 times the odds to develop metritis than cows without RP (Dubuc et al., 2010; Mellado et al., 1994). Twinning cows had two times the odds to develop metritis and 2.50 times the odds to have retained placenta (Kinsel et al., 1998; Markusfeld-Nir, 1997; Nielen et al., 1989).

**Nutritional Status**

Cows that developed metritis had less DMI and greater NEFA concentrations 1 week before calving when compared to healthy cows (Hammon et al., 2006). In addition, some studies reported an association between elevated NEFA concentrations and diseases during the post-partum period (Dohoo et al., 1984a; LeBlanc et al., 2005; Ospina et al., 2010a; Duffield et al., 2002; Ribeiro et al., 2013; Martinez et al., 2012). Galvão et al., (2010) reported that cows that develop metritis had greater NEFA and BHB concentrations at calving than cows without metritis. Two different studies reporting the association between elevated NEFA and the increased risk of developing metritis. Ospina et al., (2010) observed that cows with NEFA ≥ 0.36 mmol/L during the post-partum period had 17 times the risk of developing metritis. Another study from
Dubuc et al., (2010) only observed an association between pre-partum NEFA concentration and metritis. In Dubuc et al. (2010), NEFA concentration of $\geq 0.6$ mmol/L 1 week before calving increased 1.6 times the odds of developing metritis. In addition, LeBlanc et al., (2004) showed that cows with a pre-partum NEFA concentration of $\geq 0.5$ mmol/L had increased odds by 1.8 times to have retained placenta. Cows that developed metritis were more likely to be in a subclinical hypocalcemia state (4.0 vs. 7.9%) and had elevated NEFA concentration (13.3 vs. 18.1%) as was observed by Ribeiro et al. (2013).

**Immunosuppression**

The temporary impairment of the immune function is a common event in a transition period and it is during this period that increase the diseases incidence (Goff and Horst, 1997; Kimura et al., 2002). The act of parturition is a ‘stressful event’ that induces the production of glucocorticoids accompanied by signaling and coordination from the hypothalamus, pituitary gland and adrenal glands. Galvão et al., (2010) reported that cows with metritis had greater plasma concentration of cortisol than healthy cows at calving. Also, impaired neutrophil and lymphocyte activity observed in cows during the periparturient period is thought to be primarily due to the effects of glucocorticoids (Cai et al., 1994; Kimura et al., 2002).

The primary functions of neutrophils are phagocytosis and degradation of antigens. Reactive oxygen species (Elsbach et al., 1985) and proteolytic proteins stored in cytoplasm granules (Faurschou et al., 2003) and extracellular traps (Brinkmann et al., 2004) can degrade phagocytized antigens. Neutrophils are recruited from the peripheral circulation to the site of infection through chemotaxis. Blood PMN function begins to decline before to parturition, reaches a nadir shortly after parturition, and slowly returns...
to pre-partum values by about 4 weeks post-partum (Kehrli et al., 1989; Hammon et al., 2006).

Calcium is a crucial component of neutrophil activation and successful phagocytosis. Cows with hypocalcemia in the transition period have increased plasma concentration of cortisol (Horst and Jorgensen, 1982), reduced proportion of neutrophils with phagocytic activity (Ducusin et al., 2003; Martinez et al., 2012) and impaired mononuclear cell response to an antigen-activating stimulus (Kimura et al., 2006). In addition, glucose is a major factor that is required by neutrophils for proliferation, survival and differentiation (Pithon-Curi, 2004). The inability of neutrophils to produce glucose through gluconeogenesis (Stjernholm, 1972) forces them to acquire glucose via a glucose transporter (GLUTs) that is insulin independent or by the breakdown of stored glycogen (Pessin et al., 1992; Barghouthi et al., 1995).

The impaired PMN function may be a risk factor for severe uterine infections. Galvão et al. (2010) observed that cytosolic neutrophil glycogen was correlated with blood glucose concentration. In addition, cows that developed metritis had less concentration of glycogen in neutrophils than healthy cows. Furthermore, because neutrophil glycogen reduction was observed before the establishment of metritis, the authors suggested that a reduction in PMN glycogen may be a risk factor for the establishment of uterine diseases in dairy cows.

Increased blood concentrations of BHB and NEFA also have been associated with decreased proliferation of monocytes, and increased NEFA concentrations have been shown to negatively affect neutrophil respiratory burst (Ster et al., 2012). Different studies have reported a relationship between periparturient PMN function suppression
during the parturient period and RP (Gunnick, 1984; Kimura et al., 2002) and metritis in dairy cows. Cai et al., (1994) reported that cows with RP, mastitis and metritis had lower myeloperoxidase activity from 1 week prior to 4 weeks after calving compared to healthy cows (Cai et al., 1994). Neutrophils from cows with RP have decreased migration ability as reported by Gunnink (1984) and decreased myeloperoxidase activity as reported by Kimura et al., (2002).

**Environment**

Few studies evaluated the direct effect of environmental on bacterial contamination and the development of metritis. Erb et al., (1987) reported the associations between calving in most times of the year and an increase in the incidence of metritis, the metritis incidence was higher during late summer and fall. Grohn et al., (1989) reported highest incidence in winter and an elevation during spring. Chapinal et al., (2011) also found higher incidence of metritis during the cool months, September through December. Bartlett et al., (1986) found no clear seasonal pattern in incidence of metritis. Other associations between environment and metritis incidence have been made indirectly, for example, heat stress being found as a predisposing factor for retained fetal membranes and therefore metritis (Dubois et al., 1980). In addition, environmental hygiene may have an effect on the bacterial flora of the uterus, but in the study reported by Noakes et al., (1991) there was no significant difference in the uterine bacterial profiles of the cows between one farm with poor hygiene and another one with good hygiene.
Uterine Microbiota in Cows with Uterine Disease

Culture-dependent Studies

Uterine bacterial contamination in cattle is a dynamic and complex process that involves a broad range of bacterial species (Sheldon et al., 2004a; Sheldon et al., 2002a; Griffin et al., 1974; Földi et al., 2006; Santos et al., 2011). Many species have been isolated from the uterus, among which are *Escherichia coli* (*E. coli*), *Trueperella pyogenes* (*T. pyogenes*), *Prevotella* spp., *Fusobacterium necrophorum* (*F. necrophorum*), *Bacillus licheniformis*, *Clostridium* spp., *Prevotella* spp, *Pasteurella multocida*, *Staphylococcus* spp., and *Streptococcus* spp. (Williams et al., 2005; Griffin et al., 1974; Olson et al., 1984). The uterine microbiota changes throughout the first weeks post-partum because of the spontaneous contamination, clearance, and recontamination of the uterus (Griffin et al., 1974; Földi et al., 2006). Mainly, *E. coli*, *T. pyogenes*, *F. necrophorum*, and *Prevotella melaninogenica* (*P. melaninogenica*) were isolated from cows with metritis (Bonnett et al., 1991; Bondurant et al., 1999; Huszenicza et al., 1999; Gilbert et al., 2007). These four main bacteria were believed to work synergistically to cause uterine disease; however, most of the work was done in cows with endometritis (Griffin et al., 1974; Ruder et al., 1981; Bonnett et al., 1991). It was shown that *E. coli* increases the susceptibility of the endometrium to subsequent infection with *T. pyogenes* (Olson et al., 1984; Gilbert et al., 2007; Williams et al., 2007), and *T. pyogenes* acts synergistically with *F. necrophorum* and *P. melaninogenica* to enhance the severity of uterine disease (Griffin et al., 1974; Ruder et al., 1981; Bonnett et al., 1991). Recent work has highlighted the importance of *E. coli* on the development of metritis and endometritis (Sheldon et al., 2010; Bicalho et al., 2010; Bicalho et al., 2012; Machado et al., 2012a; Machado et al., 2012b); especially the fact that *E. coli*
predisposes to infection with other pathogenic bacterium such as *F. necrophorum* and *T. pyogenes* (Bicalho et al., 2012; Machado et al., 2012b), increases the likelihood of developing metritis and endometritis, and decreases the likelihood of conception (Bicalho et al., 2010; Bicalho et al., 2012; Machado et al., 2012b).

The most prevalent bacteria isolated from the uterine lumen of cows with metritis are *E. coli* and *T. pyogenes* (Sheldon et al., 2010a). In addition, Sheldon et al., (2002) found *T. pyogenes, Prevotella species, E. coli, F. necrophorum, and F. nucleatum* to be the most prevalent bacterial isolates from the uteri of dairy cows with metritis. In a study reported by Huszenicza et al. (1999), cows with metritis 10 to 14 DPP had increased prevalence of *A. pyogenes, E. coli* and Gram-negative obligate anaerobes (especially Bacteroides species) compared with cows without metritis.

**Culture-independent Studies**

LeBlanc et al., (2006) reported that pathogenic bacterial infection is a major factor that plays a role in the epidemiologic triad that determines disease that also involves the immune status of the host and the environment. Studies based on non-cultured techniques, show the complexity of the uterine microbiota that changes rapidly after parturition (Jeon et al., 2015; Santos et al., 2011; Santos et al., 2012). Jeon et al., (2015) reported that healthy and cows that developed metritis show bacterial contamination soon after calving, and also cows with different health status (metritis vs. non-metritis) share a similar uterine microbiota after calving, that contains diverse phylotypes such as Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, and Tenericutes and, the microbiota changes from a day 0 to day 6 post-partum, with a decrease in the abundance of Proteobacteria and an increase in the abundance of Bacteroidetes and Fusobacteria, which were dominant in cows that develop metritis.
There is a positive association between uterine discharge score and the relative abundance of *Bacteroides* and *Fusobacterium* (Jeon et al., 2015). Several authors have reported the negative association between pathogenic bacterial infection and reproductive performance (Földi et al., 2006; LeBlanc et al., 2002; Borsberry et al., 1989; Williams et al., 2008). In addition, pathogenic bacterial infection can affect reproductive performance are a delay of uterine involution, inflammation, and erosion of the uterine epithelium (Azawi, 2008; Elliott et al., 1968). Another factor that affects the normal ovarian cell function is the production of bacterial lipopolysaccharide that alters subsequent ovulation (Williams et al., 2008).

**Importance of Metritis to the Dairy Industry**

Numerous studies have demonstrated both direct and indirect negative impacts of uterine disease on overall dairy herd performance and profitability (Rajala and Grohn, 1998; Fourichon et al., 1999; Gilbert et al., 2005). Deluyker et al., (1991) observed that cows with metritis averaged 2.3 kg/day less milk over the first 120 DIM compared with healthy herdmates, in the same way Daetz et al., (2016) observed that cows with metritis averaged 5.7 kg/day less milk over the first 30 DIM compared with cows without metritis. Goshen and Shpigel, (2006) observed that metritis when left untreated resulted in loss of 337 kg of milk per lactation in primiparous and 498 kg in multiparous cows. Even when metritis was treated, the loss in milk was estimated at $83 per affected cow. Overton and Fetrow, (2008) evaluated the cost of a case of metritis based on a large study with 500 affected cows. They computed milk loss, treatment costs, mortality, increased culling, and loss in reproductive performance, and concluded that a case of metritis costs $358. Given the US dairy cow population (9 million) and the typical incidence in US herds of 20%, the estimated cost of metritis to the US dairy industry is
approximately $650 million. In the European Union, the cost of metritis has been estimated at $1.8 billion (Sheldon et al., 2009). Obviously, the disease process also affects the well-being of the cow. Metritis is an inflammatory disease that, to a large extent, has similar pathogenesis to pelvic inflammatory disease in women, which is extremely painful (Williams et al., 2008).

**Immune Response in the Uterus**

Uterine bacterial contamination is ubiquitous in cows. However, the interesting point is that bacterial contamination does not always imply disease. The uterus has a series of mechanisms to prevent and defend itself against bacterial contamination and tissue damage. The innate immunity is the most important defense system of the bovine uterus, and neutrophils are the main phagocytic cells in the uterus, but their recruitment and activation are controlled by resident tissue macrophages and dendritic cells (Galvão et al., 2012). Phagocytes such as neutrophil and macrophages recognize pathogen-associated molecular patterns (PAMPs) present in microbial invaders through pattern-recognition receptors (PRRs). Examples of PAMPs include lipopolysaccharide from gram-negative bacteria, lipotechoic acid, peptidoglycan from gram-positive bacteria, and zymosan from yeast (Medzhitov and Janeway, 2002). The most important group of PRRs is the toll-like receptors (TLRs) family. There are ten well characterized and widely expressed TLRs in mammals (Akira et al., 2006); TLR1, TLR2, and TLR6 recognize lipids found in bacteria and fungi; TLR3, TLR7, TLR8, and TLR9 recognize nucleic acids from viruses and bacteria; TLR4 recognizes lipopolysaccharide and polysaccharides found in gram-negative bacteria, fungi (mannan), and parasites (glycoinositolphospholipids from Trypanosoma); TLR5 recognizes flagellin in flagellated bacteria; and TLR10 still has no recognized ligand (Akira et al., 2006). After tissue injury
the body produces endogenous factors that function as PAMPs such as heat-shock protein 70 (HSP-70), which can bind to PRRs such as nucleotide-binding oligomerization domain (NOD) receptors (Medzhitov and Janeway, 2002).

Resident macrophages and endometrial cells are involved in the initial recognition of pathogens and initiation of the immune response by production and release of pro-inflammatory cytokines and chemokines including tumor necrosis factor alpha (TNFα), interleukin (IL) -1, IL-6, and IL-8, which lead to a massive influx of leukocytes into the uterine tissue and lumen, but particularly neutrophils, which are responsible for most of the bacterial clearance after uterine infection (Galvão et al., 2011). Nonetheless, during the transition into lactation, dairy cows experience a reduction in leukocyte function, including a reduction in neutrophil phagocytosis and killing capacity, and a reduction in cytokine production and proliferation by mononuclear cells (Kehrli and Goff 1989; Hammon et al., 2006). This reduction is particularly evident in cows that develop metritis and endometritis (Hammon et al., 2006; Jeon et al., 2016). Cows that develop retained placenta, metritis or endometritis have been shown to have a dampened immune response shortly after calving with downregulation of pro-inflammatory cytokines (i.e. TNFα) by monocytes and endometrial cells (Galvão et al., 2011; Galvão et al., 2012), decreased production of TNFα by E. coli-stimulated monocytes (Galvão et al., 2012), decreased production of IL-8 and decreased chemotaxis of neutrophils to the sites of placental attachment (Kimura et al., 2002), and decreased influx of neutrophils into the uterine lumen (Gilbert and Santos, 2016). Several factors might account for such reduction in function. The transition into lactation is a period of energy, amino acids, mineral, and vitamin deficiency in which cows have
to rely on their stored nutrients for their normal functions. This period is characterized by a decrease in feed intake, leading to a sharp decrease in blood concentrations of glucose and minerals, especially calcium. Phagocytes depend on anaerobic glycolysis for the energy required for chemotaxis, phagocytosis, and microbial killing. Neutrophils depend mainly on extracellular glucose to generate ATP for chemotaxis, but they depend primarily on intracellular glycogen to generate glucose for phagocytosis and intracellular killing (Kuehl and Egan 1980; Galvão et al. 2010). Calcium is also a key mediator of phagocyte activation, phagocytosis and killing. Ionized calcium is required for initiation of phagocytosis and formation of the phagolysosome (Jaconi et al., 1990). Therefore, it is believed that decreases in glucose and calcium contribute to the observed immunosuppression, which in turn increases the risk of metritis (Galvão and Santos, 2014).

Cytokines are produced as part of the acute phase reaction that takes place in animal following infection or tissue damage, and that is associated with inflammation. The liver is affected by cytokines and and produces and secretes acute phase protein (APP). Haptoglobin is part of the major APP in ruminants and is synthesized in the liver in response to pro-inflammatory cytokines such as TNFα and interleukin (IL)-6 IL-6 (Oliviero et al., 1989). Several biological functions have been described for Hp. The primary function of Hp during intravascular hemolysis is to form a Hp-hemoglobin (Hb) complex which prevents the loss of iron, haptoglobin inhibits bacterial proliferation such as E.coli by reducing the availability of iron (Eaton et al., 1982). Haptoglobin also acts as an anti-oxidant to reduce the oxidative damage to Hb, albumin, lipids and to the tissues (Ceciliani et al., 2012; Baumann et al., 1994). Haptoglobin has an anti-
inflammatory role by inducing heme oxygenase-1 (HO-1) and IL10 when it forms Hb-Hp complexes to CD163 (Philippidis et al., 2004).

The increase in Hp is an indicator of the degree of bacterial contamination and inflammation as previously reported (Herath et al., 2009). Haptoglobin is usually used as an inflammation marker in cattle because of its greater concentration in disease animals compared with healthy animals (Eckersall et al., 1988) and the longer half-life compared with other APP, the half-life of bovine haptoglobin is around 10 hours (Osada, 1987). Cows that developed metritis, which is an acute and more severe process, had increased haptoglobin levels around the time of metritis diagnosis (Skinner et al., 1991). Some studies showed that cows with metritis had the Hp concentration higher than health cows (Huzzey et al., 2009; Galvão et al., 2010). Furthermore, cows with a Hp concentration ≥ 0.8 mg/mL had decreased cyclicity at 21 days post-partum (Dubuc et al., 2012).

The key to a successful immune response is in maintaining a coordinated balance between generating enough activity to achieve pathogen clearance and not causing extensive damage to the surrounding tissue. The interleukin-10 is an anti-inflammatory cytokine that is a modulator of overproduction of pro-inflammatory cytokines, and has been shown in vitro to inhibit neutrophil and macrophage phagocytic activity (Steinhauser et al., 1999). IL-10 production starts several hours after an initial acute immune response.

**Treatment of Metritis**

The two main objectives of treating cows that suffer from metritis is first to improve the cow’s welfare and second to maintain the reproductive and productive standards of the cow (LeBlanc, 2008).
Traditionally, antibiotics have been used to prevent or treat uterine disease (Risco and Hernandez, 2003; Chenault et al., 2004; Galvão et al., 2009a; Dubuc et al., 2011a; McLaughlin et al., 2013). The most common antibiotics that have been used for treatment of uterine disease are amoxicillin administered intra muscular (i.m.) (Armengol et al., 2015), procaine penicillin administered i.m. (Drillich et al., 2001) ampicillin (Lima et al., 2014), tetracycline (Smith et al., 1998), oxytetracycline administered i.m. or as intrauterine infusion (Smith et al., 1998; Armengol et al., 2015; Königsson et al., 2001; Goshen and Shpigel, 2006) and ceftiofur hydrochloride (CH) administered i.m. (Chenault et al., 2004). There are only three approved antibiotics for treatment of metritis in dairy cows in the USA: ceftiofur hydrochloride (Excenel®, Zoetis, Florham Park, NJ) and ceftiofur crystalline-free acid (Excede®, Zoetis), which are broad-spectrum third-generation cephalosporins, and Liquamycin® LA-200® (Zoetis) a long-acting oxytetracycline. Because of the long withdrawal time for milk (4 days) and meat (28 days) for Liquamycin, Excenel® (no withhold for milk and 3 days for meat) and Excede® (no withhold for milk and 13 days for meat) are the antibiotics of choice. Ceftiofur has been shown to reach minimal inhibitory concentration in endometrium for uterine pathogens such as E. coli, T. pyogenes, and F. necrophorum (Okker et al., 2002; Witte et al., 2011; von Krueger et al., 2013).

The Need for Alternative Antibiotics

Antibiotic resistance is increasing steadily (Ozawa et al., 2012). When the antibiotic is used, it kills or inhibits all susceptible bacteria, but also the phenomenon called “antimicrobial selection pressure” happens, the remaining resistant bacteria will be the backbone of the next generation that can spread among the population (White et al., 2001), in other words, when the antibiotic has lost its ability to effectively control or
kill bacteria growth, the bacteria are “resistant” and continue to multiply in the presence of therapeutic levels of an antibiotic. New alternatives to disease treatment in food animals have been investigated (Machado et al., 2012a; Bicalho et al., 2010). The use of alternatives to traditional antibiotics for prevention and treatment of disease in food animals is needed, thus is possible to protect human and animal health.

Although antibiotic resistance is becoming an increasing problem, new antimicrobial development by pharmaceutical companies is declining (Boucher et al., 2013), making it incumbent upon the federal funding agencies to support the development of new antimicrobials. Chitin, a linear polymer of β-(1, 4)-linked N-acetylglucosamine, is the second most common polysaccharide found in nature. Chitosan can be derived by partial deacetylation of chitin; chitosan has shown intrinsic antimicrobial activity (Lim and Hudson, 2004). Therefore, is necessary to develop agents that retain antimicrobial activity at neutral pH without causing adverse effects on animals. Taken together, clearly there is a need for alternatives to traditional antibiotics to prevent and treat disease in dairy cows.

**Chitosan**

Chitosan can be derived by partial deacetylation of chitin; therefore, it is comprised of copolymers of glucosamine and N-acetylglucosamine (Illum, 1998; Jones and Mawhinney, 2006). Chitosan has shown promising results due to its intrinsic polycation properties including low toxicity, excellent biocompatibility, and high loading capacity for hydrophilic molecules including antibodies (Baldrick, 2010; Trapani, et al., 2011). Moreover, chitosan has shown intrinsic antimicrobial activity (Lim and Hudson, 2004). However, antimicrobial activity of chitosan is only observed in acidic conditions due to the deprotonation in the amine group and low solubility of chitosan at neutral pH.
(Liu, et al., 2004; Qi, et al., 2004). This limits the use of chitosan because most organs maintain neutral pH. Chitosan has a broad spectrum of activity against gram-negative and gram-positive bacteria (Cuero, 1999; Rabea et al., 2003; Sudarshan et al., 1992), and has been recognized as a “Generally Recognized as Safe” (GRAS) for general use in foods by the US Food and Drug Administration (FDA, 2001). Therefore, the use of chitosan is a plausible alternative to antibiotics in cattle.

**Chitosan Microparticles**

Chitosan microparticles can be manufactured by sonicating chitosan while adding 10% sodium sulfate to induce cross-linking between chitosan molecules as previously reported (Van de Lubben, 2001). The size (mean ± SD; 0.6 ±0.08 μm; range 0.43-0.86 μm) and cross-linking between chitosan molecules can be evaluated by electron microscopy (Jeon et al., 2014). CM can eliminate pathogenic bacteria in cattle organs at a neutral pH such as the GI tract, the uterus or the mammary gland (Jeong et al., 2011). This was a vital finding because previous studies had shown that antimicrobial activity of chitosan (not CM) was abolished at neutral pH due to the pKa value of the amino group in chitosan (pKa = 6.5) (Helander et al., 2001).

**Antimicrobial Activity of CM**

To understand whether the *E. coli* O157:H7 reduction observed was caused by binding and removal or by antimicrobial action, Jeon et al., 2014 determined antimicrobial activity by a standard plating method after incubation of *E. coli* O157:H7 with CM at various concentrations, ranging from 0% to 0.2%. Jeon et al., 2014 reported that CM showed a concentration dependent bactericidal activity against *E. coli* O157:H7 (Figure 1-1). Of all concentrations examined, 0.2% CM showed the most antimicrobial activity resulting in complete inhibition of *E. coli* O157:H7 during 6 h of incubation. To
further explore the effect of pH on the antimicrobial activity of CM, Jeon et al., (2014) also tested antimicrobial activity at pH ranging from 5 to 9 (Figure 1-2). Although the strongest antimicrobial activity was observed at acidic pH (pH 5), CM still had significant antimicrobial activity at pH 7. Taken together, these data show a clear dose-dependent antimicrobial activity of CM against *E. coli*, and that although the antimicrobial activity is attenuated at higher pH, the antimicrobial activity is maintained at neutral pH. This indicates that CM can be used and effective in the uterus because the uterine pH is maintained near neutral (pH 6.84-7.25) (Ozenc et al., 2010). At neutral pH, a bacterial membrane disruption was observed when *E. coli* O157:H7 was exposed to CM (Figure 1-3). Because CM has fewer amino groups due to the crosslinking process, another binding mechanism in addition to electrostatic interaction has been postulated. They concluded that CM bound to an outer membrane protein OmpA of *E. coli* O157: H7 at neutral pH. To determine if the observed binding was related to a specific bacterial gene, Jeon et al., 2014 explored the role of OmpA in the antimicrobial mechanism of CM by restoring OmpA in the ∆ompA deletion mutant and looking at antimicrobial activity against *E. coli*. They observed that CM was ineffective with ∆ompA deletion, but functional complementation of the ∆ompA mutant (∆ompA + pOmpA) restored the antimicrobial activity of CM, suggesting that OmpA is not the only molecule interacting with CM.

To understand the underlying mechanisms of CM antimicrobial activity, Jeon et al., (2014) tested if CM could bind to *E. coli* O157:H7 at neutral pH and identified the binding site for CM. Jeong’s group concluded that a direct contact between CM and *E. coli* may result in a bactericidal effect, and they hypothesized that surface-exposed
molecules in *E. coli* might bind to CM. The way that chitosan interacts with bacteria is not entirely understood. The most accepted hypothesis is that the positive charge given to chitosan by its amino groups could allow it to bind to a negatively charged microbial cell membrane increasing its permeability and resulting in the leakage of cell constituents (Liu et al., 2004; Qi et al., 2004; Sudarshan et al., 1992). Another hypothesis proposed by Sudarshan et al., 1992 is that chitosan could inhibit the mRNA synthesis through penetration into the cell nuclei and binding with DNA. Chitosan also has chelating properties and could interact with essential metal divalent ions such as Ca2+, Mg2+ and Cu2+ affecting microbial growth (Chung, 2003; Kong et al., 2008), and, chitosan can enhance immune response increasing chemotaxis and ROS production in PMN (Suzuki et al., 1986).

**In vitro, ex vivo and in vivo Antimicrobial Activity of CM**

Jeong et al., (2014) tested the antimicrobial activity of CM *in vitro* and *ex vivo* using uterine fluid from cows with metritis. CM showed antimicrobial activity *in vitro* against six pathogens of importance for animal and human diseases, with different efficacy depending on the pathogen. The growth of *E. coli* O157:H7 and intrauterine pathogenic *E. coli* (IUPEC) was inhibited at 0.05%, and completely eliminated at 0.2% *in vitro*. In comparison to these *E. coli* species, *Vibrio cholerae*, *Salmonella enterica*, *Klebsiella pneumoniae*, and *Streptococcus uberis* were less sensitive to CM at 0.1 or 0.3%. *V. cholerae* was inactivated at 0.5%; whereas, *S. uberis* was inactivated at 1%. CM also inhibited the growth of IUPEC *ex vivo* at 0.2 and 0.4% but was most efficacious at ≥ 0.6%. CM have broad-spectrum of antimicrobial activity against important pathogens *in vitro* and *ex vivo* (*Figure 1-4*). In addition, Jeong et al. (2014) tested the *in vivo* antimicrobial activity of CM in cows with metritis by assessing its effect on IUPEC.
Cows were treated with ceftiofur (2.2mg/kg for 5 days i.m.) or with 8 g of CM for 5 days via intrauterine infusion. Chitosan microparticle treatment was found to be as effective as ceftiofur in controlling IUPEC growth (Figure 1-5).
Figure 1-1. Survival curve of E. coli O157:H7 during CM treatment. E. coli O157:H7 was grown with CM at 0% (circle), 0.05% (square), 0.1% (triangle), or 0.2% (inverted triangle), and each time point represents the mean values and standard error of means (SEM) of colony forming units (CFU) recovered from triplicate test tubes. Data from Jeon et al. (2014).
Figure 1-2. pH effect on antimicrobial activity of CM. E. coli O157:H7 was incubated for 6 h in the presence of 0.1% CM in LB broth at various pH (ranging from pH 5 to pH 9) and viable cells were counted using direct plating method; Mean values ± SEM are plotted from three independent experiments; *P < 0.05, t-test. Data from Jeon et al. (2014).
Figure 1-3. LIVE/DEAD viability assay. E. coli O157:H7 was incubated with 0.2% CM at 37°C for 3 h and then incubated with SYTO 9 (green, live) and propidium iodide (red, dead) for 15 minutes, and then bacteria were observed using the fluorescence microscope (Leica Microsystems Wetzlar GmbH, Germany). Fluorescent micrograph of E. coli O157:H7 treated with either 0% CM (left), 70% isopropanol (middle), or 0.2% CM. Results shown are representative of three independent experiments. Data from Jeon et al. (2014).
Figure 1-4. A broad antimicrobial activity of chitosan microparticles. Various strains including E. coli O157:H7, Intrauterine pathogenic E. coli (IUPEC), V. cholerae, S. enterica, K. pneumoniae, S. uberis were treated with CM at different concentrations, and viable cells were measured after 6 h treatment; Mean values ± SEM are plotted from three independent experiments. Data from Jeon et al. (2014).

Figure 1-5. Comparison of CM and ceftiofur antibacterial activity against IUPEC. Data from Jeon et al. (2014).
CHAPTER 2
INTRODUCTION

Metritis, an acute inflammatory disease of multiple layers of the uterine lining with systemic implications, affects 20 to 40% of the postpartum dairy cows in the first 21 DIM (LeBlanc et al., 2002; Bell et al., 2007; Sheldon et al., 2009; Dubuc et al., 2010). In cows with risk factors such as dystocia, delivery of twin calves, retained fetal membranes (RFM) or stillbirth the incidence of metritis ranges from 30% to 45% (Markusfeld, 1987; Bell et al., 2007; Benzaquen et al., 2007). The consequences of metritis include reduced welfare (Stojkov et al., 2015), increased incidence of other diseases, decreased productive and reproductive performance, increased culling, and decreased profitability (Erb et al., 1980; Sheldon et al., 2004a; Kossaibati et al., 1997).

The dairy cow is unique in the sense that virtually all cows are infected with bacteria in the first days following calving (Sheldon and Dobson., 2004; Jeon et al., 2015); however, cows with risk factors for metritis are more likely to have pathogenic bacteria than cows without risk factors (Bicalho et al., 2010; Giuliodori et al., 2013). Failure to eliminate pathogenic bacteria leads to the establishment of disease. A broad-spectrum third-generation cephalosporin, namely ceftiofur, is the antibiotic of choice for treating metritis primarily because it does not require milk withdrawal. The Food and Drug Administration (FDA), citing concerns for human health because of development of antibiotic resistance, has banned the use of third generation cephalosporins for disease prevention in cattle, swine, chickens, and turkeys. If this action is not enough to control antibiotic resistance, the next step might be to ban its use for disease treatment. This highlights the need for alternatives to traditional antibiotics for prevention and treatment of diseases in food animals. Chitosan, which is categorized as GRAS
(Generally Recognized as Safe) by the FDA, is made out of chitin. Chitin is the second most abundant biopolymer on the earth after cellulose. It is part of structural components of the exoskeleton of arthropods and the cell walls of fungi and yeast. Chitosan is a linear polysaccharide produced by deacetylation of chitin and is non-toxic, bioadhesive, biocompatible, and biodegradable. Because of these desirable characteristics, chitosan has been used broadly in the food, pharmaceutical, textile, agriculture and cosmetics industries and for water treatment. Chitosan microparticles (CM) have broad-spectrum antimicrobial activity at acidic and neutral pH (Jeong et al., 2014). Therefore, CM seems as a promising alternative to traditional antibiotics for the treatment of disease in dairy cows.

In this study, CM was evaluated as an alternative treatment for metritis. The rationale for this study was that development of an effective alternative to current antibiotic treatments could improve animal health, welfare, and fertility, which would enhance farm profitability and sustainability. Furthermore, reducing dependence on traditional antibiotics is expected to delay development of bacterial resistance to antibiotics used in human medicine such as third generation cephalosporins, improving public sentiment toward animal agriculture and the sustainability of the dairy industry.

We hypothesized that cure of metritis for CM treatment would be similar to treatment with EXD and both treatments would be superior to CON. In addition, we hypothesized that blood analytes and rectal temperature would decrease quicker in CM and EXD treated cows than in CON cows. Finally, we hypothesized that an effective treatment for metritis would have a positive impact on milk yield, reproductive performance, and survival of dairy cows. The objective of this experiment was to
evaluate the efficacy of intrauterine administration of CM for the treatment of metritis in dairy cows, and a secondary objective was to evaluate the effect of CM treatment on milk yield, reproductive performance, and survival of dairy cows.
CHAPTER 3
MATERIALS AND METHODS

Cows, Housing and Feeding Management

The study was conducted in three dairy herds located in North Central Florida from May, 2016 to June, 2017. Herds A, B, and C had 4,400, 1,800, and 450 lactating cows, respectively. All herds milked only Holstein cows. In herds A and B, cows were milked three times daily and in herd C cows were milked twice a day. The rolling herd average milk yield ranged from 10,500 to 12,000 kg. Postpartum pens had sand-bedded stalls and were equipped with sprinklers over the feeding areas that were activated when the environmental temperature rose above > 21 °C. The postpartum diet was formulated to meet or exceed the dietary nutrient requirements for a lactating cow weighing 680 kg and producing 45 kg of 3.5% fat-corrected milk and 3.0% protein (NRC, 2001), and it was delivered as a TMR twice daily.

Sample Size Calculation

For evaluation of the efficacy of intrauterine administration of CM for the treatment of metritis; the sample size was calculated to detect treatment differences of 8 PU assuming a cure rate of 75% for EXD (McLaughlin et al., 2012), 67% for CM and 55% for the CON treatment (McLaughlin et al., 2012); therefore, if differences in cure rates are less than 8 PU, no statistical differences were expected. With alpha of 5% and 80% power, a sample size of 250 cows per group would be needed [75% (187/250) vs. 67% (167/250); P = 0.049; 67% vs. 55% (137/250); P = 0.006].

With a sample size of 250 cows per group after accounting for attrition, differences of approximately 8 PU in pregnancy per artificial insemination (AI) to first service, or overall culling when these outcomes range from 27 to 35%, or a difference of
8 PU in cyclicity by 50 DIM when cyclicity ranges from 67 to 75% would be statistically significant (P ≤ 0.05). Based on previous studies (Bittar et al., 2014; Vieira-Neto et al., 2014), with a sample size of 250 per group, differences of approximately 35 days in time to culling or time to pregnancy would be statistically significant (P ≤ 0.05). With a sample size of 250 per group, differences of 1.2 kg, standard deviation of 4 kg, would be statistically significant (P ≤ 0.05). Individual milk weights were recorded on farms A and C, and monthly on farm B. Production and reproduction data were collected from the farm management software (PCDart® and AfiFarm®).

**Eligibility Criteria, Metritis Diagnosis, and Enrollment**

Cows calving within the withdrawal period for any antimicrobial, cows treated with any antimicrobial, steroidal and/or nonsteroidal anti-inflammatory, or antipyretic agents after calving, cows submitted to caesarian section or fetotomy, cows that aborted (< 260 days of gestation), cows that had uterine prolapse after delivery, and cows diagnosed with infectious (e.g. mastitis, gastroenteritis, pneumonia, peritonitis) or metabolic (i.e. displaced abomasum) diseases at the time of metritis diagnosis were not eligible for enrollment in the experiment.

Cows were examined for diagnosis of metritis at 5, 7 and 9 DIM using the Metricheck® device (Simcro, Hamilton, NZ). Discharge retrieved from the vagina was scored as: 1 = not fetid normal lochia, viscous, clear, red, or brown; 2 = cloudy mucoid discharge with flecks of pus; 3 = not fetid, mucopurulent discharge with < 50% pus; 4 = not fetid mucopurulent white, yellow or reddish-brownish discharge with ≥ 50% pus; and, 5 = fetid, thin, serous, or watery, may have been reddish-brownish, with or without pieces of necrotic tissue present (adapted from Chenault et al., 2004). Cows with a vaginal discharge score of 5 were classified as having metritis. At 5 DIM all cows were
scored for body condition (BCS; 1 = thin, 5 = obsess; Ferguson et al., 1994) and vaginal-vulvar laceration (VLS; 0 = no laceration; 1 = laceration < 2 cm at dorsal commissure or internal vaginal wall; 2 = vaginal-vulvar laceration > 2 cm; Viera-Neto et al., 2013).

At enrollment, all cows had rectal temperature measured with a thermometer that was calibrated daily before use and cows with rectal temperature ≥ 39.5 ºC were considered febrile (Upham, 1996). Cows diagnosed with metritis were blocked by parity (primiparous or multiparous) and, within each block, were randomly assigned to one of three treatments. Cows assigned to the chitosan microparticle (CM; n = 276) treatment received intrauterine infusion of 24 g of CM dissolved in 40 mL of sterile distilled water on D0 (diagnosis/enrollment), D2, and D4. According to Jeon et al. (2014) a CM concentration of 0.6% was the most efficacious at reducing the bacterial count in the uterine fluid from cows with metritis. Therefore, the amount of CM infused in the uterus was 24 g to give a final concentration of at least 0.6% assuming a uterine content in cows with metritis of 4 L (24/4000*100 = 0.6%). In addition, CM was administered every other day for a total of three treatments to increase the practicality of treatment administration. Chitosan was purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO) and a 1% (wt/vol) chitosan solution was prepared in 2% acetic acid (v/v) and 1% Tween 80 (v/v). To facilitate cross-linking, the chitosan solution was stirred and sonicated with the addition of 2 mL of sodium sulfate (10% [wt/vol]) into 100 mL of chitosan solution. The total sonication time and sonication power was varied to obtain the desired chitosan microparticle size (∼0.6 μm). Chitosan microparticle was collected by centrifugation (12,000 x g) for 10 minutes, washed three times with sterile water,
 aliquoted, and stored at 4 °C to be used in the experiments. The intrauterine infusions were performed after the vulva was cleaned using alcohol 70%. A single rounded tip pipette (Uter flush pipetes10 G01, VAN BEEK®, Orange City, IA) was used to deliver the solution into the uterine lumen. Cows assigned to the Excede treatment (EXD; n = 275) were treated with subcutaneous injections of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) in the base of the ear at D0 and D3. Live body weight was estimated using a heart girth measuring tape (Nasco Inc., Atkinson, WI). Cows assigned to the control treatment (CON; n = 275) did not receive any treatment. A larger sample size was enrolled to allow for up to 10% attrition. Cows with a vaginal discharge ≤ 3 were classified as Healthy (n = 2,441).

Cure of Metritis and Escape Therapy

At 12 d after enrollment all cows enrolled in the CM, EXD, and CON treatments and 275 healthy cows were examined by Metricheck. Cows previously diagnosed with metritis that had vaginal discharge score < 5 on d 12 after enrollment were considered to have been cured. Thus, cows with vaginal discharge score = 5 and cows that were sold or died ≤ 12 d after enrollment were considered a treatment failure. The post-enrollment exam on d 12 was performed by a veterinarian from the research team that was unaware of treatment assignment.

Escape therapy with an antibiotic of choice was allowed for all groups one day after enrollment. Criteria for escape therapy were severe dehydration, anorexia, weakness, severe depression, systemic shock, or any other clinical signs that were attributable to metritis (McLaughlin et al., 2012). Presence of vaginal discharge score 5 and rectal temperature ≥ 39.5 °C alone were not cause for escape therapy. The escape therapy was initiated by the herdsmen in each farm following the criteria described
above, but without consultation with the research team. The escape therapy followed each herds’ standard treatment protocols. Cows that received escape therapy were considered a treatment failure. Cows that did not receive antibiotics but received support therapy with hypertonic saline, oral electrolytes, dextrose, aspirin, or flunixin meglumine were not considered to have received escape therapy.

The reason for culling (sold or died) within 60 DIM was recorded for all enrolled cows in the experiment. The reasons listed were peritonitis, mastitis, injury to teat/udder, lung abcess, fatty liver, trauma, broken leg and low milk yield. A physical examination was performed by the herd veterinarian or by a veterinarian from the food animal in reproduction and medicine service from University of Florida in all cows that were sold. The diagnosis of peritonitis antemortem and was defined as the presence of granular, gritty feel, with an impression of an abnormal freedom of movement within the abdominal cavity at rectal palpation and pelvic inflammation. A necropsy examination was performed by the herd veterinarian or a veterinarian from the food animal reproduction and medicine service from University of Florida in all the cows that died within 60 DIM.

**Reproductive Management and Reproductive Performance**

In herd A the voluntary waiting period was 48 DIM, and cows were artificially inseminated when detected in estrus by visual observation or by the use of a heat detection device (Kamar®, Kamar Inc., Steamboat Springs, CO). Cows that had not been inseminated by 55 ± 3 DIM received one injection of PGF$_{2\alpha}$ and were inseminated upon estrus detection thereafter. Cows not observed in estrus by 72 ± 3 DIM were submitted to the Ovsynch protocol and were fixed-time AI at 82 ± 3 DIM. The diagnoses of pregnancy were performed by transrectal ultrasonography (Easi-Scan
linear bovine ultrasound machine, IMV Imaging, North America, Inc., Rochester, MN) at 33 ± 3 d after service. Nonpregnant cows were resynchronized using the Ovsynch protocol. In herd B the voluntary waiting period was 50 DIM, and cows not detected in estrus by the use of tail paint heat detection aid and inseminated by 70 DIM were enrolled in an Ovsynch protocol. The diagnoses of pregnancy were performed by the farm personnel through rectal palpation of the uterine contents at 42 ± 3 d. In herd C, cows were were enrolled in a Double-Ovsynch protocol at 54 ± 3 DIM. The diagnoses of pregnancy were performed by transrectal ultrasonography (Easi-Scan linear bovine ultrasound machine, IMV Imaging, North America, Inc., Rochester, MN) at 33 ± 3 d after service. All the breeding and pregnancy diagnosis were recorded on the on-farm management software by the herd personnel. Weekly backups were saved and used to retrieve reproductive performance data. Ultrasonography was performed at 36 ± 3 and 50 ± 3 DIM to determine cyclicity status based on the presence of corpus luteum in herd A. Pregnancy per breeding to first service and time to pregnancy up to 300 DIM were calculated for all cows enrolled in the experiment.

**Blood Metabolite Concentrations**

Blood samples were collected by puncture of the coccygeal vein or artery in evacuated tubes (Vacutainer, Becton Dickinson and Company, Franklin Lakes, NJ) containing K2 EDTA at D0, D3, D6, D9 and D12. On D0 and 3, blood was sampled before treatments with CM and EXD. Blood samples were maintained on ice until plasma was harvested by centrifuging tubes at 2000 x g for 10 minutes (Heraeus Megafuge 1.0; Thermo Fisher Scientific, Inc., Waltham, MA). Plasma was transferred to two polypropylene vials and stored at -80 °C until assayed for NEFA, BHB and haptoglobin (Hp).
Concentrations of NEFA were analyzed by a chemistry analyzer (RX Daytona+; Randox Laboratories Ltd.) in a single assay and the inter-assay CV was 5.1%. Concentrations of BHB in plasma were analyzed by a chemistry analyser (RX Daytona+; Randox Laboratories Ltd.) in a single assay and the inter-assay CV was 4.2%. Plasma Hp concentration was determined using a colorimetric assay via quantification of the haptoglobin/hemoglobin complex by the estimation of differences in peroxidase activity. Assays were performed in 16 x 100 borosilicate tubes. Briefly, 5 μl of plasma sample or deionized water (blank) was added to 7.5 mL of a solution containing 0.6 g/l of the reagent O dianisidine, 13.8 g/l of sodium phosphate monobasic, and 0.5 g/l EDTA. After, 25 μl of 0.3 g/l bovine haemoglobin solution was added to each assay, followed by water bath incubation at 37°C for 45 minutes. After incubation, 100 μl of freshly prepared 156 mM hydrogen peroxidase solution was added to each assay. Samples were incubated at room temperature for 60 minutes. Then, 200 μl of each assay was transferred to a 96-well polystyrene flat-bottom microplate. Optical density (OD) at 450 nm was measured on the Epoch2 Microplate Spectrophotometer (BioTek, Winooski, VT). Finally, the final OD of each assay was subtracted by the blank assay OD. Optical density data was converted to a concentration unit (μg/mL) using standard curves generated by serial dilutions of a sample of known concentration determined by ELISA (Life Technologies; West Chester, PA) as previously described (Cooke and Arthington, 2013). The intra- and inter-assay CV were 6.9% and 7.7%, respectively.

**Statistical Analysis**

Binary outcomes such as cure of metritis, sold within 60 DPP, died within 60 DPP, culled within 60 DPP were analyzed by mixed logistic regression using the GLIMMIX procedure of SAS version 9.3 (SAS Institute, Cary, NC). The model included
the fixed effects of treatment (CM, EXD, CON), season (cool vs. hot), fever (yes vs no), parity (primiparous vs. multiparous), BCS (high (BCS ≥ 3.75) vs. moderate (BCS > 3 and < 3.75) vs. low (BSC ≤ 3)), dystocia (yes vs. no), RFM (yes vs. no) and VLS (< 2 vs. 2) and two-way interactions between treatment and other covariates. Herd was included as a random effect. Differences detected at P ≤ 0.05 were considered significant.

Continuous outcomes such rectal temperature, plasma concentrations of NEFA, BHB, Hp were analyzed by ANOVA for repeated measures using the MIXED procedure of SAS. Models also included the fixed effects of treatment, season, parity, BCS, dystocia, RFM, VLS and day of enrollment. Rectal temperature or analyte concentrations at the metritis diagnosis day were included in the repeated measures models as a covariate. Farm and ID were included as a random effect. Differences detected at P ≤ 0.05 were considered significant.

For the Evaluation of the effect of CM treatment on milk yield, reproductive performance, and survival; dichotomous outcomes such as pregnancy per AI was compared using logistic regression using the GLIMMIX procedure of SAS. Differences in daily milk yield during the first 60 DIM and differences in monthly milk yield during the first 300 DIM was analyzed by ANOVA for repeated measures using the MIXED procedure of SAS. Hazard of pregnancy up to 300 DIM was analyzed by Cox’s proportional hazard model using the PHREG procedure of SAS. Models included, as appropriate, the fixed effects of treatment, season, parity, BCS, dystocia, RFM, VLS, and interactions between treatment and other covariates. Herd was included as random (GLIMMIX and MIXED) or strata (PHREG). P ≤ 0.05 were considered significant.
CHAPTER 4
RESULTS

Effect of Treatment on Metritis Cure

Treatment had an effect \((P < 0.01)\) on metritis cure on D12. Cows that received EXD had greater \((P \leq 0.01)\) cure compared with cows that received CM and CON on D12; however, no difference \((P = 0.45)\) was observed between CM and CON treatments (Figure 4-1). In addition, the proportion of cows that were cured from metritis was less for cows with VLS = 2 compared with cows with VLS < 2 (58.9 vs. 72.3%; \(P < 0.01\)) and for cows with fever at the time of metritis diagnosis compared with cows without fever at the time of metritis diagnosis (65.9 vs. 71.7%; \(P = 0.04\)).

Effect of Treatment on the Proportion of Cows Culled by 60 DIM

Treatment had an effect \((P < 0.04)\) on the proportion of cows culled by 60 DIM. The proportion of cows culled by 60 DIM was greater \((P < 0.01)\) for cows that received CM compared with cows that received CON or EXD (Figure 4-2); however, no difference \((P = 0.55)\) was observed between CON and EXD treatments. The proportion of cows culled by 60 DIM in the HTH group was 4.4% and it was less \((P < 0.01)\) than all treatments of cows with metritis (Figure 4-2). In addition, the proportion of cows culled by 60 DIM was greater \((P < 0.01)\) for multiparous compared with primiparous cows (6.9 vs. 5.5%), and for cows with VLS = 2 compared with cows with VLS < 2 (8.6 vs. 6.0%; \(P = 0.04\)).

Effect of Treatment on the Proportion of Cows Sold by 60 DIM

Treatment affected \((P = 0.01)\) the proportion of cows sold by 60 DIM. The proportion of cows sold by 60 DIM was greater \((P < 0.03)\) in cows that received CM compared with cows that received CON or EXD (Figure 4-3); however, no difference \((P$$}
was observed between CON and EXD treatments. The proportion of cows sold by 60 DIM in the HTH group was 3.5%, and it was less ($P < 0.01$) than all treatments of cows with metritis (Figure 4-3). In addition, the proportion of cows sold by 60 DIM was greater for multiparous compared with primiparous cows (5.3 vs. 4.5%; $P = 0.01$).

**Effect of Treatment on the Proportion of Cows that Died by 60 DIM**

Treatment had no effect ($P = 0.13$) on the proportion of cows that died by 60 DIM (Figure 4-4). In addition, the proportion of cows that died by 60 DIM was greater for multiparous compared with primiparous cows (1.6 vs. 0.9%; $P < 0.01$), for cows with VLS = 2 compared with cows with VLS < 2 (3.1 vs. 1.1%; $P < 0.01$), for cows that calved in the hot season compared with cows that calved in the cool season (2.2 vs. 1.1%; $P < 0.01$), and for cows with BCS $\geq$ 3.75 compared with BCS $> 3$ and $< 3.75$ (1.9 vs. 0.9%, $P = 0.02$). The proportion of cows that died by 60 DIM in the HTH group was 0.9%, and was less ($P < 0.01$) than all treatments of cows with metritis (Figure 4-4).

**Effect of Treatment on Reason for Culling**

Treatment had an effect ($P < 0.01$) on the reason for culling in cows with metritis by 60 DIM (Figure 4-5). The proportion of cows with metritis culled by 60 DIM with peritonitis was greater ($P < 0.01$) in cows that received CM compared with cows that received CON or EXD treatments (Figure 4-5). There was no effect of treatment ($P = 0.15$) on the proportion of cows culled by 60 DIM for other reasons (combination of mastitis, injury to teat/udder, lung abcess, fatty liver and low milk yield), and it was 10.1, 6.9, and 5.8% for CM, CON and EXD, respectively.

**Effect of Treatment on Rectal Temperature**

Treatment had no effect on rectal temperature on D0 ($P = 0.62$) or up to D12 ($P = 0.58$; Figure 4-6); however, there was an interaction between treatment and time on
rectal temperature up to D12 ($P = 0.04$), which showed that rectal temperature was greater for the CON treatment compared with CM and EXD treatments on D12. There was also an effect of time ($P < 0.01$), which showed that rectal temperature decreased steadily from D3 until D12. In addition, rectal temperature was greater for cows that calved in the hot season compared with cows that calved in the cool season (39.1 ± 0.03 vs. 39.0 ± 0.02 °C; $P < 0.01$) and greater for primiparous compared with multiparous cows (39.1 ± 0.03 vs. 39.0 ± 0.03 °C; $P = 0.02$). The HTH group had smaller ($P < 0.01$) rectal temperature than all treatments of cows with metritis on D0. The rectal temperature did not differ ($P = 0.45$) between the HTH group and treatments of cows with metritis up to D12.

**Effect of Treatment on Plasma NEFA Concentration**

Treatment had no effect on plasma NEFA concentration on D0 ($P = 0.96$) or up to D12 ($P = 0.23$; Figure 4-8); however, there was an effect of time ($P < 0.01$), which showed that plasma NEFA concentration decreased steadily from D3 until D12. There was no interaction between treatment and time on NEFA concentration ($P = 0.11$). In addition, plasma NEFA concentration was greater for multiparous compared with primiparous cows on D0 (1.10 ± 0.18 vs. 0.64 ± 0.18; mmol/L; $P < 0.01$). The plasma NEFA concentration did not differ among the HTH group and all treatments of cows with metritis on D0 and up to D12 ($P \geq 0.20$) (Figure 4-9).

**Effect of Treatment on Plasma BHB Concentration**

Treatment had no effect on plasma BHB concentration on D0 ($P = 0.52$) or up to D12 ($P = 0.54$; Figure 4-10); however, there was an effect of time ($P < 0.01$), which showed that plasma BHB concentration decreased steadily from D3 until D12 for the EXD and CON treatments and increased from D3 until D12 for the CM treatment. There
was no interaction ($P = 0.17$) between treatment and time on plasma BHB concentration. In addition, plasma BHB concentration was greater for multiparous compared with primiparous cows (1.14 ± 0.06 vs. 0.63 ± 0.05; mmol/L; $P < 0.01$) and was greater for cows with BCS ≥ 3.75 compared with BCS > 3 and < 3.75 (1.05 ± 0.08 vs. 0.73 ± 0.047; mmol/L; $P < 0.01$) on D0. The plasma BHB concentration on D0 was greater ($P < 0.02$) for all treatments of cows with metritis compared with the HTH group, however, the plasma BHB concentration did not differ among the HTH group and all treatments of cows with metritis up to D12 ($P = 0.38$) (Figure 4-11).

**Effect of Treatment on Plasma Hp Concentration**

Treatment had no effect on plasma Hp concentration on D0 ($P = 0.30$) or up to D12 ($P = 0.16$; Figure 4-12); however, there was an effect of day ($P < 0.01$), which showed that plasma Hp concentration decreased steadily from D3 until D12. There was no interaction ($P = 0.61$) between treatment and time on plasma Hp concentration. In addition, plasma Hp concentration was greater ($P = 0.02$) for cows that calved in the hot season compared with cows that calved in the cool season (1079 ± 134 vs. 800 ± 135 μg/mL). The HTH group had smaller ($P < 0.01$) plasma Hp concentration than all treatments for metritis on D0 ($P < 0.01$). The plasma Hp concentration did not differ among the HTH and metritic groups up to D12 ($P = 0.22$).

**Effect of Treatment on Daily Milk Yield Within 60 DIM**

Treatment had an effect ($P < 0.01$) on milk yield up to 60 DIM (Figure 4-14); the HTH group had greater milk yield than all treatments for metritis and they averaged 37.12 ± 0.19, 38.22 ± 0.19, 39.52 ± 0.19, and 42.26 ± 0.06 kg/day for CM, CON, EXD and HTH respectively. There was an interaction between treatment and DIM ($P < 0.01$), which showed that milk yield increased for all treatments until the occurrence of the
metritis, but after the CM treatment, the milk yield was less for the CM treatment compared with CON or EXD treatments and HTH group. In addition, milk yield up to 60 DIM was greater for multiparous compared with primiparous cows (45.24 ± 0.92 vs. 34.07 ± 0.089 kg/day, P < 0.01) and for cows calving in the cool season compared with cows calving in the hot season (40.19 ± 0.074 vs. 39.12 ± 0.11 kg/day P < 0.01).

**Effect of Treatment on Daily Milk Yield Within 30 Days after Enrollment**

When the different treatments of metritis were analyzed separately and the day of diagnosis was used as a covariate, treatment had no effect on milk yield on D0 (P = 0.65); however, treatment had an effect on milk yield up to D30 after enrollment (P < 0.01; Figure 4-15). There was an effect of time (P < 0.01) on milk yield, which showed that milk yield increased steadily from D0 until D30 of enrollment. In addition, milk yield was greater for multiparous compared with primiparous cows up to D30 of enrollment (37.64 ± 0.67 vs. 32.30 ± 0.67; kg/day; P < 0.01) and was greater for cows with BCS ≥ 3.75 compared with cows with BCS > 3 and < 3.75 and BCS ≤ 3 (35.56 ± 0.69; 35.48 ± 0.65 and 33.87 ± 0.76; kg/day, respectively; P = 0.02).

**Effect of Treatment on Monthly Milk Yield**

Treatment had an effect on milk yield up to 10 months (P < 0.01; Figure 4-16). HTH group had greater milk yield than all treatments of cows with metritis and it was 35.52 ± 0.44, 35.79 ± 0.43, 36.46 ± 0.43 and 37.43 ± 0.25 kg/day for CM, CON, EXD and HTH respectively. There was an interaction between treatment and DIM (P < 0.01), which showed that milk yield was greater for the HTH group compared with all treatments of cows with metritis up to 6 months and then was less for the HTH group compared with all treatments of cows with metritis up to 10 months. In addition, milk yield was greater for multiparous compared with primiparous cows up to 300 DIM (39.49
± 0.25 vs. 33.62 ± 0.27 kg/day; \( P < 0.01 \) and for cows with BCS ≥ 3.75 compared with cows with BCS > 3 and < 3.75 and BCS ≤ 3 (37.46 ± 0.26; 37.07 ± 0.23 and 35.15 ± 0.41 kg/day; respectively; \( P < 0.01 \)). Finally, milk yield was greater for cows with VLS = 2 compared with cows VLS < 2 (36.25 ± 0.24 vs. 36.87 ± 0.30 kg/day; \( P < 0.01 \)).

**Effect of Treatment on Pregnancy per breeding Risk**

Treatment had an effect (\( P = 0.04 \)) on pregnancy per breeding. Cows that received EXD had greater (\( P = 0.01 \)) proportion of pregnancy per breeding at the first breeding compared with cows that received CM (35.8 vs. 24.7%); however, no difference (\( P = 0.24 \)) on the proportion of pregnancy per breeding at the first breeding was observed between the CM and CON treatments (24.7 vs. 29.2%). HTH cows resulted in greater (\( P < 0.01 \)) rate of pregnancy per breeding at the first breeding than CM treatment (35.7 vs. 24.7%) (Figure 4-17).

**Effect of Treatment on Hazard of Pregnancy**

Treatment affected (\( P = 0.01 \)) the hazard of pregnancy up to 300 DIM. It was greater for EXD than CM or CON. Kaplan-Meier analysis showed that cows treated CM had greater (\( P < 0.01 \)) median days to pregnancy when compared with CON and EXD treatments and they were 149, 137 and 131 days for CM, CON and EXD, respectively (Figure 4-18). Median days to pregnancy was 113 days for the HTH group (Figure 4-19).
Figure 4-1. Proportion of cows cured of metritis on day 12 after enrollment according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM, intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON, cows did not receive any treatment; or EXD, a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. Overall effect of treatment in the logistic regression, \( P \leq 0.01 \). a,b,cSuperscripts indicate significant differences \( (P \leq 0.05) \) among treatments after Tukey-Kramer adjustments.
Figure 4-2. Proportion of cows culled within 60 DIM according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM, intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distillated water on days 0, 2 and 4 after diagnosis of metritis; CON, cows did not receive any treatment; or EXD, a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. In additional, the healthy (HTH) group. Overall effect of treatment in the logistic regression, $P \leq 0.01$. a,b,cSuperscripts indicate significant differences ($P \leq 0.05$) among treatments after Tukey-Kramer adjustments.
Figure 4-3. Proportion of cows sold within 60 DIM according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM, intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON, cows did not receive any treatment; or EXD, a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. In addition, the healthy (HTH) group. Overall effect of treatment in the logistic regression, \( P \leq 0.01 \). a,b,c Superscripts indicate significant differences (\( P \leq 0.05 \)) among treatments after Tukey-Kramer adjustments.
Figure 4-4. Proportion of cows died within 60 DIM according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM, intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distillated water on days 0, 2 and 4 after diagnosis of metritis; CON, cows did not receive any treatment; or EXD, a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. In additional, the healthy (HTH) group. Overall effect of treatment in the logistic regression, P ≤ 0.01. a,b,cSuperscripts indicate significant differences (P ≤ 0.05) among treatments after Tukey-Kramer adjustments.
Figure 4-5. Proportion of cows culled within 60 DIM with peritonitis according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM, intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON, cows did not receive any treatment; or EXD, a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. Overal effect of treatment in the logistic regression, $P \leq 0.01$. a,b,cSuperscripts indicate significant differences ($P \leq 0.05$) among treatments after Tukey-Kramer adjustments.
Figure 4-6. Least squares means (± SE) of rectal temperature (°C) for cows diagnosed with metritis according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM (solid line), intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON (dashed line), cows did not receive any treatment; or EXD (dashed line), a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. TRT: P = 0.62; Day: P < 0.01; TRT x Day: P = 0.04.
Figure 4-7. Least squares means (± SE) of rectal temperature (°C) according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM (\(\triangledown\) solid line), intruterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON (● dashed line), cows did not receive any treatment; or EXD (▲ dashed line), a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. In additional, the healthy group (HTH ○ dotted line). TRT: P = 0.45; Day: P < 0.01; TRT x Day: P = 0.02.
Figure 4-8. Least squares means (± SE) of plasma concentrations of NEFA (mmol/L) for cows diagnosed with metritis according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM (\(\nabla\) solid line), intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON (● dashed line), cows did not receive any treatment; or EXD (▲ dashed line), a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. TRT: \(P = 0.23\); Day: \(P < 0.01\); TRT x Day: \(P = 0.11\).
Figure 4-9. Least squares means (± SE) of plasma concentrations of NEFA (mmol/L) according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM (solid line), intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON (dashed line), cows did not receive any treatment; or EXD (dashed line), a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. In addition, the healthy group (HTH dotted line). TRT: P ≥ 0.20; Day: P < 0.001; TRT x Day: P = 0.06.
Figure 4-10. Least squares means (± SE) of plasma concentrations of BHB (mmol/L) for cows diagnosed with metritis according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM (△ solid line), intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON (○ dashed line), cows did not receive any treatment; or EXD (◆ dashed line), a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. TRT: P = 0.52; Day: P < 0.01; TRT x Day: P = 0.17.
Figure 4-11. Least squares means (± SE) of plasma concentrations of BHB (mmol/L) according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM (▼ solid line), intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON (● dashed line), cows did not receive any treatment; or EXD (▲ dashed line), a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. In addition, the healthy group (HTH ○ dotted line). TRT: P = 0.38; Day: P < 0.02; TRT x Day: P = 0.07.
Figure 4-12. Least squares means (± SE) of plasma concentrations of haptoglobin (μg/mL) for cows diagnosed with metritis according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM (▼ solid line), intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON (● dashed line), cows did not receive any treatment; or EXD (▲ dashed line), a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. TRT: \( P = 0.16 \); Day: \( P < 0.01 \); TRT x Day: \( P = 0.61 \).
Figure 4-13. Least squares means (± SE) of plasma concentrations of haptoglobin (μg/mL) according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM (▼ solid line), intruterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON (● dashed line), cows did not receive any treatment; or EXD (▲ dashed line), a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. In additional, the healthy group (HTH ○ dotted line). TRT: P = 0.22; Day: P < 0.01; TRT x Day: P = 0.01.
Figure 4-14. Least squares means (± SE) of milk yield (kg/d) in the first 60 DPP according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM (▼ solid line), intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON (● dashed line), cows did not receive any treatment; or EXD (▲ dashed line), a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. In additional, the healthy group (HTH ○ dotted line). TRT: P < 0.01; Day: P < 0.01; TRT x Day: P < 0.01.
Figure 4-15. Least squares means (± SE) of milk yield (kg/d) in the first 30 days relative to enrollment (diagnosis day), according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM (\(\triangledown\) solid line), intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON (● dashed line), cows did not receive any treatment; or EXD (▲ dashed line), a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. TRT: \(P < 0.01\); Day: \(P < 0.01\); TRT x Day: \(P = 0.01\).
Figure 4-16. Least squares means (± SE) of milk yield (kg/d) in the first 10 months according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM (solid line), intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON (dashed line), cows did not receive any treatment; or EXD (dashed line), a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. In addition, the healthy group (HTH dotted line). TRT: P < 0.01; Day: P < 0.01; TRT x Day: P = 0.01.
Figure 4-17. Proportion of cows pregnant at first breeding 33 days after breeding according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM, intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON, cows did not receive any treatment; or EXD, a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. In addition, the healthy (HTH) group. Overall effect of treatment in the logistic regression, $P \leq 0.01$. a,b,cSuperscripts indicate significant differences ($P \leq 0.05$) among treatments after Tukey-Kramer adjustments.
Figure 4-18. Kaplan-Meier survival curves for proportion of non-pregnant cows for cows diagnosed with metritis according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM, intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON, cows did not receive any treatment; or EXD, a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. Overall effect of treatment from the Cox’s proportional model: $P = 0.01$. 

![Kaplan-Meier survival curves](image)
Figure 4-19. Kaplan-Meier survival curves for proportion of non-pregnant cows according to treatment used. Cows diagnosed with metritis were assigned randomly to receive: CM, intrauterine infusion of 24 g of chitosan microparticles dissolved in 40 mL of sterile distilled water on days 0, 2 and 4 after diagnosis of metritis; CON, cows did not receive any treatment; or EXD, a subcutaneous injection of 6.6 mg/kg of ceftiofur crystalline-free acid (Excede®, Zoetis) at the time of diagnosis of metritis and 3 days later. In addition, the healthy group. Overall effect of treatment from the Cox's proportional model: \( P = 0.01 \).
The experiment was designed to evaluate different treatments for metritis in dairy cow. The main focus was to assess the efficacy of an intrauterine administration of CM. Overall, cows treated with CM had cure similar to CON, which were less than EXD. Daetz et al., (2016) evaluated the efficacy of CM in preventing metritis in dairy cows, and reported that treatment with CM resulted in reduced incidence of metritis up to 7 DIM compared with untreated control cows; however, no differences were observed when cows were followed up to 14 DIM. Preliminary experiments from our group had indicated that CM could be a viable treatment for metritis (Jeon et al., 2014; Jeon et al., 2016). Chitosan microparticles have been shown to have broad-spectrum of antimicrobial activity in vitro (Jeon et al., 2014), and the same was expected in vivo. Indeed, Jeon et al., (2016) showed that CM was more effective than ceftiofur at reducing the relative abundance of Bacteroidetes, and was as effective as ceftiofur at reducing the relative abundance of Fusobacteria from D0 to D6. Bacteroidetes and Fusobacteria are the dominant bacteria in cows with metritis (Santos et al., 2011; Jeon et al., 2015; Jeon et al., 2016b); therefore, these results were promising. One aspect missing from our previous experiments was the effect of CM to on absolute bacterial abundance (i.e. Bacterial counts); therefore, it is possible that CM was not effective in reducing bacterial counts, which could have accounted for reduced cure rate compared with EXD.

Treatment with CM resulted in greater culling than EXD or CON. Increased culling in CM cows is likely a result of an exacerbation of the inflammation after CM infusion. Although, CM did not result in increased concentrations of Hp or an increase in
rectal temperature after infusion, it did increase the proportion of cows culled because of peritonitis. More specifically, culling rate due to peritonitis was 81% and 76% greater for CM compared with EXD or CON, respectively. Therefore, it is possible that the degree of inflammation was not fully represented by the rectal temperature in all cows and in the subset of cows sampled for plasma concentrations of Hp, but could be captured when all cows were evaluated for culling reason. We had no previous indication that CM could lead to an exacerbated inflammatory state, so this hypothesis remains to be unsolved. One of the main differences between previous and the current experiment is the dose used. For the present experiment, the dose used was greater than previously used, from 8g to 24g/infusion, in order to achieve an estimated 0.6% CM concentration in the uterine content, which was the concentration shown to be effective against IUPEC ex vivo. Chitosan can be derived by partial deacetylation of chitin; therefore, it is comprised of copolymers of glucosamine and N-acetylglucosamine. Chitosan is generally recognized as safe by the FDA, it is non-toxic, bioadhesive, biocompatible, and biodegradable. Because of these desirable characteristics, chitosan is promising as antimicrobial and have been used broadly in pharmaceutical industries. Nonetheless, Lanctôt et al. (2017) reported that infusion of chitosan hydrogel in the mammary gland of cows caused a sharp increase in somatic cells, indicating a rapid activation of an inflammatory response. However, in the present experiment, CM was used. The microparticles are derived from the chitosan molecule by sonication to break the chitosan molecule and addition of 10% of sodium sulfate to induce cross-linking between the chitosan molecules. After the sodium sulfate is added to the chitosan solution, it dissociates into Na$^{2+}$ + SO$_4^{2-}$. The SO$_4^{2-}$ binds to the chitosan
molecule and the Na\(^{2+}\) are washed away during particle preparation. To our knowledge, there is no previous study evaluating the inflammatory response to CM in cows or its cytotoxicity. It is also not known if the sulfate binding CM molecules is safe and cannot cause any tissue damage.

Another indication that CM was detrimental was seen in milk yield. Cows in the CM treatment had less milk yield than EXD and CON. Again, this is likely a result of exacerbated inflammation in CM cows. Inflammation redirects nutrients such as glucose away from anabolic processes that support milk and muscle synthesis towards the inflammatory response (Johnson, 1997; Horst et al., 2018). Adequate fueling of immune cells is a critical component in successfully mounting an effective immune response (MacIver et al., 2018). Improving glucose availability can increase longevity and function of activated leukocytes (Sagone et al., 1974; Garcia et al., 2015). The energetic cost of immune activation has been estimated using a LPS-euglycemic clamp model, and observed that approximately 1 kg of glucose is used by the immune system during a 12-hour period in lactating dairy cows (Kvidera et al., 2017). The amount of glucose utilized by LPS-activated immune system in lactating dairy cows is 0.66 g glucose/kgBW0.75/h, (Kvidera et al., 2016a). Additionally, cows in the CM group had decreased pregnancy per AI at the first service and decreased proportion of pregnant cows by 300 DIM compared with EXD or CON. An increased uterine inflammatory response in the CM group would have led to more uterine tissue damage because of generation and release of reactive oxygen species and the release of proteolytic enzymes (Lacy et al., 2006).

A secondary objective was to compare EXD and CON in regards to culling, milk yield and fertility because these outcomes had not been previously reported in the
experiments used for approval of ceftiofur hydrochloride (Chenault et al., 2004) and ceftiofur crystalline-free acid (McLaughlin et al., 2012) for treatment of metritis. As shown herein, cows treated with EXD had increased cure compared with CON (McLaughlin et al., 2012). Additionally, the proportions of cows cured of metritis for the ceftiofur and control treatments were in agreement with previous reports, which ranged from 75 to 78% for cows treated with ceftiofur and from 55 to 62% for untreated control (Chenault et al., 2004; McLaughlin et al., 2012). Recently, an experiment with grazing cows compared the use of Excede for treatment of metritis using a single subcutaneous administration compared with untreated controls and reported that the ceftiofur treatment was effective in reducing the adverse effects on reproductive performance but not on milk yield (Piccardi et al., 2016). Cows with metritis treated with a single subcutaneous administration of ceftiofur had increased pregnancy per AI to first service and hazard of pregnancy compared with untreated controls (Piccardi et al., 2016).

Although not a randomized experiment, we included a convenience HTH group as a reference to understand the impact of metritis and respective treatments on production, reproduction and survival of cows. Cows in HTH group had less culling, greater milk yield, and better fertility than cows with metritis. In addition, HTH cows had less concentration of plasma Hp and no differences on plasma NEFA and BHB concentrations were observed when compared with cows with metritis. These findings confirmed that cows with metritis have reduced milk yield, approximately 4.5 kg/day, compared with HTH group. These results corroborate those of Huzzey et al. (2007) and Daetz et al., (2016) which reported that cows with metritis have reduced milk production, approximately 5.7 kg/day, compared with cows without metritis. The
interaction between treatment and DIM was significant, on a case the two explanatory variables (DIM and treatment) interacts with the response variable (milk production), in our results the magnitude of the milk production was increased after treatment in the first 60 DPP, also the same interaction was observed when the monthly milk production was evaluated. The monthly milk yield was statistically different between the treatments.

Cows in the HTH group resulted in greater fertility than metritic groups. The effect of the treatment on fertility was analyzed in the Cox model analysis, and the hazard of pregnancy up to 300 DPP was greater for EXD than CM and CON, which all were lower for the HTH group. LeBlanc et al., (2002) and Overton and Fetrow, (2008) reported that cows with metritis have reduced reproductive performance. Ribeiro et al., (2016) reported that inflammatory disease such metritis before breeding reduced fertilization of oocytes and development to morula, one reason for that is that cows suffered metritis had delayed growth of the first dominant follicle and reduced concentrations of estradiol (Sheldon et al., 2012). In additional, Bronfield et al., (2015) reported that the presence of LPS in the follicular fluid of cows with metritis and endometritis seems to be a potential reason for compromised steroidogeneses, follicle growth, and impaired oocyte development. Ribeiro et al., (2016) also reported that metritic cows had smaller conceptus and consequently less secretion of IFN-τ in the uterine lumen. However, is known that IFN-τ might be critical for the synchrony of pregnancy, recognition and early placentation events.

When plasma Hp concentration of the metritic groups was compared with healthy cows. Plasma Hp concentration of the metritis diagnosis D0 was increased to cows with metritis when compared with healthy (no metritis) cows, which is similar to what was
reported by Sheldon et al., (2001). An increase in plasma Hp concentration was expected in cows with metritis in response to pro-inflammatory cytokines, such as TNFα and IL-6, which are produced in the presence of tissue damage or LPS release by gram-negative bacteria (Eckersall et al., 1988; Oliviero et al., 1989; Nakagawa-Tosa et al., 1995). Haptoglobin is an indicator of the degree of bacterial contamination and inflammation (Galvão et al., 2010; Herath et al., 2009), Galvão et al., (2010) reported that cows that developed metritis, which is an acute and more severe process, had increased haptoglobin levels around the time of metritis diagnosis. The Hp plasma concentration decreased after the day of diagnose in all treated groups, which might be due to metritic cows receiving treatment (Smith et al., 1998), or by a decrease in the uterine inflammation process, since the Hp concentration also was lower to the control group up 12 days after the metritis diagnosis.

In the present experiment, treatment did not affect plasma NEFA concentration. Concentration of NEFA peaked at calving in all treatments and no difference was observed between cows with or without metritis. Some studies reported an association between concentrations of NEFA or BHB in plasma either before or immediately after calving and the development of diseases such as displaced abomasum, clinical ketosis, metritis and retained placenta (Cameron et al., 1998; LeBlanc et al., 2005; Ospina et al., 2010). A lower DMI and elevated concentration of metabolic indicators such as NEFA and BHB have been associated with metritis during the peripartum period (Hammon et al., 2006). Statistical difference was observed for the concentration of BHB when the parity was compared, the plasma BHB concentration was higher for multiparous than primiparous. Several studies have reported a higher NEFA and BHB concentration in
multiparous cows than in primiparous cows during the postpartum period (Duffield et al., 1997; Dohoo et al., 1984b; Santos et al., 2001).

In conclusion, intrauterine infusion of 24 g of CM dissolved in 40 mL of sterile distilled water every other day for a total of three treatments as therapy for metritis did not improve cure rate, but it was detrimental to milk yield, culling and fertility compared with the untreated controls. Cows in the EXD treatment had increased cure, milk yield and fertility compared with CON. Treatment did not affect plasma Hp, NEFA and BHB concentrations. Plasma Hp concentration was greater in metritic cows on D0 compared with HTH cows, but there was no difference in NEFA and BHB concentrations. The results from this experiment indicate that CM used as described herein is ineffective in the treatment of metritis and is detrimental to milk production, fertility and survival in dairy cows.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Eduardo Barros de Oliveira was born in Brazil. He is the fourth generation of cattle ranchers, and he spent his childhood on his family’s farm. That was where he had the opportunity to be in contact with farm animals and decided that veterinary medicine was the best field to pursue as a career.

Eduardo attended Universidade Federal de Goiás (UFG) in Goiânia, Goiás, Brazil. He received the DVM degree in 2015. In addition to his responsibilities as student, he was the teaching assistant in a reproduction class for 2 years. After graduating in 2015, Eduardo was hired by Alta Genetics Brazil as an associate veterinarian to work in the areas of medicine, production and reproduction.

In 2016, Eduardo moved to the United States and got involved in several research projects at the University of Florida. In 2018, he was accepted in the Master of Science program in Veterinary Medical Science at the College of Veterinary Medicine of the University of Florida, Department of Large Animal Clinical Sciences. Upon completion of his MSc program, Eduardo will move to the University of California Davis to continue his post-graduate studies in a clinical residency in dairy production medicine in the Veterinary Medicine Teaching and Research Center in Tulare, California. Eduardo sees his future closely associated with the dairy industry, especially in health, reproduction and management, and also his long term goal is to become a professor, and share his knowledge as other have done with him.