ROLE OF THE MICROBIOME FOR THE DEVELOPMENT OF METRITIS IN DAIRY CATTLE AND NOVEL STRATEGIES FOR PREVENTION

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ROLE OF THE MICROBIOME FOR THE DEVELOPMENT OF METRITIS IN DAIRY CATTLE AND NOVEL STRATEGIES FOR PREVENTION

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ABSTRACT

Metritis is a systemic illness that affects around 20% of dairy cattle, occurring within 21 days post-partum. Among the risk factors associated with the disease are retained placenta, stillbirth, dystocia, ketosis, and hypocalcemia. The main microorganisms associated with the disease are *Escherichia coli*, *Trueperella pyogenes* and *Fusobacterium necrophorum*. Detrimental effects caused by the disease are decreased milk production, increased calving to conception interval, and decreased pregnancy per artificial insemination, with the cost of a single case of metritis being estimated at around $513 ($240 to $884). There is a need for the development of reliable models of metritis induction to better understand the dynamics of microorganism growth and determine the metabolic and clinical alterations associated with the development of the disease. A reliable *in vivo* model of metritis is also a useful tool to test different strategies to prevent or treat the disease, without using a large number of animals, while also easily determining the physiologic alterations caused by these interventions.

This dissertation presents in vivo models of metritis induction in primiparous and multiparous Holstein cows, revealing the microorganisms and metabolic changes associated with the development of the disease. A challenge containing 10^6 cfu each of *E. coli*, *T. pyogenes* and *F. necrophorum* caused metritis in 80% of multiparous cows, increased rectal temperature and decreased dry matter intake. We also determined that *F. necrophorum* was the main microorganism associated with the development of the disease. The same dose did not significantly increase the incidence of metritis in primiparous cows, but those cows had a 2.7-times

greater hazard of being diagnosed with metritis, associated with reduction in milk production when compared to control cows.

Lasty, we present a novel strategy (recombinant bovine interleukin-8, rbIL-8) for the prevention of uterine diseases and for increased milk production as an alternative to the use of antimicrobials to treat the disease. The last chapter discusses the second administration of rbIL-8 in cows that had received the treatment two lactations before, showing that it modulates the uterine microbiome, reducing the bacterial load and controlling the growth of the pathogenic bacterial species *Fusobacterium*.

This dissertation reveals that metritis can be induced in a dose-dependent manner in healthy multiparous cows via infusion of a cocktail containing pathogenic microorganisms described to be associated with the disease, and the main pathogen associated with the disease was *Fusobacterium*. We also demonstrated the effects of a novel strategy for prevention of metritis, administration of rbIL-8, showing that it controls the growth of *Fusobacterium*, the microorganism that was found to be relevant for induction of metritis. Further research needs to be conducted to elucidate the reasons that the same model did not induce metritis in primiparous cows.

BIOGRAPHICAL SKETCH

Josiane (Josi) Cristina do Carmo Silva was born and raised in Uberaba, Minas Gerais – Brazil. Her parents are Julio Mauro da Silva and Tania Maria do Carmo Silva and her only sister is Thaciana Cristina do Carmo Silva. Her parents came from a very humble family without much education, but always invested all their energy and time to raise their two daughters with strong values and good education. Both always pushed her daughters to pursue public universities in Brazil, as a good opportunity to start a career.

She received her degree in Veterinary Medicine at the University Federal of Uberlandia, Brazil in 2010, under the supervision of Dr. Ricarda Maria dos Santos, working in heat synchronization of beef cattle. Dr. Ricarda Maria is a very dedicated mentor, who instilled on her the passion for the cattle industry and professionalism as a veterinarian.

After graduation Josi spent some time in a dairy farm in Minnesota, working with Dr. Ricardo Chebel. During her time in Minnesota Josi was exposed to different field trials that students were conducting at the farm, her interest in research started when helping a study which tested strategies to prevent uterine diseases. At that time, the need to stay with family was bigger and she went back to Brazil to start working in a bull study, which after three years brough Josi to Texas to improve skills in processing semen.

During her time working in a bull study Josi was transferred from Texas to Wisconsin and later to New York, experiencing different cultures. Although the work with bulls brought joy and aggregated experience in working in the private industry, Josi desire to work in the dairy industry was bigger, encouraging her to search for opportunities in the academia, to get better qualification to compete for jobs in a foreign country.

At that time Josi was living in Ithaca, New York, where she was introduced to Dr. Rodrigo

Bicalho, who would later become one of her PhD professors. After almost a year of internship, Josi was invited to join Dr. Bicalho's research group and by a destiny surprise, start a research with uterine diseases.

This dissertation is dedicated to my mom and my dad for their efforts to invest in my education and support all my decisions.

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To Dr. Ricarda Maria, who has taught me how to be a female veterinarian that works in a dairy farm. She has shown me that woman and man can occupy the same space in the dairy industry as long as there is professionalism and respect. Thanks for the mentorship even after college, for spending hours preparing me for presentations and exams, and the great friendship.

To Dr. Rodrigo Bicalho, who gave me the opportunity to join his research group at Cornell University and for believing in me. To all my committee members, Dr. Craig Altier, Julio Giordano, Soon Hon Cheong, and Heather Huson, for their commitment, support, and guidance during my PhD program, and efforts on making my degree competition possible. I would further like to thank Dr. Cheong, for dedicating his time in helping me understand the "researcher way of thinking", all the hours dedicated not only discussing research, but guiding me to go through moments in life. Also Dr. Giordano, for willing to guide me through end of my degree, dedicating hours in meeting and discussions.

Finally, I would like to extend my gratitude and appreciation to Dr. Altier for "adopting" and believing in me. I really appreciate the great mentorship, the patience, dedication, the long hours meetings to make me an independent researcher in such a short time, for teaching me how to handle situations in the most calm and polite way possible. I have finally learned to be confident about myself and hopefully I became a researcher. I would never have completed my program

without his help.

Finally, I thank Rimi Chowdry for the great friendship and for always be willing to review papers and help with anything I needed, and all my friends from the Animal Sciences Department for making my PhD program easier, for weekends studying together, for great happy hours just to distract each other and make all the stress go away. The department is full of great researchers and good people.

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ALP, Alkaline phosphatase

ALT, Alanine transaminase

APP, Acute phase protein

AST, Aspartate transaminase

BCS, Body condition score

BHB, Beta-hydroxybutyrate

BMR, biometrics representative

BW, Body weight

CaSR, calcium-sensing receptor gene

CBC, Complete blood count

cfu, Colony forming unit

CK, Clinical ketosis

CURC, Cornell University ruminant center

DM, Dry matter

DMI, Dry matter intake

DNA, Deoxyribonucleic acid

DWP, Dairy wellness profit

FA, Fatty acids

US FDA, United States Food and Drug Administration

FSH, Follicle-stimulating hormone

GGT, Gamma-glutamyl transferase

GMILK, Genomic enhanced predicted transmitting ability for milk yield

GnRH, Gonadotropin-releasing hormone

IL, Interleukin

LAL, Limulus amebocyte lysate

LB , Luria-Bertani

LBP, Lipopolysaccharide binding protein

LH, luteinizing hormone

LKV, Laked kanamycin vancomycin

LPS, Lipopolysaccharide

NEB, Negative energy balance

NEFA, Non esterified fatty acids

OTU, Operational taxonomic unit

PBS, Phosphate-buffered saline

PCR, polymerase chain reaction

rbIL-8, Recombinant bovine interleukin 8

RT, Rectal temperature

SAA, Serum amyloid A

SCH, Subclinical hypocalcemia

TLR, Toll-like receptor

TMR, Total mixed ration

TNFα, Tumor necrosis factor alpha

TPI, Total Performance Index

VDS, Vaginal discharge score

Z_MET, Genomic standardized transmitting ability for metritis risk

Z-RP, Genomic standardized transmitting ability for retained placenta risk

CHAPTER ONE: INTRODUCTION

The reproductive tract and metritis definition

The reproductive tract is composed of the vulva, vagina, cervix, uterus, oviduct, and ovaries. The vulva and cervix are the first anatomical defense barrier to pathogenic microorganisms that invade the vagina towards the uterine lumen. During and after parturition these barriers are breached, allowing bacteria to ascend from the environment, the animal's skin, feces or through the blood stream into the uterus (RISCO et al., 2007, Jeon et al., 2017, Rosales and Ametaj, 2021). Uterine contamination is ubiquitous in cattle; however, it can either be transient, when cows are able to eliminate bacteria by uterine defense mechanisms during the puerperium; or it can be persistent, depending on the pathogenicity of the bacteria and the inability of the host immunity to control its growth. When the contamination is persistent, uterine diseases can occur, leading to negative impacts on productivity and reproduction (Sheldon and Dobson, 2004, RISCO et al., 2007, Molina-Coto and Lucy, 2018, Rosales and Ametaj, 2021).

The definition of uterine diseases is based on characteristics of the uterine discharge, the days post-partum and the clinical signs (RISCO et al., 2007). Unfortunately, the definition of uterine diseases was not clear in the past, creating some confusion among clinicians and researchers, consequently, the incidence of diseases was not precisely reported, creating an underestimation of their effects to dairy farmers (RISCO et al., 2007, Perez-Baez et al., 2021). Aware of this limitation, Sheldon et al. (2006) proposed a definition for all uterine diseases that could be adopted in the field and by researchers. The authors proposed that uterine diseases be classified as puerperal metritis, clinical endometritis, subclinical endometritis and pyometra. For the purpose of this dissertation, we will focus on metritis and explore our findings.

According to Sheldon et al. (2006), puerperal metritis is defined to occur in an animal with an abnormally enlarged uterus and a fetid watery red-brown uterine discharge, associated with signs of systemic illness (decreased milk yield, dullness or other signs of toxemia) and fever >39.5 °C, within 21 days postpartum. Clinical metritis is defined as presenting an abnormally enlarged

uterus and a purulent uterine discharge detectable in the vagina, within 21 days after parturition, but without systemic illness. In our research we simply used the term metritis, which includes cows diagnosed with puerperal and clinical metritis.

Incidence and risk factors

Although metritis occurs in the first 21 days postpartum, the disease is most common within 10 days. The disease occurs on average in 20% of lactating cows, ranging from 8 to 40% in some farms (LeBlanc, 2008, Galvão, 2012).

Metritis is a multifactorial disease, making it impossible to isolate a single cause (Galvão, 2018). Many researchers associate the disease with breaches in the barriers of the reproductive tract, such as vulva and endometrium, also affecting the production of mucus, an important mechanic barrier, facilitating the path for bacteria to access the uterine tissue and consequently cause infection. Among the risk factors associated with the disease are parity, dystocia, male offspring, twins, stillbirth, abortion, prolapsed uterus, retained placenta and vulvovaginal laceration (Markusfeld, 1984, 1987, Kimura et al., 2002, Dubuc et al., 2010, Ghavi Hossein-Zadeh and Ardalan, 2011, Vieira-Neto et al., 2016). According to Vieira-Neto et al. (2016) primiparous cows tend to have increased odds for the development of metritis, possibly explained by the high incidence of parturition problems. Among other factors considered risky for the development of metritis: cows with dystocia have 2.3 greater odds to have metritis, and cows with retained placenta have 46.3 greater odds to have metritis as well. For that specific study, the authors wanted to bring attention to vulvovaginal laceration, which was not yet described as a risk factor for metritis. They found that cows with more than 2 centimeters of vulvovaginal laceration had 2.6 greater odds to have metritis ($P < 0.001$), while cows with less than 2 centimeters tended to have metritis when compared to cows that did not present any vulvovaginal laceration ($P = 0.10$). According to Ghavi Hossein-Zadeh and Ardalan (2011), cows with dystocia, stillbirth, retained placenta, twin births, primiparity, and male calves had 4.32, 6.26, 27.74, 6.57, 1.68, 2.41 greater odds to have metritis,

and had a delay in uterine involution when compared to cows without metritis.

Although the hygiene of the environment is not associated with the incidence of uterine diseases, Schuenemann et al. (2011) demonstrated that the association of perineal hygiene at the time of calving is. Using a scale that ranges from 1 to 3, where 1 is a perineum free of manure and dry; 2 is a perineum slightly wet, with dirt-manure on less than 10% of the surface; 3 a perineum moderately wet, covered with dirt-manure on more than10% of the surface, the authors reported that cows with scores 3 or 2 had higher incidence of metritis when compared to cows with score 1. Therefore, they concluded that uterine contamination could result from the cow itself.

Some metabolic alterations are also known to be risk factors for the development of metritis. Cows diagnosed with hypocalcemia have a reduced percentage of neutrophils able to perform phagocytosis and the oxidative burst, consequently presenting increased risk for the development of metritis compared to normocalcemic cows (Hammon et al., 2006, Martinez et al., 2012). Also, the relative risk for the development of metritis decreased 22% for every increase of 1mg/dL of serum calcium.

Ketosis is also associated with increased incidence of metritis (Dohoo and Martin, 1984, Duffield et al., 2009, Suthar et al., 2013). Suthar et al. (2013) discussed that it possibly happens due to the decrease in dry matter intake (DMI) during the first 2 weeks post-partum, consequently increasing non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB) levels. This reduction in DMI also impairs the immune response, as immune activation requires glucose availability (Kvidera et al., 2017).

Bacteria associated with metritis

It was well stablished that the uterus of pregnant cows is sterile, with its microbiome being established right after parturition (Sheldon et al., 2009). Recently, Moore et al. (2017) tested the hypothesis that the uterus of virgin heifers and pregnant cows has a resident microbiome by 16S rRNA sequencing, reporting that the three most abundant phyla were *Firmicutes*, *Bacteroidetes*,

and *Proteobacteria*. That finding suggested that the uterine microbiome is established with the beginning of reproductive maturity, and that pregnancy could be established and maintained in the presence of a resident uterine microbiome. Therefore, it is now understood that the uterus possesses its resident microbiome, and with parturition there is a blooming in growth of pathogenic bacteria in the uterus that may help the establishment of disease (Sheldon et al., 2019).

There is an interesting change in the knowledge about microorganisms associated with the development of metritis. This happened because before 2010 most studies relied on culture-dependent studies; after that most studies relied on PCR techniques, therefore finding microorganisms that were previously unknown to be associated with the disease (Galvao et al., 2019). Based on culture-dependent studies, the most common bacteria associated with metritis were *Escherichia coli*, *Trueperella pyogenes* and *Fusobacterium necrophorum*, along with some *Prevotella* and *Bacteroides* species (Huszenicza et al., 1999, Sheldon et al., 2002, Sheldon et al., 2019). With the advance of metagenomic techniques, microorganisms that were considered difficult to culture under conventional circumstances such as *Bacteroidetes*, *Fusobacteria*, *Proteobacteria*, and *Firmicutes* were found to be associated with uterine diseases (Machado et al., 2012). Also, as an advantage of this technique, virulence factors of *E. coli* found in the uterus of cows with endometritis were identified (Sheldon et al., 2010). The strain pathogenic to the uterus is considered more adherent and invasive to endometrial cells than other strains isolated from healthy cows, due to the expression of Type 1 fibrin D-mannose-specific adhesin (commonly known as FimH). Later, in a study where bacterial species-specific virulence factors were explored, Bicalho et al. (2012b) found that cows that cows positive for *fimH* at one or three days postpartum had greater odds to be positive to *lktA* at eight days postpartum, which increased the odds of being diagnosed with metritis afterwards. The group therefore speculated that *E. coli* was a pioneer pathogen in the uterus, acting in synergy with *F. necrophorum* and *T. pyogenes* to cause metritis and clinical endometritis, as the group

Based on recent findings regarding the microorganisms associated with the development of metritis, a study was conducted to elucidate the changes in the uterine microbiome from calving until the establishment of metritis (Jeon et al., 2015). The group observed changes in the microbiome from parturition to 6 ± 2 days post-partum, finding that *Bacteroides* was the main genus associated with metritis, and was positive correlated with the genus *Fusobacterium*. The authors also found that as the abundance of *Bacteroides* or *Fusobacterium* increased, the uterine discharge score worsened. Some bacteria were described as associated with uterine health, *Escherichia*, *Sneathia*, and *Pedobacter*. The group then hypothesized that the uterus of a healthy cows maintained heterogenicity and richness compared to cows with metritis (Jeon et al., 2015, Jeon et al., 2016).

Immune response in the uterus

As bacterial contamination of the uterus occurs naturally after parturition (Jeon et al., 2015), a robust immune response must be mounted to protect the animal from pathogenic bacteria and to help tissue regeneration. The innate immune system is the main protection for the uterus, starting the response after sensing the presence of pathogens via pathogen-recognition receptors, such as toll-like receptors (TLR). An example is the recognition of LPS, an endotoxin present in gram-negative bacteria, by TLR-4; another example is lipopeptide being recognized by TLR1, TLR-2 and TLR-6. This interaction between pathogen and recognition receptors leads to production of the pro-inflammatory cytokines (interleukin) IL-1β, IL-6, the chemokine IL-8, and antimicrobial peptides (Machado and Silva, 2020). The first defensive cells to arrive in the uterus are the neutrophils. They are activated and directed to the site of infection by the chemokine IL-8. At the site of infection, neutrophils phagocyte and kill invading microorganisms, controlling the infection (Hammon et al., 2006, Donofrio et al., 2010). If any event reduces the immune response after parturition, such as the development of negative energy balance (NEB), there is an increased chance for the development of uterine diseases. If the immune response is not controlled,

an exacerbated inflammation will cause tissue damage and prolonged presence of neutrophils in the endometrium, causing damage to the endometrium and infertility (LeBlanc, 2014), not to mention that the reduced immune response will allow the dysbiosis in the uterine microbiome that can lead to diseases (Galvao et al., 2019).

The role of the adaptative immune system in controlling uterine diseases is still unclear (Sheldon et al., 2019). Some researchers have identified areas in the endometrium that are rich in T and B cells (Wagner and Hansel, 1969, Bonnett et al., 1991), others affirm that the presence of antibodies in the endometrium is associated with reduced incidence of uterine diseases (Machado et al., 2014a). Although preliminary data of a vaccine containing inactivated cells of *E. coli*, *T. pyogenes* and *F. necrophorum* had promising results in protecting against uterine diseases (Machado et al., 2014b), another study produced the opposite result (Freick et al., 2017), leading the authors to hypothesize that adaptative immunity does not provide long term protection to the uterus for unknown reasons.

A new approach was suggested by Sheldon et al. (2020) to better understand the physiology behind the development of uterine diseases in dairy cows. The approach tries to justify that although all cows are exposed to the same pathogens, some will develop the disease and others will not, based on three complementary strategies: avoidance, tolerance, and resistance. According to the authors, avoidance is a mechanism to limit the exposure to pathogens. An example is the hygiene behavior presented by cows, being typical to see cows self-grooming and choosing clean and dry bedding to rest. Tolerance is the ability to limit the tissue damage caused by pathogens without affecting the pathogen burden, including functional barriers to bacterial infection, neutralization of toxins, and repair of damaged tissues. Resistance involves the recruitment of innate and adaptative immunity to control the infection. The authors also suggest that the ideal cow should be resilient, which means a cow that prevents disease by avoidance, tolerance and resistance when exposed to pathogens.

Impacts on fertility, milk production, and costs

It is important to understand the impact on fertility of the immune response that is triggered by bacterial contamination. Sheldon et al. (2009) published a minireview with a detailed explanation of how uterine infection affects fertility in dairy cows. According to the authors, bacterial contamination after parturition leads to activation of the immune system due to the presence of LPS in gram negative bacteria' such as *E. coli* and *F. necrophorum*, that is recognized by TLR-4. The immune response triggered by this recognition can cause damage to the endometrium and therefore reduce the chance of conception. The LPS will also affect hormonal secretion. The release of gonadotropin-releasing hormone (GnRH) from the hypothalamus and luteinizing hormone (LH) from the pituitary is suppressed, reducing the ovulation of dominant follicles. On the other hand, the secretion of follicle-stimulating hormone (FSH) is not affected, so follicular waves emerge normally during the first week post-partum. The follicular fluid will also contain LPS, perturbing the estradiol secretion from granulosa cells by reducing aromatase expression. If the cows happen to ovulate, the corpus luteum will secrete less progesterone than in normal fertile animals. As inflammation will trigger secretion of prostaglandin E, the secretion of prostaglandin F will be impaired, therefore prolonging the luteal phase of metritic cows. In conclusion, uterine contamination has many detrimental effects on fertility; cows will have follicular recruitment but will fail to ovulate due to low LH concentration. If there is ovulation, this corpus luteum will probably be persistent as the secretion of prostaglandin F is impaired. The oocyte that ovulates was exposed to LPS while growing and grew under a low concentration of estradiol. If the oocyte is fertilized by an spermatozoa, it will be in contact with a damaged endometrium, therefore explaining the late resumption of cyclicity.

Based on the evidence provided above, we conclude that metritis has detrimental effects on farm profitability. Researchers have also shown that cows with metritis have reduced milk production throughout the lactation, (Perez-Baez et al., 2019), a delayed resumption of ovarian

cyclicity (Santos et al., 2010a, Ribeiro et al., 2013), reduced pregnancy per artificial insemination (Santos et al., 2010a, Lima et al., 2019), and increased calving-to-conception interval (Giuliodori et al., 2013, Vieira-Neto et al., 2016). Some researchers affirm that metitic cows are more likely to be culled (Wittrock et al., 2011) while others indicate that it does not influence culling rate (Dubuc et al., 2011).

Perez-Baez et al. (2021) estimated the cost of metritis in dairy herds from four regions of the United States. The authors found that cows with metritis had lower dry mater intake (5,769.7 ± 147.3 vs. 6,227.1 ± 143.9 kg/cow; $P < 0.01$) and lower milk yield (9,463.0 ± 318.7 vs. 10,277.0 ± 313.8 kg/cow; $P < 0.01$), which resulted in less income from milk sales. Metritic cows also had a lower proportion of pregnancies by 305 days in milk (69.2 ± 1.2 vs. 79.2 ± 1.1%; $P < 0.01$) and were more likely to leave the herd by death or being sold. The estimated cost of treatment for metritis was $117.90. In conclusion, the authors estimated that the cost of a single case of metritis in that study was around $512.80, with a 95% range from $884.10 to $240.30.

Diagnosis

It is important to precisely diagnose metritis to quantify the severity of the disease and treat it as fast as possible. Unfortunately, there is no established "gold standard" for the diagnoses of metritis (LeBlanc et al., 2002, Williams et al., 2007). Manual examination is often used for diagnosis as it is a very cheap and rapid technique that does not cause uterine contamination and does not induce production of acute phase proteins (LeBlanc et al., 2002). Other strategies include the use of vaginoscopy or instruments to withdraw the vaginal content, such as the Metricheck device (Sheldon et al., 2008).

Recently, a different approach was proposed for the diagnosis of metritis. Efforts have been made to develop products that identify animals with high risk to develop the disease or to help predict the cure and success of treatments. Among those are the use of activity monitors, biomarkers, algorithms, and immune cells profiles (Stangaferro et al., 2016, Pomeroy et al., 2017,

Barragan et al., 2018, Barragan et al., 2019, Pascottini et al., 2020).

Strategies of treatment

The only antibiotic approved by the US Food and Drug Administration (FDA) for treatment of metritis is ceftiofur, a third-generation cephalosporin with a broad spectrum of activity, that results without milk withdrawal in the reduction in rectal temperature, improvement in milk production and fertility, and increase in cure rate (Drillich et al., 2001, Chenault et al., 2004, McLaughlin et al., 2012, Jeon et al., 2021). When exploring the effects of ceftiofur on the uterine microbiome, Jeon et al. (2021) found that the treatment alters the uterine microbiome by reducing the relative abundance of *Fusobacterium*, a microorganism associated with the development of metritis (Galvao et al., 2019).

Although the effects of ceftiofur are well known, and its efficacy is confirmed, it is desired that farmers reduce the use of antibiotics to the minimum to reduce the risk of antimicrobial resistance (WHO., 2021). This approach has increased the search for new strategies to prevent or treat metritis. Among strategies that did not succeed are the use of mannose, bacteriophages, chitosan and dextrose. Some other strategies aiming to reduce the bacterial presence or modulate the immune response in the uterus are still in development and will be discussed here.

Lactic acid bacteria are tested to reduce the incidence of metritis when administered intravaginal during the pre- and post-partum periods. The effects were a reduction in the incidence of metritis, a lower incidence of total uterine infection and lower systemic inflammation as a result of lower incidence of lipopolysaccharide-binding protein and lower serum amyloid A. Based on these findings, more research is needed to elucidate the mechanism of action of these probiotics (Deng et al., 2014, Genis et al., 2018).

Efforts were also made to develop vaccines against metritis pathogens (Machado et al., 2014b, Freick et al., 2017, Meira et al., 2020). The first tested vaccine contained a combination of inactivated cells of *E. coli*, *T. pyogenes* and *F. necrophorum* and recombinant

proteins of major virulence factors (FimH, lktA, and pyolysin, Plo) and was administered to heifers 60 days before parturition with a booster 30 days later (Machado et al., 2014b). The successful vaccine reduced the incidence of metritis. The same vaccine formulation was later tested, with the formulation containing the combination of recombinant subunit proteins being successful in reducing the uterine bacterial load, decreasing the uterine load of *F. necrophorum* by nine days post-partum and increasing reproductive performance (Meira et al., 2020). A second potential vaccine containing a combination of *T. pyogenes*, *E. coli*, *Streptococcus uberis*, *Bacteroides*, and *Peptostreptococcus* species, was administered intravenous six weeks prior to parturition with a booster three weeks later. Interestingly, this vaccine formulation failed to prevent metritis (Freick et al., 2017).

Interleukin-8 (IL-8) is another potential tool to prevent metritis when produced recombinantly. The protein is a chemokine secreted by monocytes, activated neutrophils, endothelial and epithelial cells (Baggiolini et al., 1989) and when linked to its receptor on neutrophils induces its activation, increasing phagocytic and killing activity (Mitchell et al., 2003). The idea of infusing cows with recombinant IL-8 came from the finding that cows that developed retained placenta presented reduced neutrophil chemotactic activity and lower plasma levels of IL-8 around parturition (Kimura et al., 2002). Based on this, Bicalho et al. (2019) aimed to coordinate and direct the neutrophil response to the site of inflammation. Therefore, the production process of recombinant bovine Il-8 (rbIL-8) in *E. coli* host cells was standardized, and the biological activity accessed *in vitro and in vivo*. Later, the protein was intrauterine infused in different doses in post-partum cows (high-dose group received 1.125 µg of rbIL-8 and the low-dose received 11.25 µg of rbIL-8). Both doses reduced the incidence of metritis in multiparous cows, and both groups produced more milk (Zinicola et al., 2019b). After the interesting result of increased milk production, the possible cause was explored in a subsequent field trial, where different routes of administration of the protein were also tested. Cows receiving intrauterine administration of rbIL-

8 presented greater milk production and increased DMI, associated with lower odds of developing diseases, supporting the conclusion that the use of rbIL-8 improves health and production when administered post-partum (Zinicola et al., 2019a).

With the results achieved during the first administration of rbIL-8, a concern arose regarding to the effects of a subsequent administration of a protein produced recombinantly in *E. coli*. *E. coli* is used for production of recombinant proteins due to the broad knowledge of its genetics and physiology (Ferrer-Miralles and Villaverde, 2013), along with simple culture methods and fast growth. Although the organism presents clear benefits for its use, a big concern is that as a gram-negative bacteria, it presents LPS as a major constituent of the outer membrane, and if the purification process of the recombinant protein is not effective, the LPS contamination present in the protein will activate an immune response, resulting in anaphylaxis and autoimmunity (Mamat et al., 2015). For this reason, a cautious process of purification of proteins is required, followed by a quality control process to assure that the protein used for treatments possess LPS under the levels required by regulatory agencies (Porter, 2001).

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CHAPTER TWO: Intrauterine infusion of a pathogenic bacterial cocktail induced clinical metritis in postpartum multiparous Holstein cows.

ABSTRACT

Metritis is a uterine disorder common in dairy cattle caused by bacterial infection, with greater incidence in the early post-partum period. The disease causes uterine enlargement with a fetid watery red brown discharge, with animals presenting different clinical signs including fever, decreased milk yield, dullness, inappetence, and dehydration. The lack of a reliable animal model makes it challenging to study and establish the efficacy of preventive and therapeutic interventions. Therefore, we developed an *in vivo* model of clinical metritis in Holstein multiparous cows using a pathogenic cocktail of *Escherichia coli*, *Fusobacterium necrophorum*, and *Trueperella pyogenes*. A total of 36 multiparous cows were randomly allocated to one of three treatment groups of 12 animals each. Cows assigned to the control group received an intrauterine administration of sterile saline solution, those in the low-dose group received a bacterial inoculum containing 10^6 cfu of *E. coli*, *T. pyogenes*, and *F. necrophorum*; and those in the high-dose group received 10^9 cfu of these same organisms, all within 24 hours of parturition. Clinical observations, rectal temperature, milk yield and dry matter intake measurements, blood sampling, and vaginal discharge collection were performed from parturition until the end of the study, at 14 days in milk. Body weight and body condition score were recorded on the first and last days of the study. Cows in the low-dose group had greater incidence of metritis (83.3%) when compared to the control (8.3%) and high-dose group (25%). Bacteria challenge altered dry matter intake, with a significant reduction in the low-dose when compared to the high-dose groups and a marginal alteration when comparing low-dose to control group. Cows in the low-dose group had lower granulocyte numbers compared the control group. Likewise, the low-dose group presented higher relative abundance of bacteria from the genus *Fusobacterium* in the vaginal discharge when compared with controls. These findings thus demonstrate that clinical metritis can be experimentally induced in postpartum multiparous Holstein cows, creating a relevant and reliable model for the study of this disease.

Our model may be further used to evaluate the efficiency and effects of interventions to control metritis.

Keywords: metritis, challenge, microbiota, multiparous, dairy cow.

INTRODUCTION

Metritis is a systemic illness caused by bacterial infection of the uterus, that most commonly affects cows within 21 days post-partum. Animals that develop metritis present an abnormally enlarged uterus with a fetid watery red-brown discharge, and in some cases present inappetence, decreased milk production, dullness, toxemia, or rectal temperature > 39.5 °C (Sheldon et al., 2006). Metritis is classified as a multifactorial disease, and it can be associated with risk factors that include retained placenta, stillbirth, twins, dystocia, delivery of male offspring, caesarian section, primiparity, ketosis, and hypocalcemia. The disease occurs mainly during the first 10 days post-partum, while some cases can be diagnosed after the second week of parturition (Markusfeld, 1984, Drillich et al., 2001, Sheldon et al., 2006, Galvão, 2018).

The reported incidence of metritis ranges from 8% to more than 50% (LeBlanc, 2008, Galvão, 2012, Vieira-Neto et al., 2016). Although part of this variation might be explained by farm-to-farm variation; the wide range of incidence could also be explained by inconsistencies in disease definition among studies (Espadamala et al., 2016). As the disease impacts the productivity of cows, efforts have been made to estimate the costs associated with metritis. A stochastic analysis reported the cost associated with a single case of metritis as $513, with 95% of cases ranging from $240 to $884 (Perez-Baez et al., 2021). When comparing cows diagnosed with metritis to cows without metritis, previous studies have reported a reduction in milk production (Dubuc et al., 2011, Perez-Baez et al., 2019, Perez-Baez et al., 2021), an increase to first service by 7 days and a 20% decrease in pregnancy per artificial insemination at first service, which resulted in an increase of 19 days in the calving to conception interval (Fourichon et al., 2000, Carneiro et al., 2016).

The main microorganisms associated with the development of metritis have been *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, and other bacteria

composing the genera *Prevotella* and *Bacteroides* (Noakes et al., 1991, Dohmen et al., 2000, Azawi, 2008). It is reported that these microorganisms contaminate the uterus during parturition, when physical barriers are disrupted and bacteria ascend the genital tract from the environment, skin and feces (Sheldon and Dobson, 2004); or via the blood stream, as some pathogens such as *Prevotella* spp. and *Helcococcus* spp., are not found in vaginal samples but can be found in the uterus (Jeon et al., 2017).

Some virulent strains of the common pathogen *E. coli* have been reported to be harmful to the reproductive tract (Silva et al., 2009, Bicalho et al., 2010, Sheldon et al., 2010) because of their abilities to adhere to and invade the endometrial epithelium and stroma (Sheldon et al., 2010), possibly due to the presence of the *fimH* gene (Bicalho et al., 2010, Bicalho et al., 2012b). Thus, *E. coli* may create an environment conducive to colonization by other pathogenic bacteria such as *T. pyogenes* and *F. necrophorum* (Dohmen et al., 2000, Williams et al., 2007). *T. pyogenes* is an opportunistic pathogen associated with uterine diseases in dairy cows (Bicalho et al., 2012a, Zastempowska and Lassa, 2012, Rzewuska et al., 2019). This bacterium is broadly used for *in vivo* models of uterine diseases induction (Farin et al., 1989a, b, Del Vecchio et al., 1992, Cester et al., 1996, Santos et al., 2010b, Amos et al., 2014, Carneiro et al., 2016, Piersanti et al., 2019b) because it causes severe damage to the endometrium tissue through a cholesterol damaging cytotoxin called pyolysin. *F. necrophorum* is another opportunistic pathogen present in necrotic conditions (Tan et al., 1996) found in both the healthy and metritic uterus (Santos and Bicalho, 2012, Jeon et al., 2015, Jeon et al., 2016). The key virulence factor in *F. necrophorum* is leukotoxin (*lktA* gene), which is cytotoxic to immune cells, protecting the bacteria against phagocytosis by neutrophils in cattle and sheep (Tan et al., 1996, Jeon and Galvao, 2018).

The development of metritis depends upon both the pathogenicity and number of bacteria that invade the uterine tract, and upon the immune response of the cow. Pathogenic bacteria must overwhelm the uterine defenses to cause infection (Sheldon et al., 2006). After parturition, the

cow relies upon the innate immune response to protect the uterus against bacterial infection, with neutrophils being the main cells involved in the process of bacterial clearance after parturition (Pohl et al., 2015, Machado and Silva, 2020, Pascottini and LeBlanc, 2020). The stimulation of the innate immune system is accompanied by pro-inflammatory cytokines that stimulate the liver to produce acute phase proteins, including haptoglobin and serum amyloid A, that are frequently used as early indicators of uterine diseases (Humblet et al., 2006).

In vivo models of uterine disease as endometritis and pyometra have been created previously (Rowson et al., 1953a, Farin et al., 1989b, Piersanti et al., 2019a, Dickson et al., 2020) using *E. coli* and/or *T. pyogene*s. None of the models described were designed for induction of metritis in post-partum cows, nor were able to determine metabolic changes caused by such induced uterine diseases. Therefore, we tested an *in vivo* model for metritis induction in healthy postpartum dairy cows by the infusion of a low (10^6 cfu per infusion) and a high (10^9 cfu) dose of *E. coli*, *T. pyogenes* and *F. necrophorum*, to test whether the model would create classic clinical signs associated with the natural occurrence of the disease. Also, analysis of blood parameters and the microbiome were used to determine metabolic and inflammatory changes, and dynamics of microorganisms from challenge to the development of metritis.

MATERIALS AND METHODS

Ethics Statement

The research protocol was reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (protocol number 2019-0040). The methods were carried out in accordance with the approved guidelines.

Sample Size Estimation

Assuming a desired type I error rate of 5%, a power of 80%, a two-sided statistical test,

and a baseline probability of metritis of 16% (level of metritis in control group), a sample size of 12 cows per group was required to detect a 75% effectiveness in inducing metritis.

Animals, Facilities, Management

Cows enrolled in this study, conducted between September 2019 and February 2020, were purchased from a commercial farm located in Scipio Center, NY. Initial experimental procedures until the day of calving were conducted at the commercial farm. Thereafter, cows were transported to the Dairy Unit of the Cornell University Ruminant Center (**CURC**). The commercial farm used to source cows had approximately 4,100 lactating cows in free-stall facilities bedded with manure solids. Dry and lactating cows had free access to *ad libitum* water and total mixed ration **(TMR)** to meet nutritional requirements according to stage of lactation. Cows presenting signs of parturition were moved to a maternity pen assisted by trained farm staff. Calving data (i.e., date and time of calving, cow and calf ID, calf sex and weight, calving ease score, and personnel present at the time of parturition) were recorded by farm personnel.

The dairy unit of the CURC located in Harford, NY is structured to house approximately 600 lactating cows in naturally ventilated free-stalls and tie-stall barns. Cows for this experiment were housed in the tie-stall barn equipped with stalls deep-bedded with sawdust, forced ventilation was provided by ventilation fans placed on the ground in the feed and manure lane whereas exhaust fans were placed on one of the lateral barn walls.

At the dairy unit of the CURC, cows had free access to water and TMR in individual water bowls and feed bins. Cows were individually fed to allow measurement of feed intake. Feeding was performed once a day, during the morning (between 0700 and 0800), by providing 110% of their expected daily consumption, therefore, individual feed intake was measured by weighing the amount of feed offered and refused daily. Measurements of TMR consumption were performed once a week and aliquots dried in 55 °C for 48 hours to estimate dry matter **(DM)**. Daily feed

consumption and average weekly DM were used for calculation of dry matter intake **(DMI)**. TMR was grounded once a week to a pass 2-mm screen of a Wiley mill (Arthur H. Thomas, Philadelphia, PA), and once a month the composites of dried TMR were sent to a commercial laboratory for analysis (Cumberland Valley Analytical Services, Waynesboro, PA). Ingredients and chemical composition (DM basis) of the postpartum diet are described in supplemental materials (Table 2.1 and Table 2.2).

Table 2.1. Ingredients (DM basis) of postpartum diet.

Ingredient	% of DM
Corn silage	44.96
Hay crop silage	16.89
Corn grain, finely ground	16.2
Soybean meal	4.66
Amino Plus[1]	3.7
Soybean Hulls, ground	3.64
Blood meal	2.24
Dextrose	1.61
Calcium carbonate	1.53
Palmit 80[2]	0.98
Sodium sesquicarbonate	0.78
Salt	0.47
Molasses	0.32
Bypass fat[3]	0.32
Magnesium oxide	0.23
Calcium sulfate	0.22
Mono-Dicalcium phosphate	0.11
Smartamine[4]	0.04
Dairy ADE[5]	0.02
Vitamin E[6]	0
Rumensin[7]	0.01
Corn distillers, ethanol	0.98
Mineral mix[8]	0.1
Mineral oil	0.02

[1]Heat-treated soybean meal; Ag Processing Inc. (Omaha, NE).

[2]Commercial high palmitic acid fat; Global Agri-trade Corporation (Rancho Dominguez, CA).

[3]High stearic fat; Cargill Animal Nutrition (Minneapolis, MN).

[4]DL-Met, physically protected with pH-sensitive coating; Adisseo (Antony, France).

[5]Vitamin mix; Cargill Animal Nutrition (Minneapolis, MN). Contains 30,073 kIU/kg vitamin A, 5,783 kIU/kg vitamin D, and 92,534 IU/kg vitamin E.

[6]Contains 510,750 IU/kg vitamin E.

[7]Premix contained 26,400 g/t of monensin; Elanco Animal Health (Greenfield, IN)

[8]Mineral mix contained 75,000 mg/kg Zn, 64,920 mg/kg Mn, 10,006 mg/kg Cu, 1,023 mg/kg Co, 1,200 mg/kg I, and 293 mg/kg Se.

Table 2.2. Chemical composition (DM basis) of postpartum diet.

Energy and chemical compostion[1]	Mean	SD
NE_L, Mcal/Kg	1.67	0.27
NDF, %	31.25	1.04
NFC, %	43.75	0.97
ADF, %	19.66	0.45
Starch, %	28.43	1.42
Crud fat, %	3.95	0.54
CP, %	14.38	0.23
Ash, %	7.54	0.38
Lignin, %	2.28	0.25
Ca, %	1.01	0.04
P, %	0.30	0.005
K, %	1.32	0.02
Na, %	0.49	0.03
Mg, %	0.30	0.01
Fe, mg/kg	342.33	34.31
Zn, mg/kg	77.33	3.32
Cu, mg/kg	19.16	1.32
Mn, mg/kg	74.83	4.49

[1]Values represents averages of 6 monthly composited samples. Chemical composition analysis was performed by Cumberland Valley Analytical Services, Waynesboro, PA.

Experimental Design

The experiment followed a randomized complete block design with one-way treatment structure. The blocking factor was based on numerical order (order of enrollment). Briefly, after selecting eligible cows, groups of three cows were allocated in treatments according to the ear tag number, following an ascending order. The treatment randomization was generated using the SAS statistical package (version 9.4; SAS/STAT, SAS Institute Inc., Cary, NC) that utilizes a random number generator function created by the Biometrics Representative **(BMR)** from Zoetis Animal Health (Kalamazoo, MI).

After parturition (4 to 22 hours), eligible cows were challenged at the maternity pen at the commercial farm by research personnel with the bacterial cocktail corresponding to the experimental treatment group and immediately transported to the dairy unit of the CURC where cows stayed until the end of the experimental period (14 DIM). Cows were eligible for enrollment in the experiment if they were in their second through six lactations, had a gestation length of 270 to 285 days, gave birth to a single offspring without assistance, did not present the placenta at the time of challenge and did not receive antibiotic treatment 30 days before calving. Also, cows were enrolled only if had no clinical signs of disease including lameness, dehydration, respiratory distress, empty rumen, recumbence, dullness, depression, displaced abomasum, mastitis, and/or vaginal tear. A total of 36 cows were selected and assigned to one of the following treatment groups: high-dose group (n = 12) received an intrauterine infusion with 120 mL of an inoculum containing 10^9 cfu of each bacterial strain (*E. coli*, *T. pyogenes* and *F. necrophorum*); low-dose group (n = 12) received an intrauterine uterine infusion with 120 mL of an inoculum containing 10^6 cfu of each bacterial strain (*E. coli*, *T. pyogenes* and *F. necrophorum*); and control group (n = 12) received an intrauterine infusion with 120 mL of sterile saline solution (TEKnova, Hollister, CA). One cow enrolled in the control group was excluded from the experiment due to aggressive behavior. Therefore, 35 cows completed the experimental period.

Milking was performed three times a day at approximately 8 hours intervals. Milk weights were recorded at each milking for each cow using milk meters which transferred data to the dairy herd management software (Dairy Comp 305, Valley Ag Software, Tulare, CA).

Inoculum preparation

The bacterial challenge used for metritis induction was composed of *E. coli*, *T. pyogenes* and *F. necrophorum*. All strains were obtained from a well-characterized bacterial collection belonging to the Bicalho Laboratory from Cornell University, Ithaca-NY. Strains were previously isolated from uterine content of cows diagnosed with metritis and selected to the present study according to virulence genes presence (Bicalho et al., 2010, Santos et al., 2010b, Bicalho et al., 2012b, Machado and Bicalho, 2014).

E. coli was cultured under aerobic conditions, with agitation (3 x *g*), in Luria-Bertani **(LB)** broth (Sigma-Aldrich) at 37°C for 24 hours. Following the 24-hour incubation, the culture was harvested by centrifugation (2500 x *g* for 10 min, room temperature). The cell pellet obtained was resuspended in LB-broth supplemented with 25% v/v of glycerol to a final count of 10^9 cfu/mL and aliquoted in 2 mL cryogenic vials. Vials were flash frozen in liquid nitrogen and stored at -80°C.

T. pyogenes was cultured in VersaTREK REDOX 1 media broth (Thermo Fisher Scientific, Kansas) in 5% CO_2 supplemented incubator, at 37°C for 48 hours. Following the 48-hour incubation, the culture was harvested by centrifugation (2429 x *g* for 10 min, room temperature); and the cell pellet resuspended in microbial freeze-drying buffer (OPS Diagnostics, Lebanon, NJ) to a final count of 10^9 cfu/mL and aliquoted in 2 mL cryogenic vials. The bottled culture was lyophilized in Advantage Pro lyophilizer (SP scientific, Warminster, PA) according to manufacturer instructions, sealed, and stored at 4°C. *T. pyogenes* was lyophilized as a strategy to achieve the concentration needed for challenge.

F. necrophorum was cultured in VersaTREK REDOX 2 Media broth (Thermo Fisher Scientific, Kansas), in BACTRON 300 anaerobic incubator (Sheldon Manufacturing INC, Cornelius, OR) for 48 hours at 37°C. Following the 48-hour incubation, the culture was harvested by centrifugation (2429 x *g* for 10 min). The resuspension of the cell pellet was performed using VersaTREK REDOX 2 (Thermo Fisher Scientific, Kansas) media broth supplemented with 25% v/v of glycerol to a final count of 10^9 cfu/mL and aliquoted in 2 mL cryogenic vials. Vials were flash frozen in liquid nitrogen and stored at -80 °C.

Within the interval of two to four hours before use in the field, each stock was transferred to 40 mL bottles containing the transport media, and dilutions were carried to achieve the specific concentration for each challenge. The inoculum containing *E. coli* and *T. pyogenes* was transported in the Versa TREK REDOX 1 media, whereas *F. necrophorum* was transported in Versa TREK REDOX 2 Media. Once diluted, challenge material was transported to the farm on ice and used upon arrival.

Evaluation of bacterial cultures purity

Prior to the field trial study, bacterial stocks were produced, and quality control assessments were performed between September 2019 and February 2020 to assure the purity and bacterial count (cfu/mL) of each strain. DNA was extracted from the pure bacterial stocks, the 16S rDNA gene was amplified and submitted to Sanger sequencing (Cornell University Biotechnology Institute, NY) to confirm the bacterial species cultured according to the methods previously described (Rodrigues et al., 2016).

Bacterial counts of the stocks were performed using the technique of agar droplets with some modifications. Briefly, for each bacterial strain, serial dilution was prepared (10^{-1} to 10^{-8}) using sterile 1X PBS (pH 7.4). Next, three drops of each dilution were inoculated onto each quadrant of agar plates. All stocks were incubated in aerobic, anaerobic, and 5% CO_2

supplemented conditions, to assure that stocks were not contaminated with any other microorganism. After incubation (for approximately 48 hours at 37 ºC), the features of bacterial growth were assessed and the number of cfu was determined.

For *E. coli* culturing, blood agar and a selective medium plate (Mastitis GN, CHROMagar, Paris, France) were used, incubating at 37 ºC for 24 hours in an aerobic incubator. Blood agar and LKV agar plates (Laked Blood with Kanamycin and Vancomycin, Anaerobe Systems, California) were used for culturing *F. necrophorum* for 48 hours at 37 ºC under anaerobic conditions. Lastly, blood agar plates were used to culture *T. pyogenes* in a 5% CO_2 supplemented incubator for 48 hours at 37 ºC.

In vivo experimental challenge

Cows were restrained in a headlock stanchion at the maternity pen; the perineal area and the vulva were cleaned with paper towels and then disinfected with 70% ethanol. The inoculum was administered using a sterile gilt foam tip catheter (QC supply, Schuyler, NE) attached to a 60 mL syringe (Air-tite products Co., Inc., Virginia Beach, VA), introduced into the cranial vagina and manipulated through the cervix, into the uterine lumen. A total volume of 40 mL of each inoculum (total of 120 mL) was infused into the uterus using different syringes. The catheter was flushed out with 10 mL of sterile saline solution to assure that all challenge material was infused. Control cows were infused with 120 mL of sterile saline solution using a single catheter.

Sample collection

Blood samples were collected from coccygeal vessels using 10 mL vacutainer tubes with spray-coated silica (BD Vacutainer, Franklin Lakes, NJ) for serum separation, and 3 mL vacutainer K2 EDTA (BD Vacutainer, Franklin Lakes, NJ) blood collection tubes for complete blood cell

count and genomic testing (CLARIFIDE, Zoetis Animal Health, Kalamazoo, MI). Samples were collected before challenge administration and daily after feeding from study day 1 to 14.

Complete blood cell count was performed using Vet hemogram instrument (Heska – Hemature^{tm}, Loveland, CO). Serum was obtained after centrifugation of 10 mL blood collection tubes at 2,000 x *g* for 15 min at room temperature and frozen at -80 °C. Serum concentration of calcium, non-esterified fatty acids **(FA)**, β-hydroxybutyrate **(BHB)**, total protein, bovine serum albumin, glucose, alkaline phosphatase **(ALP)**, gamma glutamyl transferase **(GGT)**, aspartate aminotransferase **(AST)**, alanine aminotransferase **(ALT)**, and lactate were measured using an automated clinical chemistry analyzer (Daytona, Randox Laboratories Ltd., Kerneysville, WV), using reagents provided by the same company.

Frozen serum samples were sent to a Zoetis Research and Development facility located in Kalamazoo (Michigan, USA) for measurement of acute phase proteins using commercial ELISA kits: lipopolysaccharide binding protein (**LBP**, Hycult Biotech, Wayne, PA), haptoglobin (Life Diagnostics, West Chester, PA), serum amyloid A (**SAA**, Life Diagnostics, West Chester, PA). Cytokines levels in serum consisting of Tumor Necrosis Factor alpha **(TNFα)**, interleukin **(IL)**-6, IL-2, IL-8/CXCL8, and IL-10 were analyzed using two custom U-Plex assays (Meso Scale Diagnostics, LLC, Rockville, MD) developed for bovines. The TNFα, IL-2, IL-6, IL-10 analysis was performed in a 4-Plex assay format, while bovine IL-8/CXCL8 was performed in a 1-Plex format. In brief, biotinylated antibodies were diluted with Diluent 100 (Meso Scale Diagnostics, LLC, Rockville, MD) to a final concentration of 10 µg/mL and individually linked to specific MSD Linkers (Meso Scale Diagnostics, LLC, Rockville, MD) according to manufacturer's recommendations. Linked antibodies were diluted in MSD Stop Solution (Meso Scale Diagnostics, LLC, Rockville, MD): 4-Plex: TNFα (1:20), IL-2 (1:40), IL-6 (1:10), and IL-10 (1:40); 1-Plex: IL-8/CXCL8 (1:40); 4-Plex linked antibodies were combined into one antibody capture solution and 1-Plex linked antibody was prepared as a separate antibody capture solution.

A total of 50 µL/well of capture antibody solution was added to the respective U-Plex assay plates and incubated for 1 hour at room temperature shaking. Following incubation, plates were washed 3 times with 0.05% Tween-20 in PBS. Samples for 4-Plex measurements were diluted to 1:2 while samples for 1-Plex measurements were diluted to 1:100 in SeaBlock (Thermo Fisher, Rockford, IL) and duplicates were added to each plate. Plates were incubated with samples and standards at room temperature for an interval of 1 to 1.5 hours with shaking. Following incubation, plates were washed 5 times with 0.05% Tween-20 in PBS, and 30 µL/well of detection antibody solutions. Antibodies used for detection were conjugated with MSD SULFO-TAG (Meso Scale Diagnostics, LLC, Rockville, MD) according to manufacturer prior to use in U-Plex assays. were added, followed by 1 hour incubation at room temperature, shaking. Plates were then washed one time with detection antibodies, followed by three washed with 0.05% Tween-20 in PBS. Finally, 150 µL/well of 2x MSD Read Buffer (Meso Scale Diagnostics, LLC, Rockville, MD) was added to plates and plates were read immediately on a MESO SECTOR S 600MM instrument (Meso Scale Diagnostics, LLC, Rockville, MD). For each cow, rectal temperature **(RT)** was measured daily from enrollment to the end of study period. Body weight **(BW)** and body condition score (**BCS**, Edmonson et al., 1989) were recorded at enrollment and study day 14 and used for calculation of BCS loss and BW loss. During the study conduct, the scale used to weight the study animals was damaged. Therefore, the BW of nine animals (three from each treatment group) on study day 0 was not collected. The BW of animals in question were not used for analysis.

Vaginal discharge sample collection

Vaginal discharge was evaluated daily from enrollment to study day 14 using a Metricheck device (Metricheck, SimcroTech, Hamilton, New Zealand). The perineal area and vulva were cleaned with paper towels and disinfected with 70% ethanol. The Metricheck device was disinfected before use by immersion in a solution containing ammonium persulfate and cleaned

with sterile distilled water. Once disinfected, the Metricheck device was introduced into the cranial extent of the vagina and retracted caudally bringing material adhered to its silicone hemisphere.

Vaginal samples were collected for bacterial load determination, by culture-dependent and culture-independent approaches (plate counting and 16S rRNA). A total of 3 swabs of vaginal content were collected per cow (one used for culture-independent methods and two used for bacterial culture). After collection, a swab collected for culture independent methods was placed in sterile tubes and stored on ice during transport. At the laboratory, the swab was placed in labeled 2 mL sterile microtubes and stored at -80°C. Two swabs were collected for cfu counts and placed immediately after sampling into specific transport media (Versa TREK REDOX 1 and Versa TREK REDOX 2, Thermo Fisher Scientific, Kansas). Samples in transport media were immediately refrigerated and upon laboratory arrival an individual vaginal swab was placed in conical centrifuge tubes, previously labeled with cow ID and study day, containing 2 mL of specific culture medium (same used for transport) and serial dilutions were carried out. Dilutions were inoculated in triplicates onto different culture media using the agar droplets technique as previously described.

Metritis definition

Metritis examination was performed daily by two research group members. Evaluation was based on vaginal mucus retrieved using the Metricheck device and scored according to characteristic of the content. The vaginal discharge score **(VDS)** varied from 0 to 3, being 0 normal lochia with viscous discharge or no discharge observed; 1 was discharge with clear mucus with <50% of purulent or mucopurulent material, 2 was discharge with clear mucus with ≥50% of purulent or mucopurulent material, and 3 was fetid watery, red-brownish uterine discharge. Metritis was defined as VDS equal to 3 regardless of the presence of fever (Sheldon et al., 2006, Sheldon et al., 2008).

DNA extraction and microbiome analysis

DNA extraction was performed adding 1 mL of UltraPure™ distilled water (DNAse and RNAse free, Invitrogen, Grand Island, NY) into 2 mL microtube containing the swab, followed by homogenization for 10 minutes in a vortex mixer (Fisher Scientific, Hampton, NH). Swabs were then removed and the remaining liquid centrifuged for 5 minutes at 15.7 relative centrifugal force (RCF) at room temperature. The pellet obtained was submitted to DNA extraction using a DNeasy PowerFood Microbial Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

For DNA sequencing, a polymerase chain reactions **(PCRs)** were performed for amplification of V4 hypervariable region of the bacterial/archaeal 16S rDNA gene using primers 515F and 806R, according to a previously optimized method for the Illumina MiSeq platform (Caporaso et al., 2012). All DNA samples were amplified using different 12-bp error-correcting Golay barcodes for 16S rRNA gene PCR (http://www.earthmicrobiome.org). PCR was performed using 10 µM of each primer, EconoTaq Plus Green 1x Master Mix (Lucigen®, Middleton, WI), 10 ng–100 ng of individual DNA and UltraPure™ distilled water (DNAse and RNAse free, Invitrogen, Grand Island, NY) to bring the final reaction volume to 50 µL. PCR conditions were: initial denaturing at 94 °C for 3 min; 35 cycles of 94 °C for 45 s, 50 °C for 1 min, and 72 °C for 90 s; and final elongation at 72 °C for 10 min. After amplification, samples were loaded in agarose gel (1.2%, wt/vol) containing 0.5 mg/ml ethidium bromide for verification of the amplicon presence. Amplified DNA was purified using Mag-Bind® Total Pure NGS (Omega Bio-Tek Inc., Norcross, Georgia) according to manufacturer's instructions. Determination of DNA concentration was performed by spectrophotometric estimation (A_{260} of 1.0 = 50µg/ml pure dsDNA). Then, DNA samples were diluted to the same concentration and pooled for library preparation and sequencing, which was performed using Reagent MiSeq V2 300 cycles on the MiSeq platform (Illumina Inc., San Diego, CA). Procedures as PCR, purification, normalization and pooling were all automated using the OT-2 robot pipetting (Opentrons, New York, NY).

Operational Taxonomic Unit **(OTU)** tables were generated through MiSeq Reporter Metagenomics Workflow, which is based on the Greengenes database (http://greengenes.lbl.gov/). The output used from this workflow is a classification of reads at multiple taxonomic levels (phylum, genus, and species).

Data Analysis

Descriptive statistics were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC). General linear mixed models using the MIXED procedure of SAS version 9.4 (SAS Institute Inc.) were created to analyze continuous data collected over time (e.g., RT, milk production, cfu count, molecular analyses, and blood parameters). Normality and homoscedasticity of residuals were assessed using residual plots. Models included the fixed effects of treatment (control, low-dose, and high-dose), day after challenge and the interaction term between treatment and days after challenge, and random effects of block and animal within block

Continuous data such as cfu counts were log10-transformed as residuals did not follow a normal distribution. Based on Akaike information criterion, the covariance structure with the lowest AIC was selected for each variable.

Multivariate logistic regression models were applied using the binary distribution of the GLIMMIX procedure (SAS version 9.4) to evaluate dichotomous outcomes such as incidence of metritis and fever. The model included fixed effect of treatment and random effect of block.

For all models, variables were considered statistically significant when a P-value ≤ 0.05 was detected, and a tendency to significance was considered if the P-value was between 0.05 and 0.10. In all models, Fisher's Protected Least Significant Difference was used for multiple comparisons such that pairwise treatment comparisons were performed if the treatment effect or treatment by time-point effect (only for repeated measures models) was significant at 0.05 level.

RESULTS

Inoculation with bacterial pathogens induces clinical metritis.

To ensure that all groups presented similar prepartum characteristics, we compared the following parameters, as shown in Table 2.3: median parity, BCS at enrollment, BW at enrollment, RT at enrollment, length of the dry period in days, days of gestation, genetic differences in total pounds of milk produced during a 305-day lactation (**GMILK**; CLARIFIDE test), genomic standardized transmitting ability for metritis risk (**Z_MET**; CLARIFIDE test), dairy wellness profit (**DWP**; CLARIFIDE test), and total performance index (**TPI**; CLARIFIDE test). No differences were detected for any variable. We also considered the average time from calving to inoculation for each group. Cows in the control group received sterile saline solution at a mean interval of 11.9 h (SD = 5.57; ranging from 3.5 to 19.7 h), whereas cows in the low-dose and high-dose groups received the bacterial inoculum at a mean interval of 10.3 h (SD = 4.61; ranging from 3.8 to 18.8 h) and 13.7 h (SD = 5.08; ranging from 5.6 to 22 h), respectively.

Table 2.3. Descriptive data for cows enrolled in the study.

Item	Controls	Low-Dose	High-Dose	*P*-value
Median parity	2	3	3	-
BCS at enrollment	3.4 ± 0.11	3.4 ± 0.11	3.4 ± 0.11	NS
BW at enrollment, kg	683.5 ± 24.1	718.2 ± 23.1	671.8 ± 23.1	NS
RT at enrollment, °F	101.7 ± 0.12	101.9 ± 0.12	101.3 ± 0.12	NS
Days of dry period	59.5 ± 2.7	58.3 ± 2.6	55.4 ± 2.6	0.49
Days of gestation	280.3 ± 2.1	279.4 ± 2.0	280.0 ± 2.0	0.96
GMILK	277.5 ± 167.9	291.4 ± 160.3	323.5 ± 153.6	0.98
Zoetis Metritis	100.6 ± 1.6	101.5 ± 1.4	100.8 ± 1.4	0.89
DWP	229.2 ± 42.2	206.7 ± 38.2	202.3 ± 36.6	0.88
TPI	2073.7 ± 53.6	2026.45 ± 51.1	2085.4 ± 48.9	0.69

BCS: Body condition score.

BW: Body weight.

RT: Rectal temperature.

GMILK Genomic Enhanced Predicted Transmitting Ability for milk yield - Describes genetic differences in total pounds of milk produced during 305-day lactation.

Zoetis Metritis (Z_MET): Genomic Standardized Transmitting Ability for metritis risk - Describes the expected disease risk of a Holstein or Jersey female of being diagnosed with metritis one or more times in a given lactation.

DWP: Dairy Wellness Profit is a selection index that expresses the expected lifetime profit of an animal. It combines the Zoetis wellness traits.

TPI: Total Performance Index is the official selection index of the Holstein breed and ranks animals on the basis of combined genetic merit for productivity, efficiency, and conformation.

We observed an effect of challenge in the incidence of metritis ($P = 0.009$) when comparing groups, the low-dose group had greater proportion of cows with metritis than the control ($P = 0.006$) and the high-dose ($P = 0.01$) group, while no difference ($P = 0.30$) was observed between

control and high-dose cows. The metritis incidence was 83% for the low-dose, compared to 25% for the high-dose, and 8% for the control group (Figure 2.1).

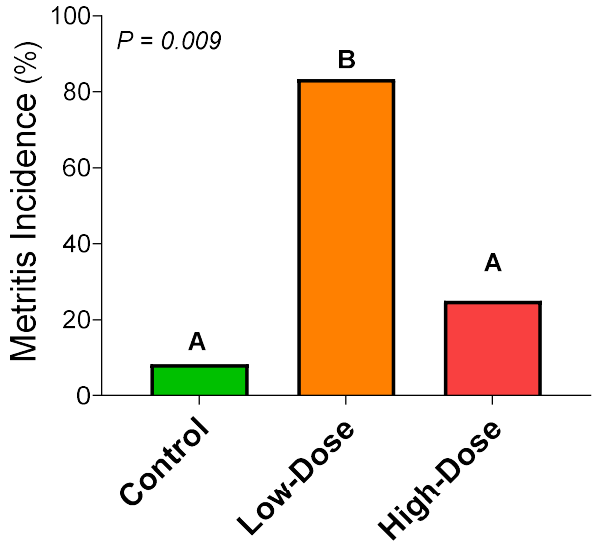

Figure 2.1. Metritis incidence during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^6 cfu (n = 12), or 10^9 cfu (n = 12) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 11).

Based on survival analysis there was an effect of challenge ($P = 0.005$) in the hazardous ratio of metritis , cows that received the low dose had a 16- ($P = 0.01$) or 5.2-times ($P = 0.03$) greater risk of being diagnosed with metritis during the study period than cows in the control or high-dose groups, respectively (low-dose vs control hazard ratio = 16, 95% CI = 1.9 to 13.4; low-dose vs high-dose hazard ratio = 5.2, 95% CI = 1.14 to 23.8). Cows in the high-dose group had a similar ($P = 0.38$) hazard of being diagnosed with metritis as the control cows (high-dose vs control hazard ratio = 3, 95% CI = 0.25 to 38.3). The time in days from inoculation to the first diagnosis of metritis for controls was 5 days, while low-dose cows were diagnosed between study days 4 and 10, and high-dose were diagnosed between days 2 and 10 (Figure 2.2).

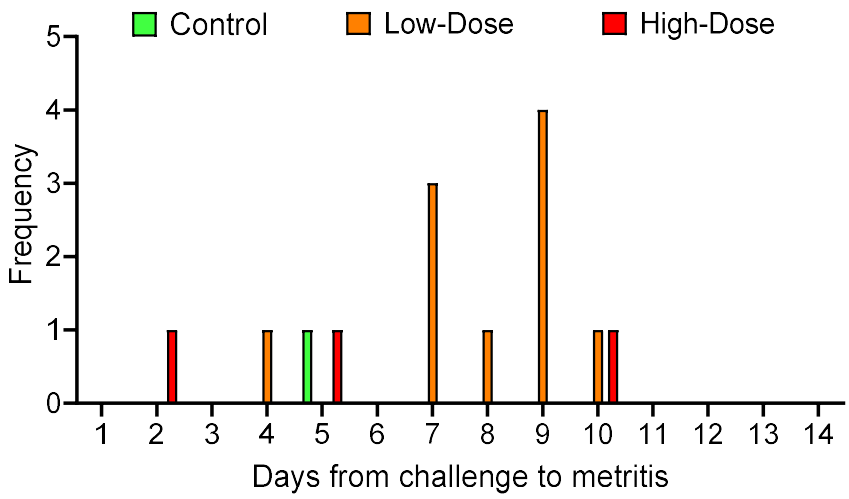

Figure 2.2. Frequency distribution of first metritis diagnosis in each group after receiving intrauterine challenge with a bacterial inoculum containing 10^6 cfu (n = 12), or 10^9 cfu (n = 12) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 11).

Challenge administration did affect RT ($P = 0.008$; control = 38.7 ± 0.12, low-dose = 38.8 ± 0.12, high-dose = 38.5 ± 0.12 °F), on the other hand, bacterial challenge did not affect ($P = 0.20$) the incidence of fever (control = 26.8, low-dose = 58.6, high-dose = 24.4 %; Figure 2.3). Fever was defined as a rectal temperature of ≥ 39.5 °C on at least one day from study days 1 to 14.

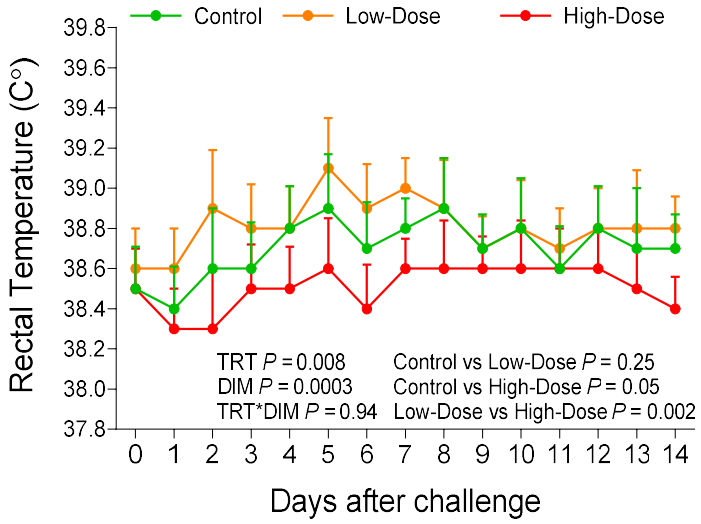

Figure 2.3. Fever incidence (A) and rectal temperature (B) during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^6 cfu (n = 12), or 10^9 cfu (n = 12) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 11).

The DMI was altered by bacterial challenge (P = 0.02). Cows that received the lower bacterial dose consumed 1.35 (P = 0.09) and 2.24 (P = 0.006) kg/d less dry matter when compared with the control and high-dose groups, respectively. No differences were observed between control and high-dose animals (P = *0.25*). (Figure 2.4A).

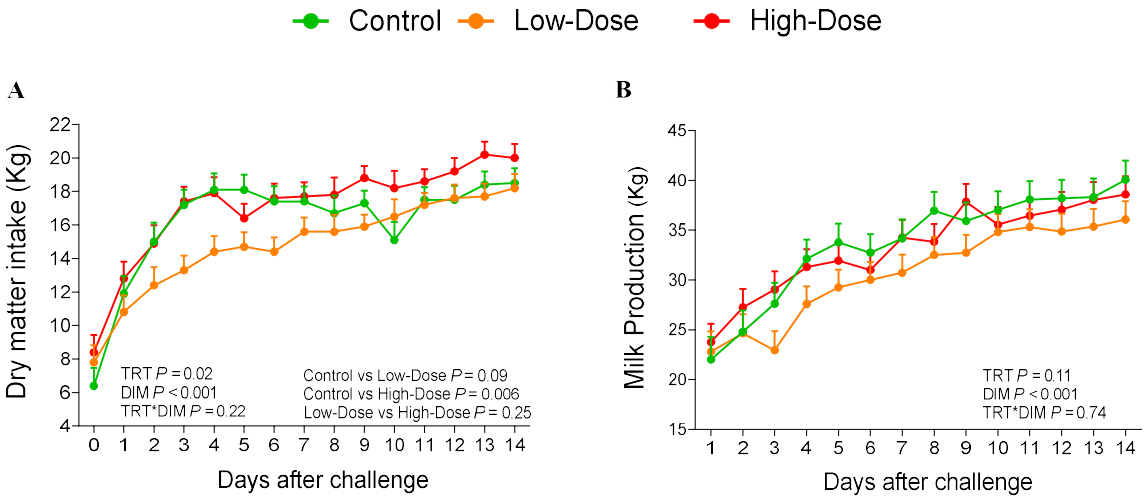

Figure 2.4. Dry matter intake (kg; A) and milk production (Kg; B) during the first 14 days of lactation for cows challenged intrauterine with a bacterial inoculum containing 10^6 cfu (n = 12), or 10^9 cfu (n = 12) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 11).

Although milk production was not altered by treatment (P = 0.26), we observed that the low-dose group produced approximately 3 kg less per day on average when compared to the other two groups (control = 33.69 ±1.94, low-dose = 30.68 ±1.82, high-dose = 33.27 ± 1.80 Kg) (Figure 2.4B). We did not find effect of bacterial challenge on BCS (P = 0.12) and BW (P = 0.50) loss

during the first 14 days of lactation (Figure 2.5).

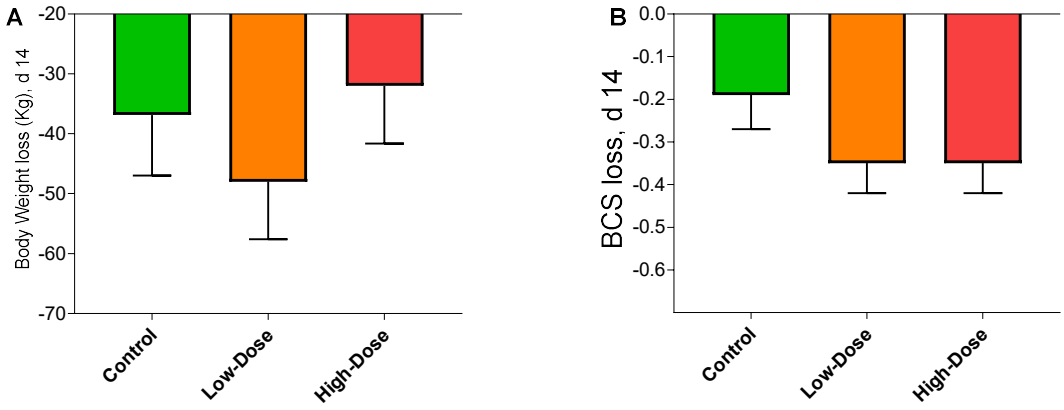

Figure 2.5. Body weight (A) and body condition score (B) loss during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^6 cfu (n = 9), or 10^9 cfu (n = 9) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 8).

Inoculation with bacterial pathogens induces inflammatory changes consistent with systemic disease.

We measured daily serum concentrations of the pro-inflammatory cytokines TNF-α, IL-2, and IL-6, the chemokine IL-8, and the anti-inflammatory cytokine IL-10 (Figure 2.6). No significant changes were observed for IL-8 ($P = 0.06$), IL-6 ($P = 0.34$, TNF-α ($P = 0.59$) and IL-2 ($P = 0.47$) levels among groups (IL-8: control = 1952.61 ± 781.5, low-dose = 1279.61 ± 526.84, high-dose = 2612.53 ± 835.17 pg/mL; IL-6: control = 3.20 ± 0.15, low-dose = 3.39 ± 0.16, high-dose = 3.31 ± 0.15 pg/mL, TNF-α: control = 47.7 ± 95, low-dose = 192 ± 100, high-dose = 109 ± 92 pg/mL; IL-2: control = 185.7 ± 372, low-dose = 830 ± 384, high-dose = 354 ± 355 pg/mL). Bacterial challenge affected ($P = 0.01$) IL-10 levels (control = 2.33 ± 0.02, low-dose = 2.39 ± 0.02, high-dose = 2.33 ± 0.02 pg/mL), with low dose animals presenting higher levels of IL-10 ($P \leq 0.05$). when compared to high-dose or control.

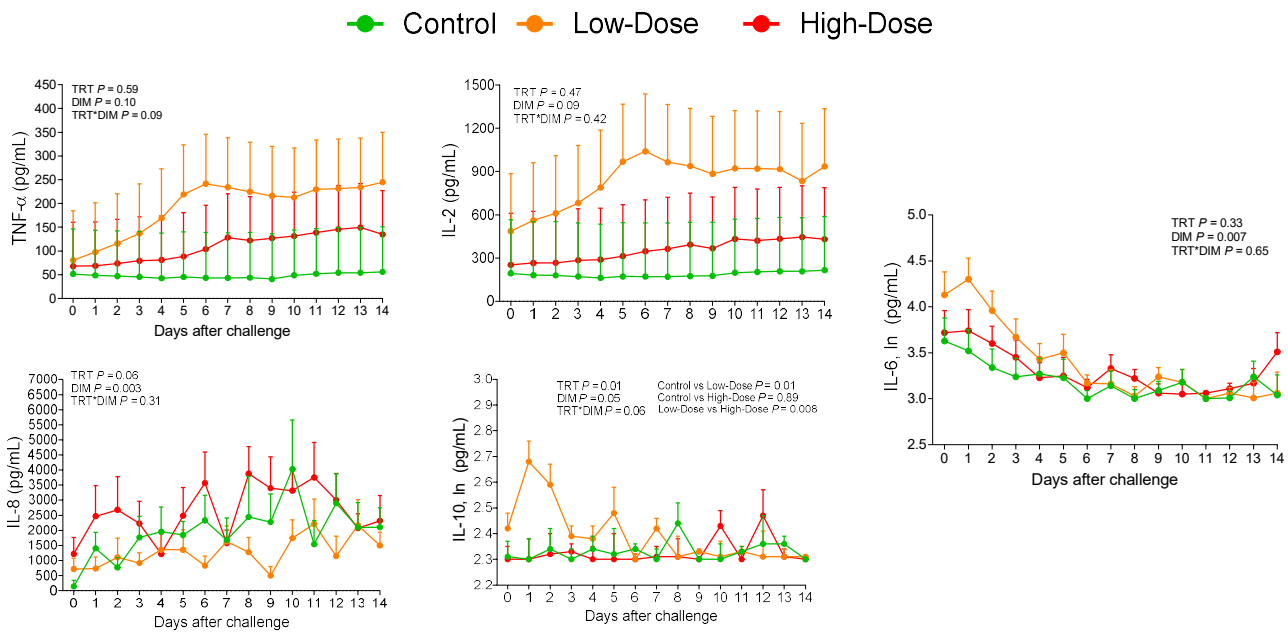

Figure 2.6. Serum TNF-α, IL-2, IL-6, IL-8, and IL-10 levels during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^6 cfu (n = 12), or 10^9 cfu (n = 12) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 11). * $P < 0.05$ (Low-Dose vs Control or High-Dose).

Bacterial challenge marginally affected SAA ($P = 0.06$) and LBP ($P = 0.08$) concentrations (SAA: control = 1573.81 ± 1100.25, low-dose = 5653.59 ± 1149.04, high-dose= 2382.93 ± 1045.55 ng/mL; LBP: control = 6883.99 ± 1104.743, low-dose = 9229.87 ± 1058.03, high-dose = 6715.19 ± 1057.16 ng/mL, Figure 2.7). No effect ($P = 0.30$) was observed in haptoglobin levels among groups (control = 1810.6 ± 1248.5, low-dose = 8148.8 ± 5381.9, high-dose = 3391 ± 2239.5 ng/mL; back-transformed; Figure 2.7).

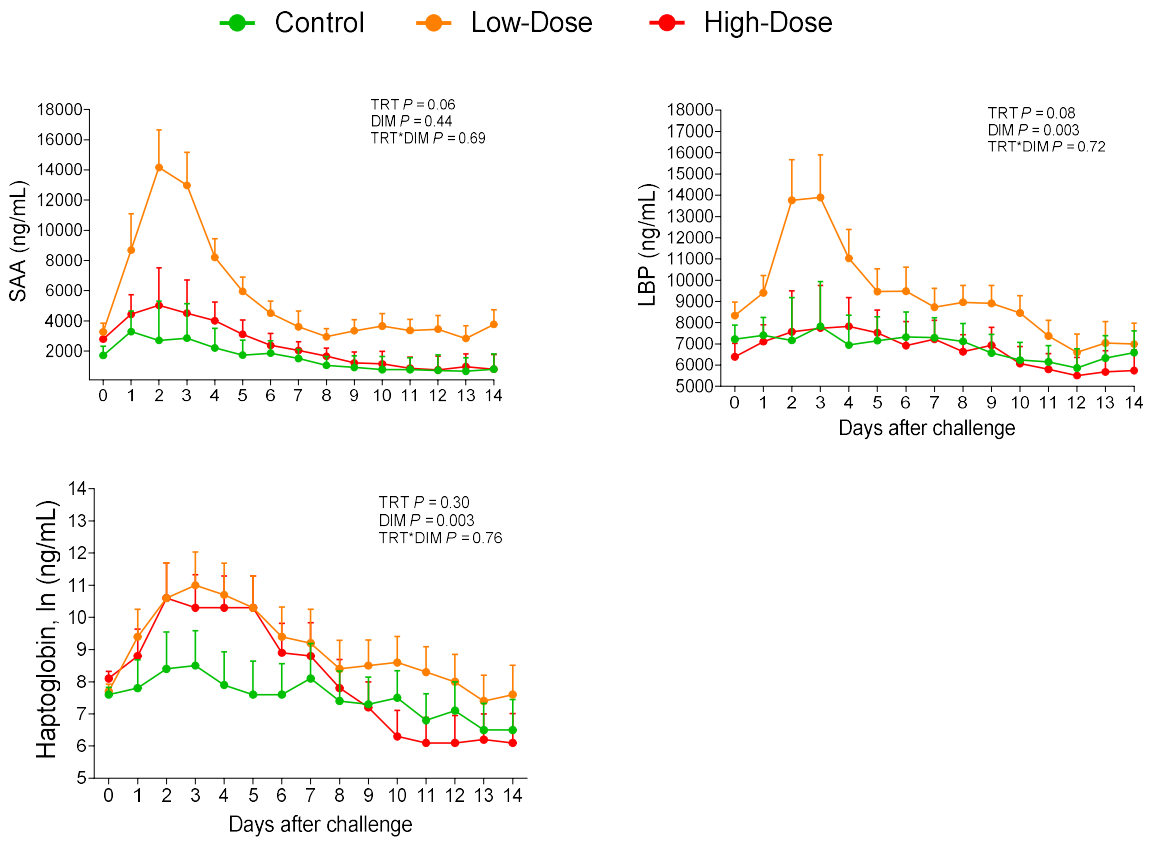

Figure 2.7. Serum SAA, LBP, and haptoglobin levels during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^6 cfu (n = 12), or 10^9 cfu (n = 12) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 11).

Bacterial challenge did not affect serum metabolites as FA (P = 0.29), BHB (P = 0.24), albumin (P = 0.51), total protein (P = 0.16), glucose (P = 0.80), and calcium levels (P = 0.13) (FA: control = 0.60 ± 0.11, low-dose = 0.67 ± 0.10, high-dose = 0.54± 0.10 mmol/L; BHB: control = 1.28 ± 0.30, low-dose = 1.28 ± 0.20, high-dose = 0.98 ± 0.11 mmol/L, albumin: control = 3.55 ± 0.11, low-dose = 3.45 ± 0.10, high-dose= 3.51 ± 0.10 g/dL; total protein: control = 7.23 ± 0.25, low-dose = 6.86 ± 0.24, high-dose = 7.33 ± 0.27 mg/dL, glucose: control = 74.31 ± 3.16, low-dose = 75.16 ± 3.03, high-dose = 76.04 ± 3.03 mg/dL; calcium: control = 9.23 ± 0.26, low-dose = 8.88 ± 0.24, high-dose = 9.22 ± 0.25 mg/dL Figure 2.8).

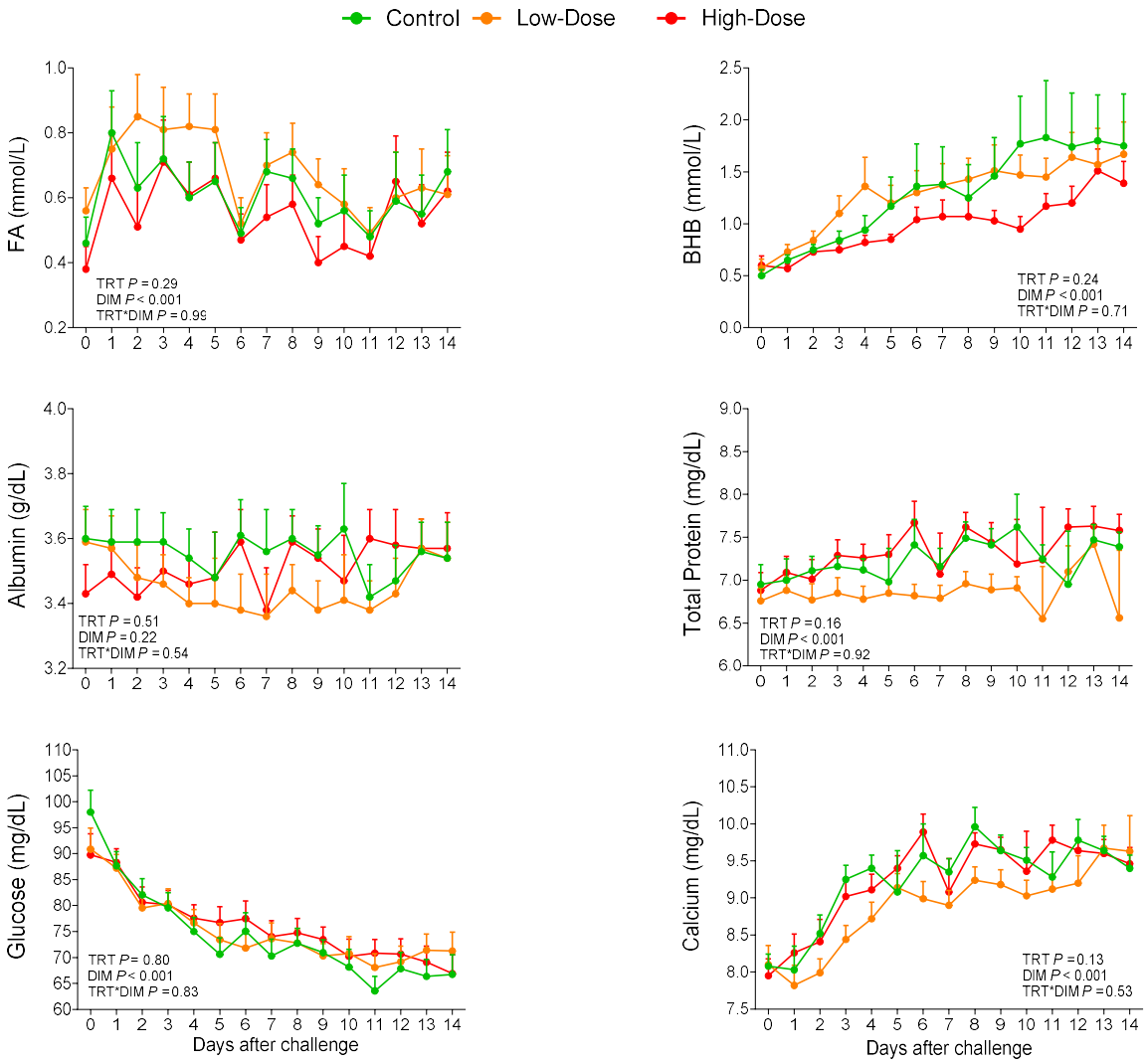

Figure 2.8. FA, BHB, albumin, total protein, glucose, and calcium levels during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^6 cfu (n = 12), or 10^9 cfu (n = 12) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 11).

We also measured other serum parameters: lactate ($P = 0.20$), and the hepatic proteins ALP ($P = 0.10$), AST ($P = 0.77$), ALT ($P = 0.26$) and did not find any effect of treatment among groups (Figure 2.9). We detected a treatment effect ($P = 0.04$) on GGT serum levels where control cows had higher GGT levels when compared with low and high-dose cows.

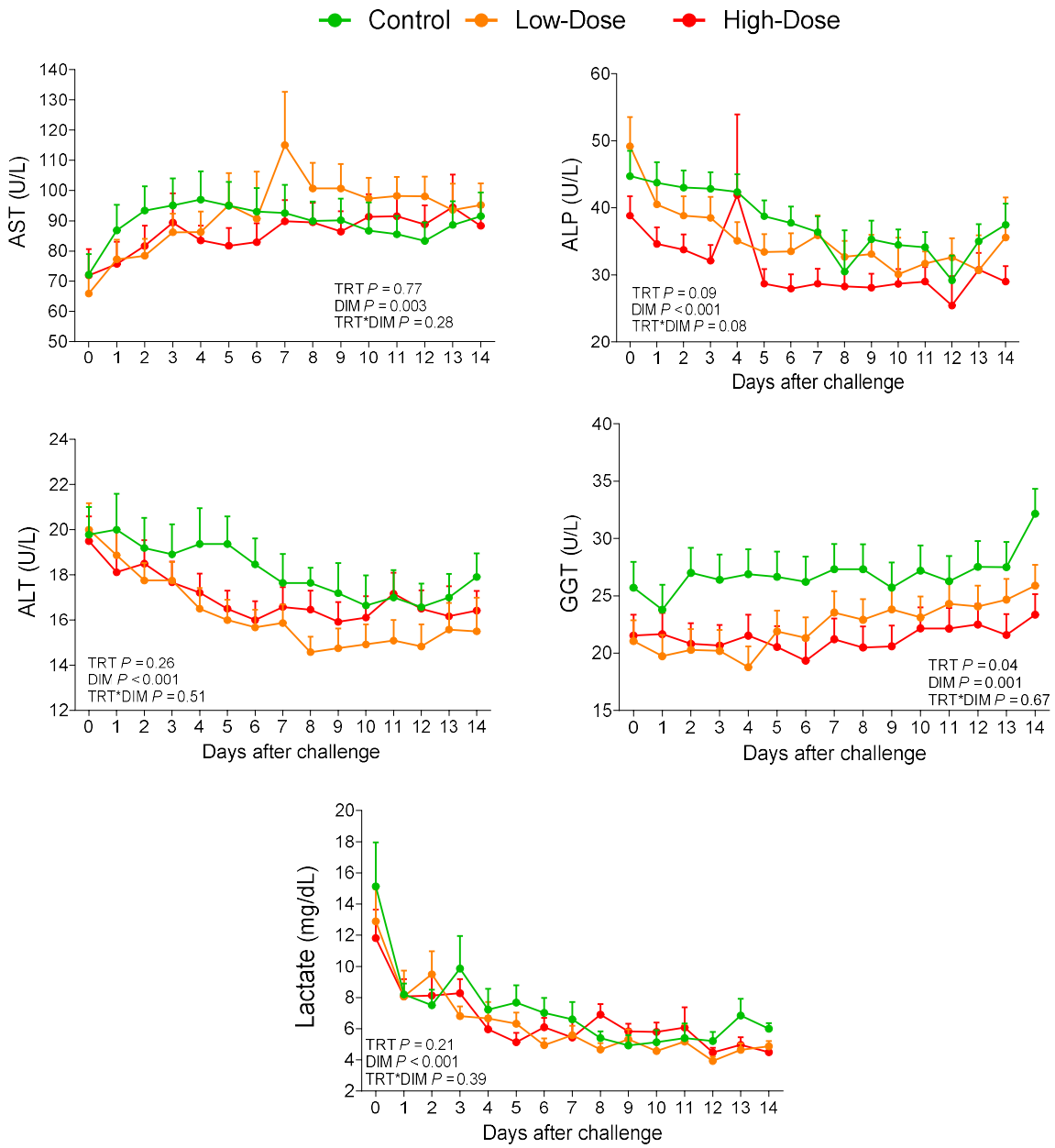

Figure 2.9. AST, ALP, ALT, GGT, and lactate levels during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^6 cfu (n = 12), or 10^9 cfu (n = 12) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 11).

Effect of bacterial challenge on granulocyte and monocyte cell numbers.

No significant differences were observed in total white blood cell (P = 0.22), lymphocyte (P = 0.31), and red blood cell (P = 0.15) counts among groups (white blood cell: control = 8.40 ± 0.65,

low-dose = 7.48 ± 0.62, high-dose = 7.41 ± 0.62 x 10^3/µL; lymphocyte: control = 3.82 ± 0.42, low-dose = 3.97 ± 0.40, high-dose = 3.27 ± 0.10 x 10^3/µL, red blood cell: control = 35.81 ± 0.17, low-dose = 5.50 ± 0.14, high-dose= 5.52 ± 0.16 x 10^3/µL). However, bacterial challenge affected granulocytes numbers (P = 0.04; control = 3.79 ± 0.30, low-dose = 2.88 ± 0.29, high-dose = 3.47 ± 0.29 ×10^3/µL). Monocytes numbers were also affected by bacterial challenge (P = 0.02; control = 0.79 ± 0.039, low-dose = 0.64 ± 0.037, high-dose = 0.67 ± 0.037 ×10^3/µL (Figure 2.10).

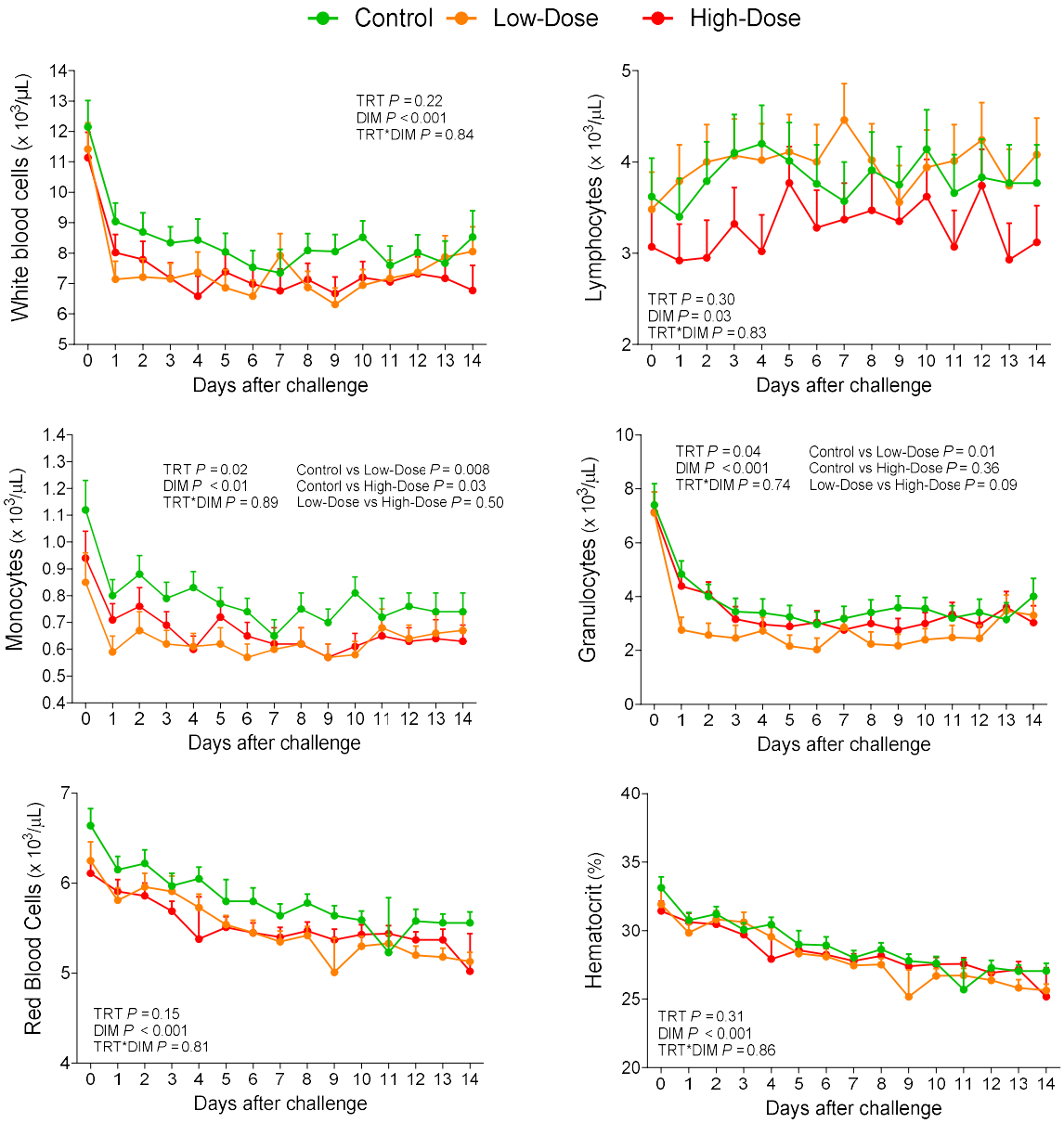

Figure 2.10. White blood cells, lymphocytes, monocytes, granulocytes, red blood cells, and percent hematocrit during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^6 cfu (n = 12), or 10^9 cfu (n = 12) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 11). * $P < 0.05$ (Low-Dose vs Control); ŧ $P < 0.05$ (Low-Dose vs High-Dose).

Bacterial challenge modified the uterine environment.

No difference was observed in cfu of total anaerobes ($P = 0.10$), while challenge marginally altered the cfu of *F. necrophorum* ($P = 0.07$) in the vaginal discharge (Total anaerobes: control = 5.58 ± 0.28, low-dose = 6.18 ± 0.26, high-dose = 5.38 ± 0.26 Log10 cfu; *F. necrophorum*: control = 2.71 ± 0.55, low-dose = 3.73 ± 0.54, high-dose = 2.42 ± 0.54 Log10 cfu; Fig. 2.11). Furthermore, challenge also altered the relative abundance of the genus *Fusobacterium*, with low-dose cows presenting greater relative abundance of the genus *Fusobacterium* ($P = 0.04$) when compared to control ($P = 0.01$) and high-dose ($P = 0.07$) cows (control = 31 ± 5.0, low-dose = 46 ± 5.0, high-dose = 36 ± 5.0 %). (Figure 2.11). We did not observe significant differences in bacterial culture (log10 cfu aerobic bacteria: $P = 0.58$, log10 cfu *E. coli*: $P = 0.38$) or relative abundance ($P = 0.53$) of the genus *Escherichia* (Figure 2.12). We also did not observe significant differences for facultative bacteria culture (log10 cfu facultative bacteria: $P = 0.37$, log10 cfu *T. pyogenes*: $P = 0.67$) or relative abundance of the genus *Trueperella* ($P = 0.55$; Figure 2.13).

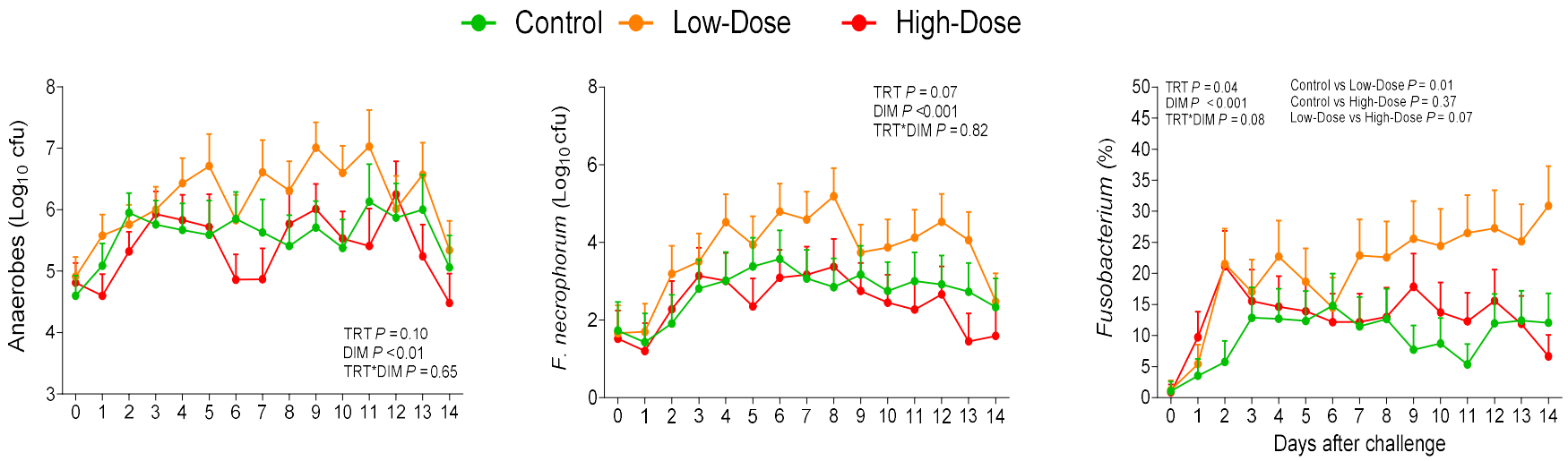

Figure 2.11. Bacterial cultures (total anaerobes and *F. necrophorum*) and microbiome (genus *Fusobacterium*) from vaginal swabs during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^6 cfu (n = 12), or 10^9 cfu (n = 12) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 11). * $P < 0.05$ (Low-Dose vs Control); ŧ $P < 0.05$ (Low-Dose vs High-Dose).

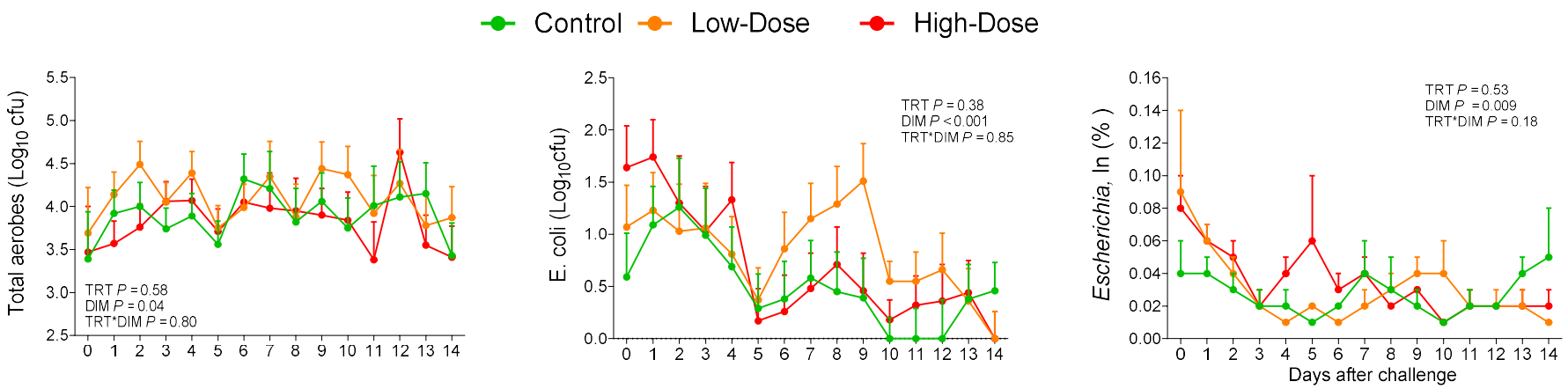

Figure 2.12. Bacterial cultures (total aerobes and *E. Coli*) and microbiome (genus *Escherichia*) from vaginal swabs during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^6 cfu (n = 12), or 10^9 cfu (n = 12) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 11). ln = natural log.

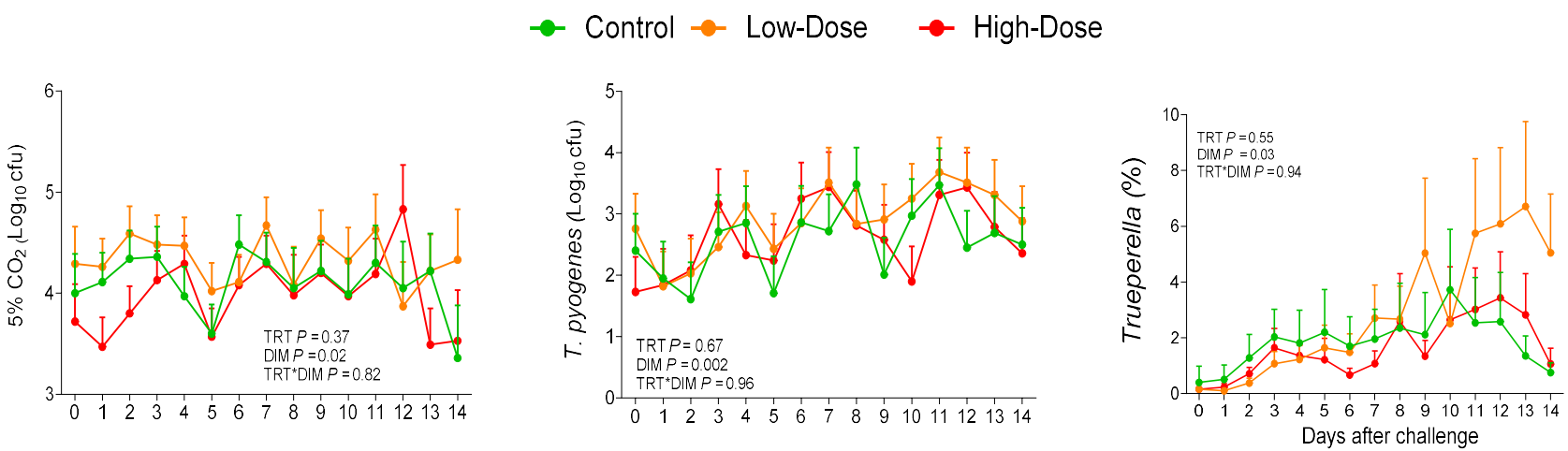

Figure 2.13. Bacterial cultures (total facultative anaerobes and *T. pyogenes*) and microbiome (genus *Trueperella*) from vaginal swabs during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^6 cfu (n = 12), or 10^9 cfu (n = 12) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 11).

To identify the microorganisms most commonly associated with metritis in infected cows regardless of the study group, we divided the cows into two groups: metritic and non-metritic. We observed that the cfu of *F. necrophorum* (metritic = 4.44 ± 0.48, non-metritis = 2.01 ± 0.42 Log10 cfu, ($P < 0.001$) and the relative abundance of the genus *Fusobacterium* (metritic = 28.57 ± 3.07, non-metritic = 10.72 ± 2.62, ($P < 0.001$)) were greater in cows diagnosed with metritis compared with cows without metritis (Figure 2.14). Metritic cows also harbored more *T. pyogenes* (metritic = 3.23 ± 0.35, non-metritic = 2.32 ± 0.28 Log10 cfu, $P = 0.05$) and had a greater relative abundance of the genus *Trueperella* (metritic = -0.31 ± 0.44, non-metritic = -1.56 ± 0.37, $P = 0.02$), when compared with non-metritic cows (Figure 2.15). Interestingly, *E. coli* was more abundant in cows with metritis compared with cows without metritis (metritic = 0.98 ± 0.16, non-metritic= 0.48 ± 0.126 Log10 cfu, $P = 0.04$), whereas we observed an increase in the relative abundance of the genus *Escherichia* in cows without metritis when compared to cows diagnosed with metritis (metritic = -3.62 ± 0.26, non-metritic = -2.55 ± 0.20; $P = 0.05$, Figure 2.16).

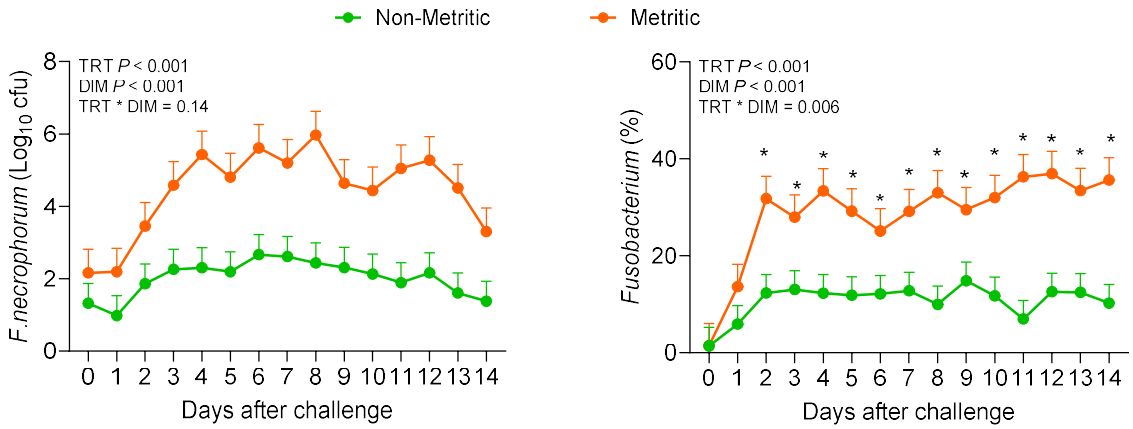

Figure 2.14. Bacterial cultures (*F. necrophorum*) and microbiome (genus *Fusobacterium*) from vaginal swabs during the first 14 days of lactation of cows diagnosed with metritis (n = 24) and cows diagnosed as healthy (n = 35).

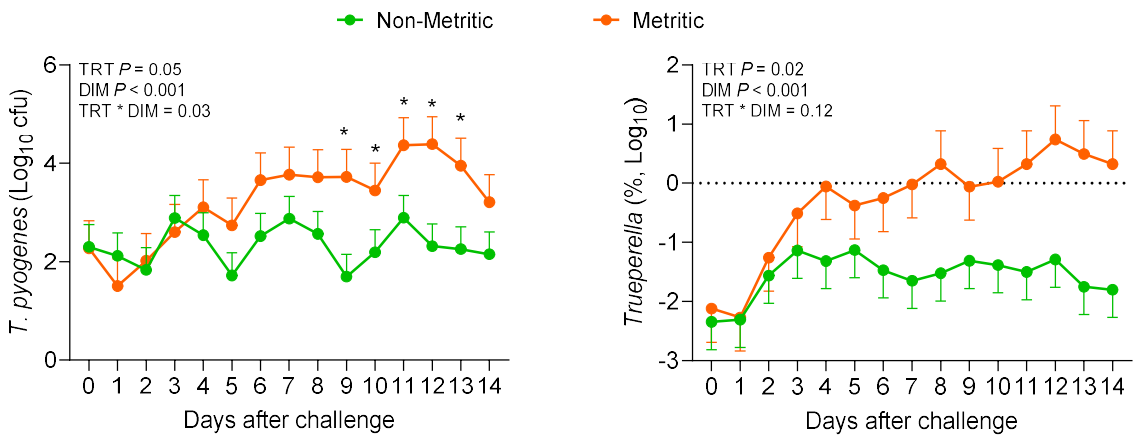

Figure 2.15. Bacterial cultures (*T. pyogenes*) and microbiome (genus *Trueperella*) from vaginal swabs during the first 14 days of lactation of cows diagnosed with metritis (n = 24) and cows diagnosed as healthy (n = 35).

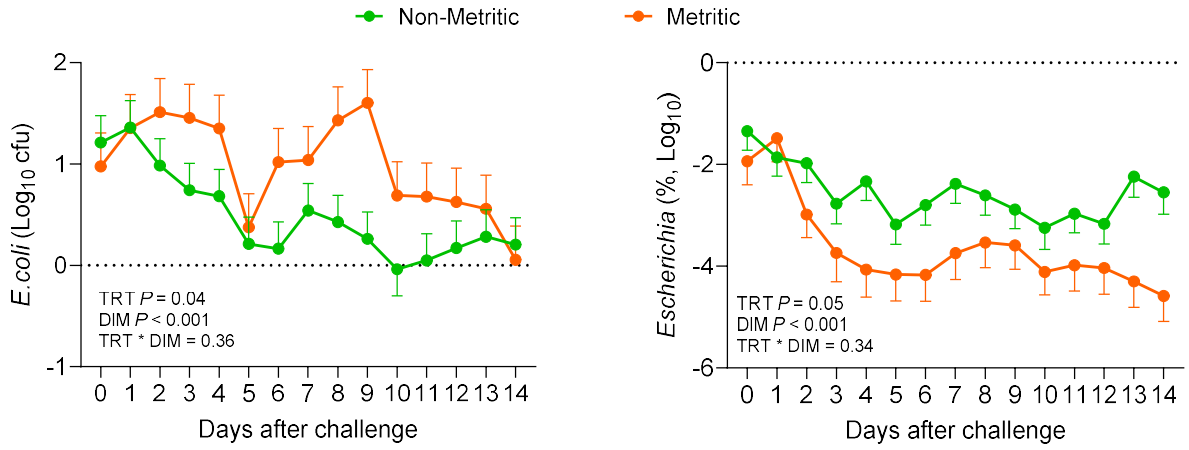

Figure 2.16. Bacterial cultures (*E. coli*) and microbiome (genus *Escherichia*) from vaginal swabs during the first 14 days of lactation of cows diagnosed with metritis (n = 24) and cows diagnosed as healthy (n = 35).

DISCUSSION

The lack of a reliable and consistent animal model of metritis in post-partum dairy cows

makes it difficult to study and establish the efficiency of certain intervention strategies. Here we presented a successful *in vivo* model for metritis induction in post-partum cows through intrauterine infusion of a combination of *E. coli*, *T. pyogenes,* and *F. necrophorum*. Our model produced a metritis incidence of 83% in multiparous cows receiving an intrauterine challenge containing 10^6 cfu of each bacterial species. Illness signs included decrease in milk production and DMI, increase in RT, all of which might be associated with the increase in pro-inflammatory interleukins and APP serum levels. The use of culture-dependent (bacterial count) and culture-independent methods (16S rRNA gene sequencing) allowed us to describe the importance of *F. necrophorum* in the development of metritis.

Previous efforts were made to develop *in vivo* models for postpartum uterine diseases in dairy cows, but there have been no reports describing an *in vivo* model of metritis. Previous reports have described induction of endometritis or pyometra in primiparous and multiparous cows during the luteal phase through the intrauterine infusion of *Trueperella pyogenes* in combination or not with anaerobic bacteria (*F. necrophorum* and *Bacteroides melaninogenicus*), or *T. pyogenes* combined with *E. coli* (Rowson et al., 1953b, Farin et al., 1989b, Piersanti et al., 2019b, Dickson et al., 2020). Although all models were able to change the vaginal discharge score in challenged cows, no clinical signs of illness were observed. Our model relied upon evidence from recent reports that characterized resident microorganisms of a metritic uterus, taking advantage of the techniques of bacterial culture, and microbiome and metagenomic analysis. These previous reports suggested that *Fusobacterium necrophorum* is a relevant microorganism for the development of metritis (Sheldon et al., 2009, Machado et al., 2012, Jeon et al., 2015, Jeon et al., 2016, Galvao et al., 2019). Thus, we administered well characterized strains of *E. coli*, *T. pyogenes* and *F. necrophorum* into the uterus of postpartum cows in one of two doses [10^6 (low-dose) and 10^9 (high-dose) cfu] within 24 hours after parturition. Surprisingly, the group presenting greater incidence of metritis (83%) received the lower dose, whereas the high-dose group had a 25%

incidence of metritis. Although the reasons for this are unknown, we propose two plausible hypotheses. It is possible that a high concentration of bacteria limited their growth and ability to colonize the uterus due to an exhaustion of nutrients within the organ. Alternatively, communication among bacteria, called quorum sensing, allows bacteria to share information and adjust gene expression accordingly. Virulence factor expression appears to be controlled by the concentration of autoinducing peptides and by bacterial density (Miller and Bassler, 2001). It is thus possible that, due to the high concentration of bacteria, the expression of virulence factors by pathogenic bacteria might have been repressed (Ng and Bassler, 2009).

The model we created accurately reproduced the clinical signs associated with naturally occurring metritis, including fever, reduced DMI, and reduced milk production. Cows in the low-dose group had higher RT on days 1, 2, 5, 6, and 7 when compared to high-dose cows, fever was diagnosed before the diagnosis of metritis. The same was reported by Benzaquen et al., (2007) who observed that the increase in RT in metritic cows with and without fever was more evident around 3 days before diagnosis of disease (Benzaquen et al., 2007). In addition to fever, low-dose cows had reduced DMI as they consumed 1.35 and 2.24 kg/day less dry matter when compared with control and high-dose cows. This is in agreement with previous observational studies (Urton et al., 2005, Bell and Roberts, 2007, Wittrock et al., 2011). Cows in the low-dose group also presented a reduction of 3 kg/day in milk production during the study period which is also in agreement with reports for natural cases of metritis (Rajala and Grohn, 1998, Bell and Roberts, 2007, Dubuc et al., 2011, Wittrock et al., 2011, Giuliodori et al., 2013). Based on these data we conclude that challenged cows presented clinical signs of disease that are similar to those reported for natural cases of metritis.

As we obtained success inducing metritis in cows challenged with the low dose of inoculum, we investigated if challenge also created signs of systemic disease. We assessed the activation of the immune system by measuring APP, production of pro-inflammatory cytokines

and alteration of serum metabolites. Previously others have described an increase in haptoglobin prior to the diagnosis of metritis (Smith et al., 1998, Sheldon et al., 2001, Chan et al., 2004, Chan et al., 2010, Dubuc et al., 2010, Galvao et al., 2010), while others have demonstrated association of SAA and haptoglobin as potential predictors of uterine diseases (Chan et al., 2010, Zhang et al., 2018). Herein, we observed a similar trend on increase in haptoglobin before day 7 post-partum in challenged animals and the same for SAA, which is in agreement with those reports.

With the present model we addressed important questions but generated additional questions for further research. Several studies have proposed that low calcium levels during early lactation is associated with increased risk of metritis occurrence (Curtis et al., 1983, Goff and Horst, 1997, Martinez et al., 2012, Rodriguez et al., 2017, Neves et al., 2018). Although we did not observe a significant difference in calcium levels among groups, our findings deserve attention. We observed that challenged and control cows had similar calcium levels at parturition, however, a numerical reduction in calcium levels in low-dose cows became evident on day 1 and persisted until study day 12. Our model agrees with the finding of Martinez et al. (2012), which reported that metritic cows had lower calcium levels when compared with non-metritic cows during the first 12 days in milk, but that calcium concentration on the day of parturition was similar between the groups (metritis vs non-metritis) (Martinez et al., 2012). We hypothesize that the reduction in calcium levels occur as a consequence of a combination of events started with parturition and contamination of the uterus. Low-dose cows had lower feed intake when compared with control cows, which may affect calcium availability and absorption in the gastro-intestinal tract. It has also been shown that proinflammatory cytokines upregulate the calcium-sensing receptor gene **(CaSR)** expression in the parathyroid gland and the kidney, resulting in decreased serum parathyroid hormone and 1,25-dihydroxy vitamin D and calcium levels (Hendy and Canaff, 2015). In the present model, low-dose cows had greater circulating concentrations of proinflammatory cytokines. Thus, high levels of proinflammatory cytokines might have upregulated the expression

of CaSR and reduced calcium levels. The numerically higher LBP levels in the low-dose group may have also contribute to calcium reduction, as it is well known that reduced blood calcium is critical for optimal LPS detoxification via noninflammatory pathways. In the absence of calcium, LPS aggregation is inhibited, which allows LBP to transfer LPS monomers to CD14 and eventually to acute-phase high-density lipoproteins for biliary excretion (Eckel and Ametaj, 2016). Therefore, it is possible that lower calcium levels are observed in animals experiencing endotoxin challenge as a protective mechanism to facilitate the elimination of LPS. In support of this concept when cows were maintained under eucalcemia after challenge with intravenous LPS, reduced milk yield, slow return to baseline DMI, and increased inflammation based on LBP levels (Horst et al., 2020) were observed as compared with cows that did not receive calcium supplementation after challenge.

By monitoring cows daily, we were able to determine the dynamics of microorganism growth during colonization of the uterus. We observed that the low-dose cows had an increased relative abundance of the genus *Fusobacterium*; in addition, the count for *F. necrophorum* was marginally increased. On the other hand, *E. coli* and *T. pyogenes* were not different among groups, leading us to question the importance of these two microorganisms for the development of metritis. When analyzing the progression of the microbiome from calving until establishment of metritis, Jeon et al. (2015) found that the genus *Fusobacterium* was the most abundant (15.7%) among 28 other genera found (Jeon et al., 2015). Similar findings were reported in two additional studies from the same group (Jeon et al., 2016, Cunha et al., 2018). Collectively, all these results support the notion that *Fusobacterium* is one of the most relevant genera, and *F. necrophorum* specifically is the most relevant species for the development of metritis. Nevertheless, it is still unclear if *E. coli* or *T. pyogenes* are important for the development of the disease. Previous work has demonstrated that the damage caused to the epithelium by *E. coli* and *T. pyogenes* is crucial to prepare the endometrium for the colonization of *F. necrophorum* (Bicalho et al., 2012a, Galvao et

al., 2019). Therefore, further investigations are needed to investigate whether the interaction among *E. coli*, *T. pyogenes*, and *F. necrophorum* is requisite for the development of the disease, or if *F. necrophorum* individually is capable of inducing metritis.

We propose that the present *in vivo* model can be used in the future to evaluate the viability of different tools used for prevention or treatment of metritis. A vaccine containing subunits of *E. coli*, *T. pyogenes* and F. *necrophorum* was developed by our group and has been demonstrated to reduce the incidence of metritis on dairy farms (Meira et al., 2020). With our model, we have established the expected incidence of metritis, and we can test the efficacy of this and other preventative measures without using a large number of animals. We can also compare the efficacy of treatments used to reduce the severity of metritis (changes in milk production, DMI), and determine whether such treatments present practically and economically justified means to control this important disease. Another important fact to consider is that most post-partum diseases happen within one month after parturition, and a post-partum cows can develop more than one disease at the same time. Using this model that we created, it might be possible to predict the effects of treatments or prevention strategies in metritis-only cases, isolating any possible confounder that can be created by the presence of other diseases.

CONCLUSION

We successfully induced metritis in post-partum dairy cows via intrauterine administration of *E. coli*, *T. pyogenes*, and *F. necrophorum*. We observed an increased incidence of disease in animals challenged with a dose of 10^6 cfu of this combination of bacteria, indicating that the development of metritis when using this particular bacterial cocktail depends on the dose of microorganisms used. We also established the dynamics of microorganisms for the development of metritis, suggesting that *F. necrophorum* plays an important role in the pathophysiology of the disease. Moreover, we did not have enough power to detect significant differences in acute phase

proteins and pro-inflammatory cytokines, as the study was designed to detect difference in metritis incidence. We did, however, observe interesting trends that are related to the observed clinical signs of natural disease. This model may be used to compare the effects of strategies to prevent or treat metritis, thus providing benefits to the dairy industry.

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CHAPTER THREE: Testing the induction of metritis in healthy postpartum primiparous cows challenged with a cocktail of bacteria.

ABSTRACT

Metritis is a postpartum uterine disease with greater incidence in primiparous cows than in cows of multiple parity. In primiparous cows the impact of the disease is not as detrimental, presumably due to a superior immune response, minimizing the harm caused by pathogenic bacteria. Here, we aimed to test whether a pre-stablished *in vivo* model of clinical metritis in postpartum multiparous Holstein cows would produce similar results in primiparous cows via intrauterine administration of pathogenic *Escherichia coli*, *Fusobacterium necrophorum*, and *Trueperella pyogenes.* We randomly allocated 36 cows into one of three groups of 12 cows each within 24h of parturition. Cows of the control group were intrauterine infused with sterile saline solution, while cows assigned to the low-dose group received an intrauterine inoculum containing 10^3 cfu of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and those assigned to the high-dose group received an inoculum containing 10^6 cfu of each bacterial species. Daily, from enrollment until the end of the study period (0 - 14 days in milk), we performed clinical observation, rectal temperature measurement, blood sampling, vaginal discharge collection, assessment of dry matter intake, and milk yield. At the time of enrollment and at the end of study period, we measured body condition score and body weight for estimation of losses. We found no significant difference in the incidence of clinical metritis among the groups, with the higher dose (10^6 cfu) inducing metritis in 64% of cows, while control and low-dose groups had 33% and 42% incidence, respectively. However, the high-dose group had a 2.7-times greater hazard of being diagnosed with metritis when compared to controls (high-dose vs control hazard ratio = 2.7, 95% confidence interval = 1.15 to 6.45). No differences were detected between low-dose group and controls. We did observe an effect of bacterial challenge on milk production, the high-dose group produced 1.76 and 2.1 kg/day less milk when compared to control or low-dose groups, whereas no differences were observed when comparing control and low-dose groups. No significant alteration in acute phase protein, plasma cytokines or

serum metabolites was observed. Interestingly, we observed higher growth of *F. necrophorum* in selective medium, suggesting the association of this microorganism with the disease. With the evidence from this study, we conclude that intrauterine inoculation with 10^6 cfu of *E. coli*, *T. pyogenes* and *F. necrophorum* elicited physical and clinical changes consistent with clinical metritis.

Keywords: Metritis, *in vivo*, microbiome, bacterial culture, dairy cows.

INTRODUCTION

The transition period, defined as three weeks before and three weeks after parturition (Grummer, 1995), is a critical period during lactation in dairy cows. As feed consumption during this period is not sufficient to meet the nutrient demands of the mammary gland, dairy cows must adapt physiologically to the burdens of lactation. This adaptation includes the uncoupling of the somatotropic axis, increasing insulin resistance, and lipolysis (Baumgard et al., 2017). Metabolic and infectious diseases occur most frequently during this period (Doepel et al., 2002, Wankhade et al., 2017), with metritis being one of them, with an incidence of 25-40% in the first week postpartum (Sheldon et al., 2009).

The standard definition of metritis was proposed by Sheldon et al. (2006), characterizing it as an abnormally enlarged uterus with a fetid, watery red-brown uterine discharge, associated or not with signs of systemic illness, including decreased milk production, dullness, and signs of toxemia. This multifactorial disease is associated with retained placenta, stillbirth, twins, dystocia, ketosis, delivery of male offspring or caesarian section, and hypocalcemia (Markusfeld, 1984, Drillich et al., 2001, Sheldon et al., 2006, Galvão, 2018). Metritis is a concern to the dairy industry as it compromises animal welfare, health, and productivity. The average cost associated with a single case of metritis is estimated to be $513, with 95% of cases ranging from $240 to $884 (Sheldon et al., 2019, Perez-Baez et al., 2021).

Postpartum uterine contamination can happen in two ways, with bacteria ascending the uterus from feces, skin, and the environment when physical barriers of the uterus are disrupted, or through blood, as some microorganisms present in the infected uterus are not found in vaginal samples (Sheldon and Dobson, 2004, Jeon et al., 2017). It has been widely described that the microorganisms typically associated with metritis are *Escherichia coli*, *Trueperella pyogenes*, *Fusobacterium necrophorum*, and bacteria of the genera *Prevotella* and *Bacteroides* (Noakes et

al., 1991, Dohmen et al., 2000, Azawi, 2008). Virulence factors known to be harmful to the reproductive tract have been described for each of these bacterial species. *E. coli* possesses *FimH* that promotes adherence and invasion of the endometrial epithelium and stroma, *T. pyogenes* carries a cholesterol-dependent cytotoxin called pyolysin that causes severe damage to the endometrial stroma, which is rich in cholesterol, and *F. necrophorum* produces leukotoxin that protects the bacteria from phagocytosis by neutrophils.

The incidence of metritis is greater in primiparous than in multiparous cows, likely due to the need for assistance during calving, increasing the likelihood of bacterial contamination of uterine lesions (Bruun et al., 2002, Bell and Roberts, 2007, Giuliodori et al., 2013). However, both animal categories have different responses to metritis, as the detrimental effects on milk production, DMI, and cull rate are more severe in multiparous than in primiparous cows (Dubuc et al., 2011, Wittrock et al., 2011). Authors speculate that this may be due to the less pronounced negative energy balance **(NEB)** observed in primiparous cows (Wathes et al., 2007) and the faster immune response (Lessard et al., 2004).

Here, we tested whether metritis could be induced in primiparous cows using a model validated for multiple parity cows (unpublished data). First lactation cows were intrauterine infused with a low (10^3 cfu) or a high (10^6 cfu) dose of *E. coli*, *T. pyogenes* and *F. necrophorum* within 24 hours after parturition and compared to control cows that received sterile saline solution.

MATERIALS AND METHODS

Ethics Statement

The research protocol was reviewed and approved by the Cornell University Institutional Animal Care and Use Committee, with the protocol number 2019-0040. All procedures were carried out in strict accordance with the approved guidelines.

Sample size calculation

Assuming a type 1 error of 5%, power of 80%, and considering an expected baseline probability of metritis of 16%, a sample size of 12 animals per group will allow us to detect a difference of 59% (75-16) in the incidence of metritis between groups.

Animals, Facilities, Management

All cows enrolled in this study were purchased from a single commercial farm located in Upstate New York (Scipio Center, NY), between June and September 2020. Right after parturition, cows that attended to inclusion and exclusion criteria were challenged at the maternity pen with the assigned challenge or control and transported to the Cornell Teaching Dairy Barn, where they stayed until the end of the study period (15 days in milk).

The commercial farm had approximately 4,100 cows in lactation, housed in free-stall barns with concrete stalls and manure solids bedding, providing access to *ad libitum* water and total mixed ration **(TMR)** according to productivity. The Cornell Teaching Dairy Farm is located in Ithaca, NY, with a capacity to house approximately 200 lactating cows in free-stall barns, with free access to water and TMR, fan ventilation and sawdust bedding. The tie stall barn has structure to allocate 8 cows, in wood shaving bedding and fan ventilation. Cows had individual access to *ad libitum* water and feed bucks that allows precise measure of daily feed intake.

Milking was performed two times a day and milk weights recorded using Dairy Comp 305 (Valley Ag Software, Tulare, CA). Feed was offered daily during the morning (between 0700 and 0800), with 110% of expected daily consumption. Feed intake was determined by the difference of weights in offered and refused feed; dry matter **(DM)** was measured daily using aliquots of TMR that were dried at 55°C for 48 hours. Daily feed intake and DM were used for calculation of dry matter intake **(DMI)**. Diet formulation and chemical composition were obtained from reports received from the nutritionist responsible for the farm and presented in Table 3.1, Table

3.2 and Table 3.3.

Table 3.1. Ingredients (DM basis) of postpartum diet.

Ingredient	% of DM
Corn silage	42.10
Haylage 1st - 3rd	14.79
Whey Blend	2.93
SS High Mix	40.17

Table 3.2. Ingredients (DM basis) of SS High Mix diet.

Ingredient	% of DM
Smartamine Lysine	0.1568
Smartamine Methionine	0.1647
MFP-BNI	0.1164
Blood Meal-BNI-II	3.3739
Express Meal-Dumond-BNI	12.2309
Amino Plus-BNI	5.1832
Soybean Meal-BNI	8.8496
Corn Gluten Feed-BNI	9.1711
Soy Hulls (P)-BNI	3.41
Corn Meal-	47.276
Berga Fat F-100	2.7166
Energizer Gold	1.1883
Limestone Ground	1.5445
S Carb	1.6177
Salt White	1.0812
SS Lact Min (1625 g/ton)	1.9227

Table 3.3. Chemical composition (DM basis) of postpartum diet.

Energy and chemical compostion[1]	DM	Supply
NE_L, Mcal/Kg	-	1.60
NDF, %	29.94	17.37
NFC, %	42.60	24.70
ADF, %	24.35	14.12
Starch, %	27.42	15.90
CP, %	15.67	9.09
Ca, %	0.73	0.42
P, %	0.37	0.21
K, %	1.43	0.83
Na, %	0.45	0.25
Mg, %	0.26	0.15
S, %	0.22	0.13
CL, %	0.56	0.32
Zn, ppm	49.14	1,292.945
Cu, ppm	12.71	334.40
Se, ppm	0.099	2.60
I, ppm	0.29	7.53

Experimental Design

The study had a randomized complete block design with one-way treatment structure, the block factor was the calving order (order of enrollment). The treatment randomization was generated using the SAS statistical package (version 9.4; SAS/STAT, SAS Institute Inc., Cary, NC) that utilizes a random number generator function created by the Biometrics Representative **(BMR)** from Zoetis Animal Health (Kalamazoo, MI). One study member was assigned for challenge administration and another study member was blinded to treatments and responsible for sample collection and metritis diagnosis.

At any sign of parturition, cows were moved to the maternity pen and assisted by trained farm personal, helping as needed. After parturition, information regarding to date and hour of calving, cow and calf ID, calf sex and weight, ease calving score, and person present at parturition, were recorded and provided to research personnel at the time of enrollment. Only healthy primiparous cows that met the inclusion criteria's (gestation length between 270 and 285 days, giving birth to a single offspring without assistance, not presenting placenta at the time of challenge, and not receiving antibiotic treatment at least 30 days before calving) were enrolled in the study. Cows were considered as healthy if not presenting lameness, dehydration, respiratory diseases, empty rumen, recumbence, dullness, depression, displaced abomasum, mastitis, and/or vaginal tear at the time of enrollment. Post enrollment exclusion happened if the animal presented recumbence due to trauma after calving (e.g. bone fracture or nerve paralysis, toxic diseases not related with metritis, and/or milk fever), developed serious diseases at the postpartum period (e.g. hardware disease, displaced abomasum, respiratory diseases, clinical hypocalcemia), was found injured or dead before completion of the 14-day postpartum observation period, received systemic antibiotics for concurrent diseases (not including mastitis treated with intramammary antibiotics), or presented retained placenta (not visible at the time of enrollment).

A total of 36 primiparous cows were selected and evenly assigned to one of the three treatments groups. The control group received an intrauterine infusion with 120 mL of sterile saline solution (TEKnova, Hollister, CA), the low-dose group receive 120 mL of an inoculum containing 10^3 cfu of *E. coli*, *T. pyogenes* and *F. necrophorum*, and the high-dose group received 120 mL of an inoculum containing 10^6 cfu of *E. coli*, *T. pyogenes* and *F. necrophorum*. One cow enrolled in the high-dose group was euthanized on study-day 4 due to severe pneumonia. Therefore, 35 cows concluded the follow-up period.

Inoculum preparation

The inoculum containing *E. coli*, *T. pyogenes* and *F. necrophorum* was used for bacterial challenge. The strains used were isolated from the uterus of cows diagnosed with metritis, belonging to our bacterial collection (Bicalho Laboratory at Cornell University, Ithaca, NY), and selected according to the virulence genes presence (Bicalho et al., 2010, Santos et al., 2010b, Bicalho et al., 2012b, Machado and Bicalho, 2014).

Under aerobic conditions we cultured *E. coli*, on Luria-Bertani **(LB)** broth (Sigma-Aldrich), with agitation (3 *g*), at 37°C for 24 hours. Following incubation, the culture was centrifuged to harvest the cells (2429 *g* for 10 min, room temperature), LB-broth supplemented with 25% of glycerol was used to resuspend the cell pellet to a final count of 10^9 cfu/mL. The final suspension was aliquoted in 2 mL cryogenic vials, flash frozen in liquid nitrogen and stored at -80°C.

An incubator supplemented with 5% CO_2 was used to culture *T. pyogenes* in VersaTREK REDOX 1 media broth (Thermo Fisher Scientific, Kansas) for 48 hours at 37°C. After incubation, the cells were harvested by centrifugation in room temperature (2429 *g* for 10 min), the pellet was resuspended to a final count of 10^9 cfu/mL in microbial freeze-drying buffer (OPS Diagnostics, Lebanon, NJ) and aliquoted in 2 mL cryogenic vials that were lyophilized in the Advantage Pro lyophilizer (SP Scientific, Warminster, PA) according to manufacturer instructions. The vials were then sealed, and stored at 4°C.

The BACTRON 300 anaerobic incubator (Sheldon Manufactoring INC, Cornelius, OR) was used to culture *F. necrophorum* in VersaTREK REDOX 2 Media broth (Thermo Fisher Scientific, Kansas), for 48-hours at 37°C. Following incubation, the culture was centrifuged in 2429 *g* for 10 min to harvest the cells. The cell pellet was resuspended to a final count of 10^9 cfu/mL using VersaTREK REDOX 2 (Thermo Fisher Scientific, Kansas) media broth supplemented with 25% of glycerol, aliquoted in 2 mL cryogenic vials, flash frozen in liquid

nitrogen and stored at -80 °C.

Before the use in the field (two to four hours) the stocks were transferred to 40-mL bottles containing the specific transport media. The Versa TREK REDOX 1 media was used to transport *E. coli* and *T. pyogenes*, while Versa TREK REDOX 2 Media was used to transport *F. necrophorum*.

Evaluation of bacterial cultures purity

The bacterial stocks of each strain used for this trial were submitted to quality control assessments between June and September 2020, to confirm the purity and bacterial count (cfu/mL). We performed DNA extraction from the stocks, amplified the 16S rRNA gene and performed Sanger sequencing (Cornell University Biotechnology Institute, NY) in order to confirm the bacterial species as previously described (Rodrigues et al., 2016).

Bacterial counts of the stocks were performed using the technique of Agar droplets with some modifications. Briefly, serial dilutions were prepared (10^{-1} to 10^{-8}) using sterile 1X PBS (pH 7.4) for each bacterial strain. Next, each quadrant of agar plates was inoculated with three drops of each dilution and incubated according to the bacterium characteristic. After this, the bacterial growth was counted and the number of cfu determined.

E. coli was cultured in blood agar and a selective medium (mastitis GN, CHROMagar, Paris, France) under aerobic conditions at 37ºC for 24 hours. *F. necrophorum* was cultured in Blood agar and LKV agar plates (Laked Blood with Kanamycin and Vancomycin, Anaerobe Systems, California) for 48 hours at 37ºC under anaerobic condition. Lastly, *T. pyogenes* was cultured in blood agar plates in 5% CO_2 supplemented incubator for 48 hours at 37ºC.

***In vivo* experimental challenge**

At the maternity pen, cows were restrained in a headlock, the perineal area and vulva were

cleaned with paper towel followed by disinfection with 70% ethanol. A sterile gilt foam tip catheter (QC supply, Schuyler, NE) was attached to a 60 mL syringe (Air-tite products Co., Inc., Virginia Beach, VA) and used for inoculum administration. The catheter was introduced into the cranial vagina, manipulated through the cervix to access the uterine lumen. One syringe was used for administration of each bacterial culture (40mL of volume) into the uterus. To assure that all the challenge was infused into the uterus, the catheter was flushed out with sterile saline solution (10 mL).

Animal sampling

Blood samples were collected during the morning from coccygeal vessels before administration of challenge and daily from study day 1 to 14. A 3 mL vacutainer K2 EDTA tube (BD Vacutainer, Franklin Lakes, NJ) was used to perform a genomic testing (CLARIFIDE, Zoetis Animal Health, Kalamazoo, MI) and a complete blood cell count **(CBC)** (Vet Hemogram instrument, Heska – Hemature^{tm}, Loveland, CO). After processing the CBC, the blood samples were centrifuged at 2,000 *g* for 15 min at room temperature for plasma separation and frozen at -80 °C. Frozen plasma samples were sent to a Zoetis Research and Development facility (Kalamazoo, Michigan) for measurement of acute phase proteins using commercial ELISA kits: lipopolysaccharide binding protein (HycultBiotech, Wayne, PA), serum amyloid A (SAA) (Life Diagnostics, West Chester, PA), and haptoglobin (Life Diagnostics, West Chester, PA). Proinflammatory cytokines such as Tumor Necrosis Factor alpha **(TNFα)**, interleukin **(IL)**-6, IL-2, IL-8/CXCL8, and IL-10 were also measured in plasma levels using two custom U-Plex assays (Meso Scale Diagnostics, LLC, Rockville, MD) developed for bovine. Analysis of TNFα, IL-2, IL-6, IL-10 were performed in a 4-Plex assay format, while a 1-Plex format was used for bovine IL-8/CXCL8. Briefly, biotinylated antibodies were diluted using Diluent 100 (Meso Scale Diagnostics, LLC, Rockville, MD) to a final concentration of 10µg/mL and individually linked to

specific MSD Linkers (Meso Scale Diagnostics, LLC, Rockville, MD) following the manufacturer's recommendations. The linked antibodies were diluted in MSD Stop Solution (Meso Scale Diagnostics, LLC, Rockville, MD): 4-Plex: TNFα (1:10), IL-2 (1:10), IL-6 (1:10), and IL-10 (1:10); 1-Plex: IL-8/CXCL8 (1:10); 4-Plex linked antibodies were combined into one antibody capture solution and 1-Plex linked antibody was prepared as a separate antibody capture solution. Capture antibody solution (50 µL/well) was added to the respective U-Plex assay plates and incubated for 1 hour shaking at room temperature. After incubation, plates were washed 3 times with PBS added 0.05% Tween-20. Samples for 4-Plex measurements were not further diluted, while samples for 1-Plex measurements were diluted to 1:100 in SeaBlock (ThermoFisher, Rockford, IL), duplicates of samples were added to each plate. Plates were incubated at room temperature for an interval of 1 to 1.5 hours shaking, with samples and standards. Following incubation, plates were washed 5 times with PBS added 0.05% Tween-20. A total of 30 µL/well of detection antibody solutions conjugated with MSD SULFO-TAG (Meso Scale Diagnostics, LLC, Rockville, MD) were added, followed by 1 hour incubation at room temperature, shaking. Plates were later washed one time with detection antibodies, followed by three washed with PBS added 0.05% Tween-20. Lastly, 150 µL/well of 2x MSD Read Buffer (Meso Scale Diagnostics, LLC, Rockville, MD) was added to plates and reading was performed immediately on a MESO SECTOR S 600MM instrument (Meso Scale Diagnostics, LLC, Rockville, MD).

A 10 mL vacutainer tube with spray-coated silica (BD Vacutainer, Franklin Lakes, NJ) was used for serum separation. Tubes were centrifuged at 2,000 *g* for 15 min at room temperature and frozen at -80 °C. Serum concentration of calcium, non-esterified fatty acids **(FA)**, β-hydroxybutyrate **(BHB)**, total protein, bovine serum albumin, glucose, alkaline phosphatase **(ALP)**, gamma glutamyl transferase **(GGT)**, aspartate aminotransferase **(AST)**, alanine aminotransferase **(ALT)**, and lactate were measure using an automated clinical chemistry analyzer (Daytona, Randox Laboratories Ltd., Kerneysville, WV), using reagents provided by the same

company

From enrollment to the end of the study period (study day 14), daily measurement of rectal temperature **(RT)** was performed. Body weight **(BW)** and body condition scores **(BCS**, Edmonson et al., 1989**)** were also performed at enrollment and at study day 14 for estimation of BW and BCS losses. Unfortunately, we experienced problems with the scale at enrollment and at the end of study for some cows and data was lost, so BW results should be interpreted with cautious as the number of cows per group is reduced.

Vaginal discharge sample collection

We performed daily evaluation of vaginal discharge (Metricheck, SimcroTech, Hamilton, New Zealand). Briefly, the perineal area and vulva were cleaned with paper towel and disinfected with 70% ethanol, the Metricheck device was disinfected by immersion in a solution containing ammonium persulfate and cleaned with sterile distilled water and then introduced in the cranial extent of the vagina and retracted caudally bringing material adhered to its silicone hemisphere.

Culture dependent (plate counting) and culture independent (16S rRNA sequencing) methods were used to determine the bacterial load in the vaginal discharge retrieved from each cow. A total of three swabs of vaginal content were collected per cow. One swab was used for culture independent methods, placed in a sterile tube and transported on ice for to the laboratory. At the laboratory, the swab was placed in 2mL sterile microtubes and stored at -80°C. Two swabs were collected for cfu counts, placed into tubes containing specific transport media (Versa TREK REDOX 1 and Versa TREK REDOX 2, Thermo Fisher Scientific, Kansas) and transported to the laboratory on ice where they were placed in pre-labeled (cow ID and study day) conical centrifuge tubes containing 2 mL of specific culture medium (same used for transportation) and processed according to the methods previously described.

Metritis definition

Evaluation of vaginal mucus was performed daily by two research members. According to the characteristics of content retrieved using the Metricheck device, the vaginal discharge score **(VDS)** varied from 0 to 3, where 0 was a clear lochia with viscous discharge or no discharge observed, 1 was a clear mucus with <50% of purulent of mucopurulent content, 2 was a clear mucus with ≥50% of purulent or mucopurulent content, and 3 as a fetid, watery, red-brownish uterine discharge disregarding of fever (Sheldon et al., 2006, Sheldon et al., 2008). Metritis was defined as animals presenting VDS equals to 3.

DNA extraction and microbiome

The DNA extraction was carried by adding 1 mL of UltraPure™ distilled water (DNAse and RNAse free, Invitrogen, Grand Island, NY) into the conical centrifuge tube containing the swab followed by 10 min of vortex mixing homogenization (Fisher Scientific, Hampton, NH). Swabs were discarded and the liquid was centrifuged in room temperature for 5 minutes at 15.7 × *g*. The pellet retrieved was submitted to DNA extraction using the DNeasy PowerFood Microbial Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

To perform DNA sequencing, a polymerase chain reactions **(PCRs)** was carried using primers 515F and 806R for amplification of the V4 hypervariable region of the bacterial/archaeal 16S rDNA gene; following the method previously optimized for the Illumina MiSeq platform (Caporaso et al., 2012). Samples of extracted DNA were amplified using different barcodes for 16S rRNA gene PCR with 12-bp error-correcting Golay (http://www.earthmicrobiome.org). For PCR, we used 10 µM of, EconoTaq Plus Green 1x Master Mix (Lucigen®, Middleton, WI), 10 ng–100 ng of DNA and UltraPure™ distilled water (DNAse and RNAse free, Invitrogen, Grand Island, NY) to achieve the final volume of 50 µL of reaction. The PCR steps were: 94 °C for 3 min for initial denaturing; 35 cycles of 45 s at 94 °C, 1 min at 50 °C, 90 s at 72 °C; and 10 min at

72 °C for final elongation. For verification of amplicon presence, the amplified DNA was loaded in agarose gel (1.2%, wt/vol) containing 0.5 mg/ml ethidium bromide. Amplified DNA was purified using the Mag-Bind® Total Pure NGS (Omega Bio-Tek Inc., Norcross, Georgia) following the manufacturer's instructions. We used spectrophotometric estimation (A_{260} of 1.0 = 50µg/ml pure dsDNA) to determine the DNA concentration. The quantified DNA was diluted to similar concentrations and pooled to prepare the library, and sequenced with the MiSeq platform (Illumina Inc., San Diego, CA) using the Reagent MiSeq V2 300 cycles. The PCR, purification and pooling procedures were automized using the OT-2 robot pipetting (Opentrons, New York, NY).

After sequencing, we used the MiSeq Reporter Metagenomics Workflow to generate the Operational Taxonomic Unit **(OTU)** tables based on the Greengenes database (http://greengenes.lbl.gov/). The classification of reads in the output used from this workflow has a classification of reads in multiple levels, we analyzed phylum, genus, and species.

Data Analysis

Analysis of descriptive statistics were performed using SAS (SAS Institute Inc., Cary, NC). Continuous data collected over time (e.g., RT, milk production, cfu count, molecular analyses, and blood parameters) was analyzed by general linear mixed models using the MIXED procedure of SAS version 9.4 (SAS Institute Inc.). Residual plots were used to assess normality and homoscedasticity of residuals. Models included the fixed effects of treatment (control, low-dose, and high-dose), day after challenge and the interaction term between treatment and days after challenge, and random effects of block and animal within block. As continuous data did not follow normal distribution, the results were log10-transformed. Based on Akaike information criterion, the covariance structure with the lowest AIC was selected for each variable.

Dichotomous outcomes such as incidence of metritis and fever were evaluated using multivariate logistic regression models, using the binary distribution of the GLIMMIX procedure

(SAS version 9.4). The model included random effect of block and fixed effect of treatment.

For all models, variables were considered statistically significant when a *P*-value ≤ 0.05 was detected, and a tendency to significance was considered if the *P*-value was between 0.05 and 0.10. In all models, Fisher's Protected LSD was used for multiple comparisons such that pairwise treatment comparisons were performed only if the treatment effect or treatment by DIM effect (only for repeated measures models) was significant at 0.05 level.

RESULTS

Descriptive characteristics of enrolled cows and time from calving to enrollment.

A detailed analysis for parity group, body condition score **(BCS)** at enrollment, body weight **(BW)** at enrollment, rectal temperature **(RT)** at enrollment, age at calving in days, days of gestation, genetic differences in total pounds of milk produced during a 305-day lactation (**GPTA**; CLARIFIDE test), genomic standardized transmitting ability for metritis risk (**Z_MET**; CLARIFIDE test), genomic standardized transmitting ability for retained placenta risk (**Z_RP**; CLARIFIDE test), dairy wellness profit (**DWP**; CLARIFIDE test), net merit (CLARIFIDE test), and total performance index (**TPI**; CLARIFIDE test) was performed, no significant differences in these variables were observed (Table 3.4). We also estimated the average time in hours from parturition to challenge for each group (Figure 3.1). Control cows were challenged with sterile saline solution in an average interval of 12.1 hours, ranging from 6.0 to 19.3 hours (SD = 4.8 h), while low-dose and high-dose cows were challenged with a mean interval of 12.4 hours, ranging from 6.2 to 18.3 hours (SD = 4.37 h), and 14.2 hours, ranging from 9.9 to 19.7 hours (SD = 3.45 h), respectively.

Table 3.4. Descriptive data for cows enrolled in the study.

Item	Control	Low-dose	High-dose	*P*-value
Parity group	1	1	1	-
BCS at enrollment	3.5 ± 0.07	3.4 ± 0.07	3.5 ± 0.08	NS
BW at enrollment, kg	574.0 ± 19.43	590.9 ± 19.43	586.5 ± 20.77	NS
RT at enrollment, °C	38.4 ± 0.18	38.5 ± 0.18	38.6 ± 0.19	NS
Age at calving, days	661.6 ± 8.2	667.7 ± 8.2	673.4 ± 8.2	0.53
Days of gestation	275.4 ± 1.2	276.2 ± 1.2	276.1 ± 1.2	0.87
GMILK	657.9 ± 149.2	720.6 ± 155.8	751.0 ± 149.2	0.90
Z_MET	100.4 ± 1.1	102.7 ± 1.1	102.8 ± 1.1	0.24
Z_RP	98.0 ± 1.3	101.0 ± 1.4	102.1 ± 1.3	0.09
DWP	284.6 ± 46.8	272.6 ± 48.8	400.4± 46.8	0.10
Net Merit	235.0 ± 35	216.0 ± 36.5	261.7± 35	0.67
TPI	2219.4 ± 43	2192.1 ± 44.9	2231.1 ± 43	0.82

BCS: Body condition score.

BW: Body weight.

RT: Rectal temperature.

GMILK: Genomic Enhanced Predicted Transmitting Ability for milk yield - Describes genetic differences in total pounds of milk produced during 305-day lactation.

Zoetis Metritis (Z_MET): Genomic Standardized Transmitting Ability for metritis risk - Describes the expected disease risk of a Holstein or Jersey female of being diagnosed with metritis one or more times in a given lactation.

Zoetis Retained Placenta (Z_RP): Genomic Standardized Transmitting Ability for retained placenta risk - Describes the expected disease risk of a Holstein or Jersey female of being diagnosed with retained placenta one or more times in a given lactation.

DWP: Dairy Wellness Profit is a selection index that expresses the expected lifetime profit of an animal. It combines the Zoetis wellness traits.

Net Merit: Net Merit index expresses the expected lifetime profit of a female compared to the breed base. Net Merit utilizes economically relevant traits related to yield, health, longevity and calving ease.

TPI: Total Performance Index is the official selection index of the Holstein breed and ranks animals on the basis of combined genetic merit for productivity, efficiency, and conformation.

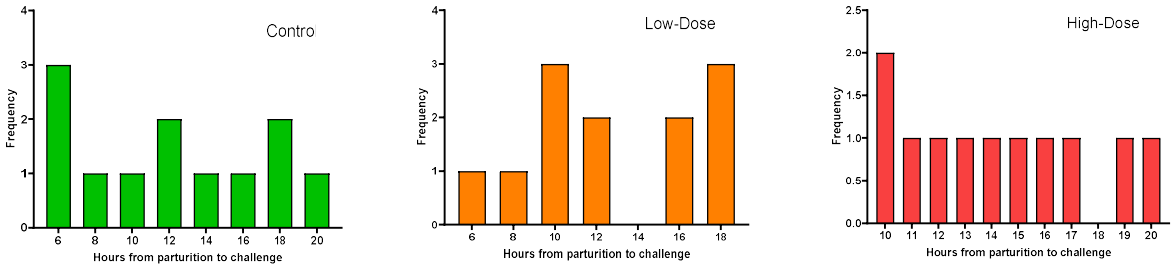


Figure 3.1. Hours from parturition to intrauterine challenge with a bacterial inoculum containing 10^3 cfu (n = 12), or 10^6 cfu (n = 11) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 12).

Bacterial challenge did not induce clinical signs associated with metritis.

We did not observe a significant difference in the metritis incidence over the course of the study among the three groups. Control cows had 33% incidence of metritis, while low-dose and high-dose groups had 42% and 64% incidence (control vs low-dose: $P = 0.67$; control vs high-dose: $P = 0.16$) (Figure 3.2). However, survival analysis reveals a difference ($P = 0.02$) among groups in the hazard of being diagnosed with metritis. The high-dose group had a 2.7-times greater hazard of being diagnosed with metritis when compared to controls ($P = 0.02$; high-dose vs control hazard ratio = 2.7, 95% confidence interval = 1.15 to 6.45) and this difference is observed starting on study-day 3; while the low-dose group had similar risk of being diagnosed with metritis than control cows ($P = 0.80$; low-dose vs control hazard ratio = 1.19, 95% confidence interval = 0.3 to 4.8). Data are presented in Figure 3.3.

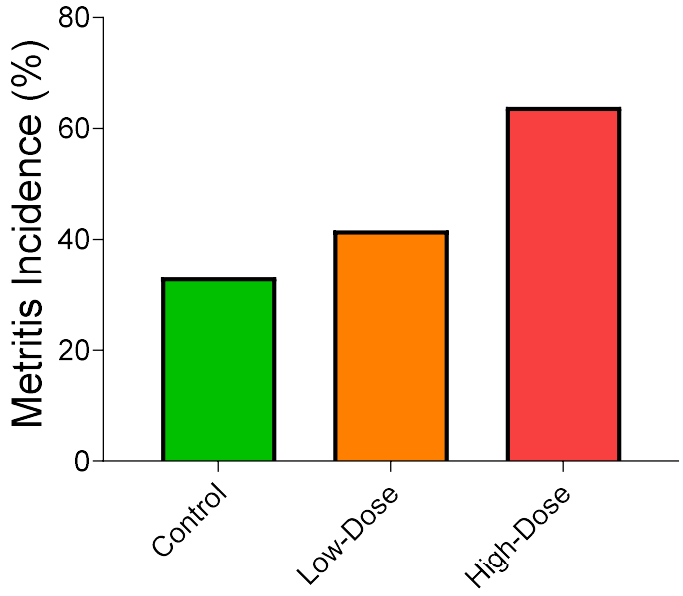


Figure 3.2. Metritis incidence during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^3 cfu (n = 12), or 10^6 cfu (n = 11) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 12).

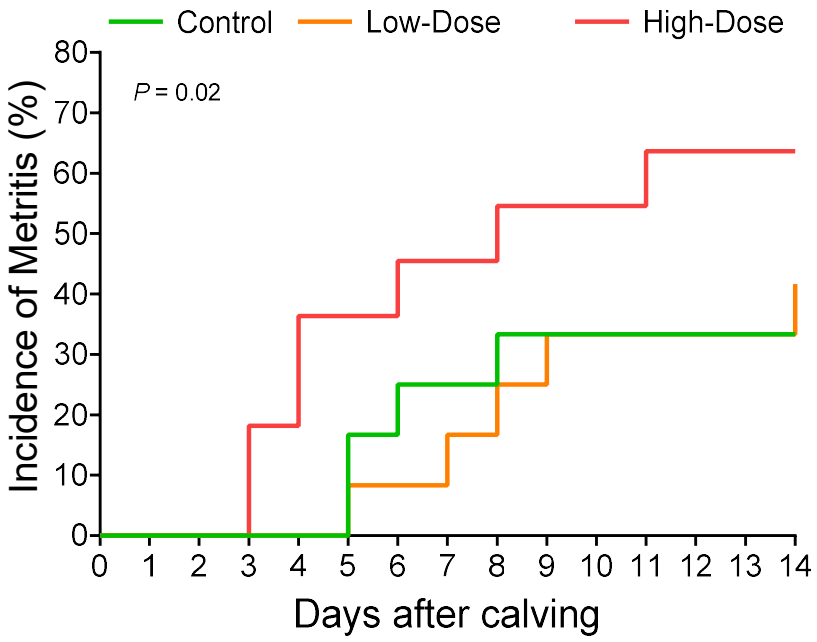


Figure 3.3. Kaplan-Meier survival curves for calving to metritis diagnosis of cows challenged intrauterine with a bacterial inoculum containing 10^3 cfu (n = 12), or 10^6 cfu (n = 11) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 12).

Bacterial challenge did not affect RT ($P = 0.17$; control = 38.6 ± 0.08, low-dose = 38.7 ± 0.08, high-dose = 38.6 ± 0.09 °C; Figure 3.4). Fever was defined as RT ≥ 39.5°C for at least one day during the study period. We observed that the control cows had a marginally lower incidence of fever when compared with low-dose cows (control = 8.3% vs low-dose = 50.0%; $P = 0.06$) but no difference when compared to high-dose cows (control = 8.3% vs high-dose = 36.4%; $P = 0.15$).

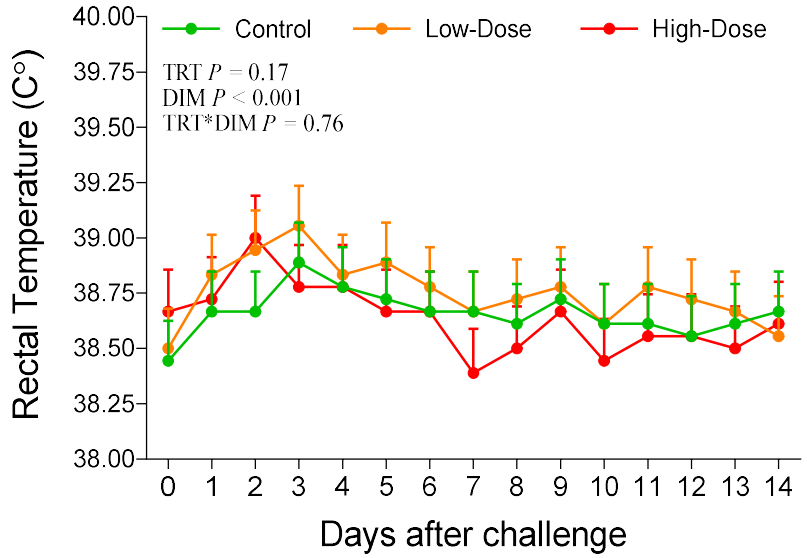


Figure 3.4. Rectal temperature (A) and fever incidence (B) during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^3 cfu (n = 12), or 10^6 cfu (n = 11) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 12).

The DMI was marginally altered ($P = 0.06$) by bacterial challenge (Figure 3.5 A). Overall, we observed that high-dose cows consumed 1.05 ($P = 0.06$) and 1.24 ($P = 0.03$) kg/d less dry matter when compared with control and low-dose cows, respectively, while no differences were observed between the control and low-dose animals ($P = 0.73$).

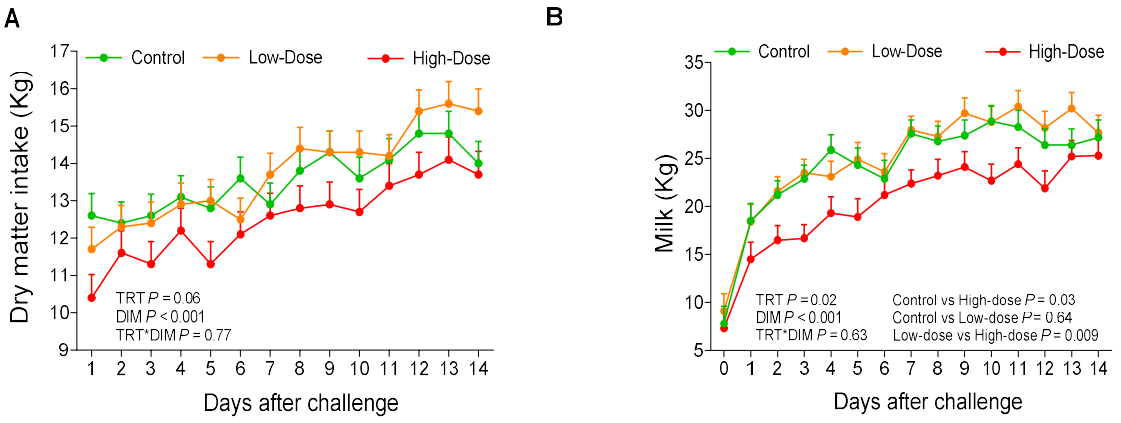


Figure 3.5. Milk production (Kg; A) and dry matter intake (kg; B) during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^3 cfu (n = 12), or 10^6 cfu (n = 11) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 12).

Milk production was reduced by bacterial challenge ($P = 0.02$; Figure 3.5 B). Overall, the high-dose group produced an average of 9.16 ± 1.2 kg/day of milk, while control and low-dose produced 10.97 ± 1.2 and 11.3 ± 1.2 kg/day, respectively. The high-dose group produced 1.76 ($P = 0.03$) and 2.1 ($P = 0.009$) kg/day less milk when compared to control or low-dose groups, whereas no differences were observed when comparing control and low-dose groups ($P = 0.64$).

We did not observe differences in BCS (P = 0.64) and BW (P = 0.56) loss between challenge groups during the first 14 days of lactation (Figure 3.6). The data regarding BW must be interpreted with caution as we lost data from several cows on the day of enrollment an at study day 14, and statistics were thus performed on a smaller sample size.

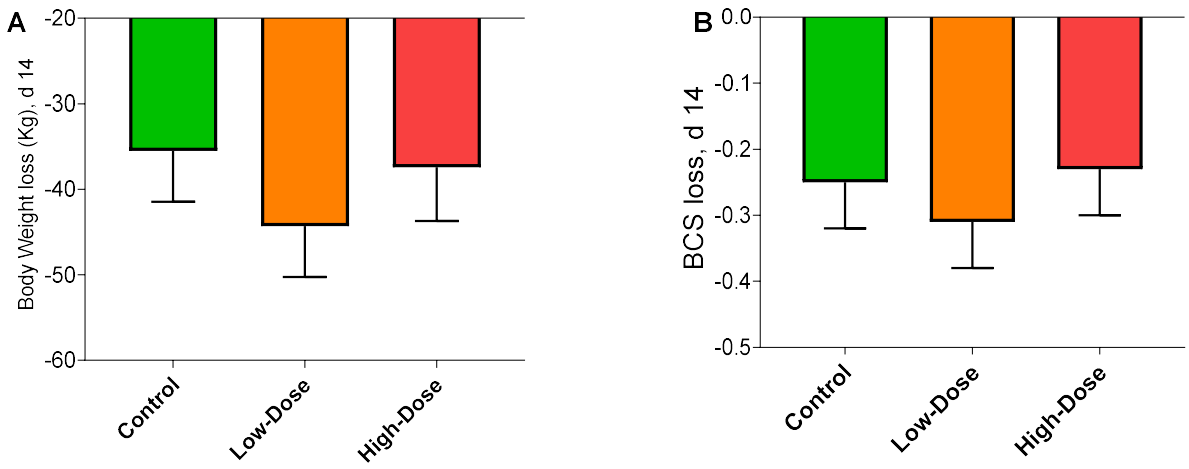


Figure 3.6. Body weight and body condition score (BCS) loss during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^3 cfu (n = 8), or 10^6 cfu (n = 8) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and placebo controls (n = 7).

Bacterial challenge did not alter acute phase proteins and cytokines levels.

We aimed to measure plasma concentrations of the pro-inflammatory cytokine tumor necrosis factor alfa **(TNF-α)**, interleukin **(IL)**-2, and IL-6; the chemokine IL-8; and the anti-inflammatory IL-10. As the majority of these were below the limit of detection for the commercial ELISA assays used, we obtained results for only IL-6 and IL-8 (Figure 3.7), and those were not affected by challenge administration (IL-6: $P = 0.96$, IL-8: $P = 0.20$).

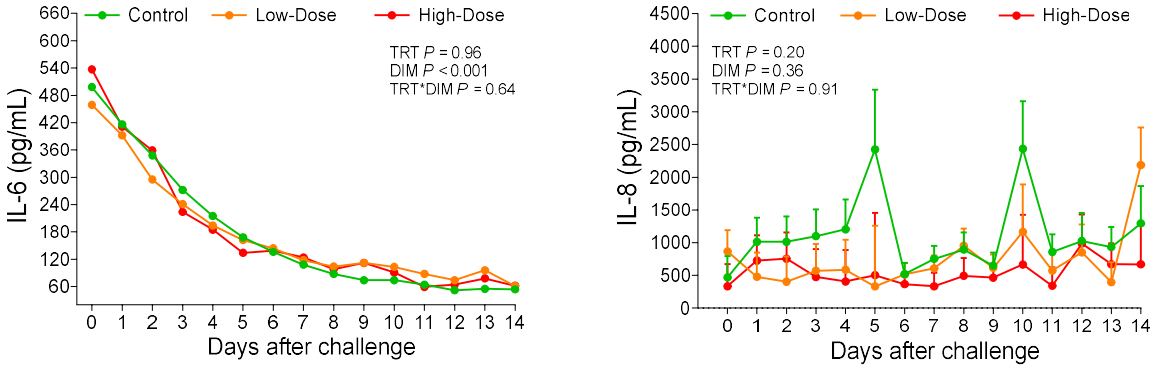


Figure 3.7. Serum IL-6 and IL-8 levels during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^3 cfu (n = 12), or 10^6 cfu (n = 11) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 12).

We did not observe significant differences in plasma lipopolysaccharide binding protein **(LPB)**, serum amyloid A **(SAA)** and haptoglobin (haptoglobin: P = 0.31, haptoglobin back transformed: P = 0.30, SAA: P = 0.26, SAA back transformed: P 0.29, LBP: P = 0.24) (Figure 3.8).

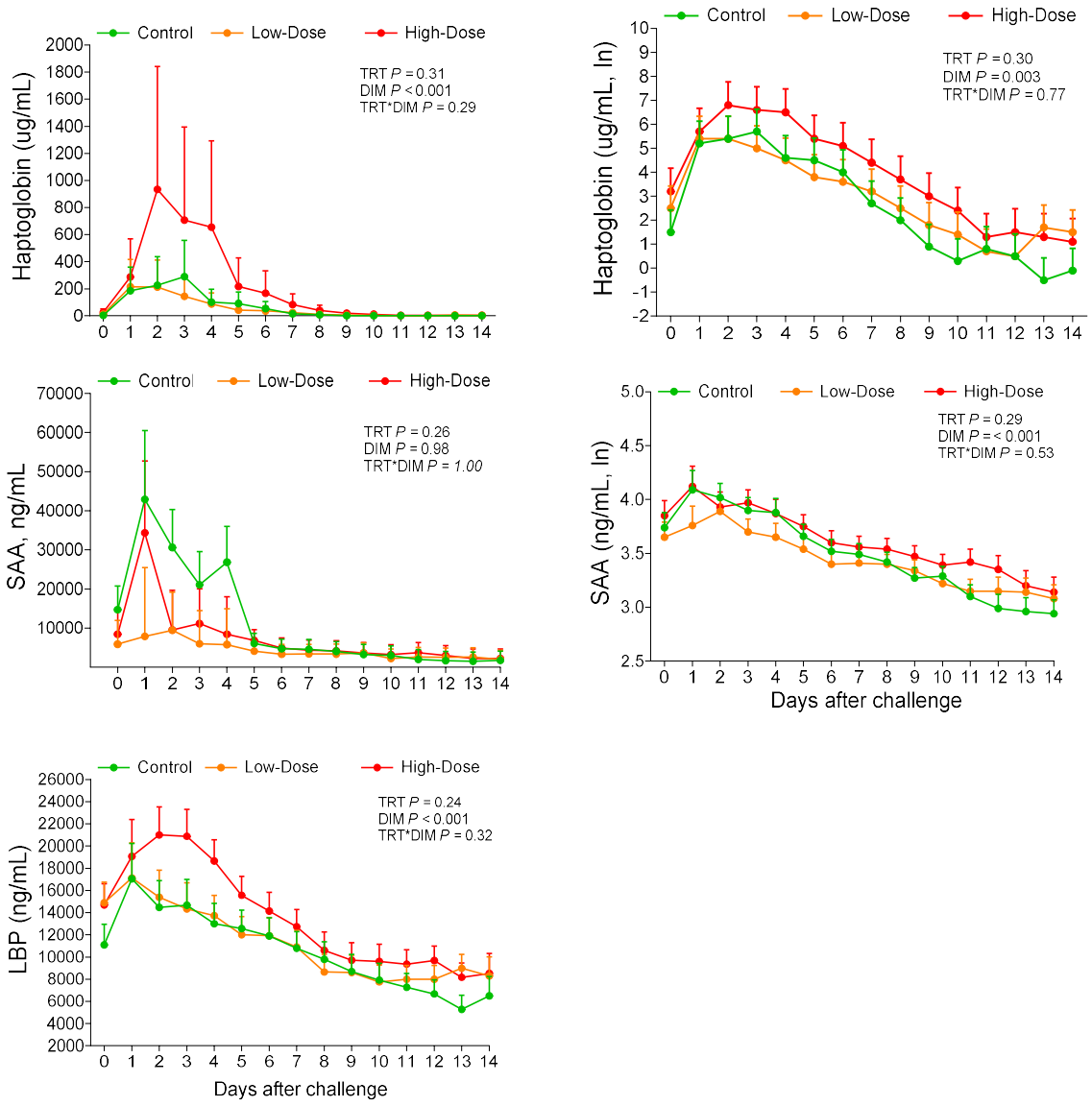


Figure 3.8. Serum haptoglobin (transformed and back-transformed), LBP, and SAA (transformed and back-transformed) during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^3 cfu (n = 12), or 10^6 cfu (n = 11) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 12).

Bacterial challenge did not affect serum metabolites, hepatic enzymes or hemogram parameters.

Bacterial challenge did not affect serum FA ($P = 0.67$), BHB ($P = 0.40$), albumin ($P = 0.42$), total

protein ($P = 0.87$), and glucose ($P = 0.19$) concentrations (Figure 3.9). We assessed liver damage by measuring serum levels of lactate ($P = 0.28$), and the hepatic proteins aspartate transferase ($P = 0.12$), alanine transferase ($P = 0.43$), alkaline phosphatase ($P = 0.44$), gamma glutamyl transferase ($P = 0.68$). None of them was affected by challenge (Figure 3.10). When analyzing the complete blood cell count, we did not observe differences in the numbers of white blood cells ($P = 0.40$), lymphocytes ($P = 0.99$), monocytes ($P = 0.38$), granulocytes ($P = 0.25$), red blood cells ($P = 0.85$), and percentage hematocrit ($P = 0.95$) among groups (Figure 3.11).

We did not detect statistical differences among calcium serum levels ($P = 0.58$). Although all cows presented similar levels of serum calcium at the time of enrollment, a treatment-by-time interaction on calcium levels on study-day 5 was detected, where high-dose cows had higher calcium when compared with low-dose cows (Figure 3.9).

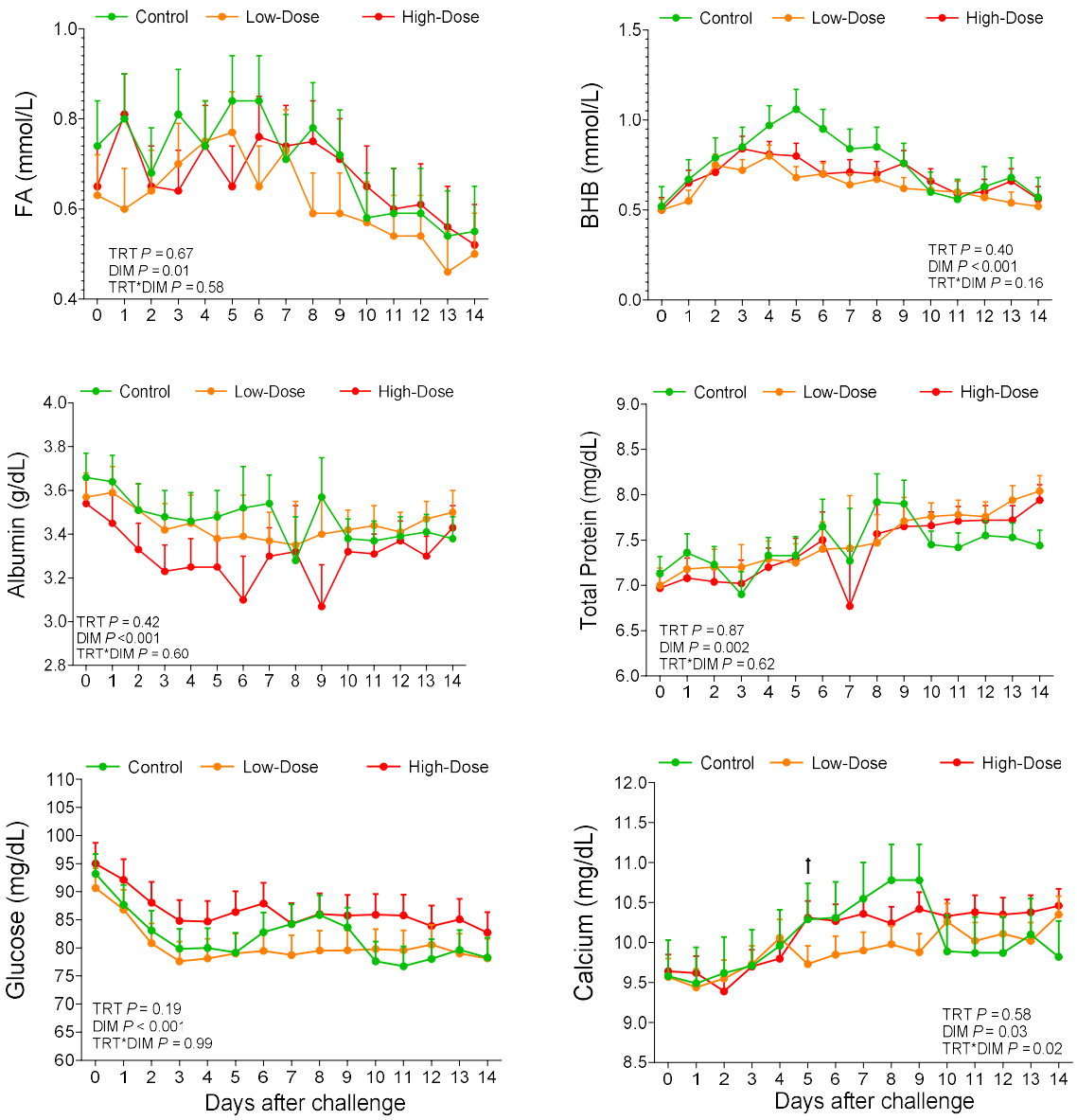


Figure 3.9. FA, BHB, albumin, total protein, glucose, and calcium levels during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^3 cfu (n = 12), or 10^6 cfu (n = 11) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and placebo controls (n = 12).

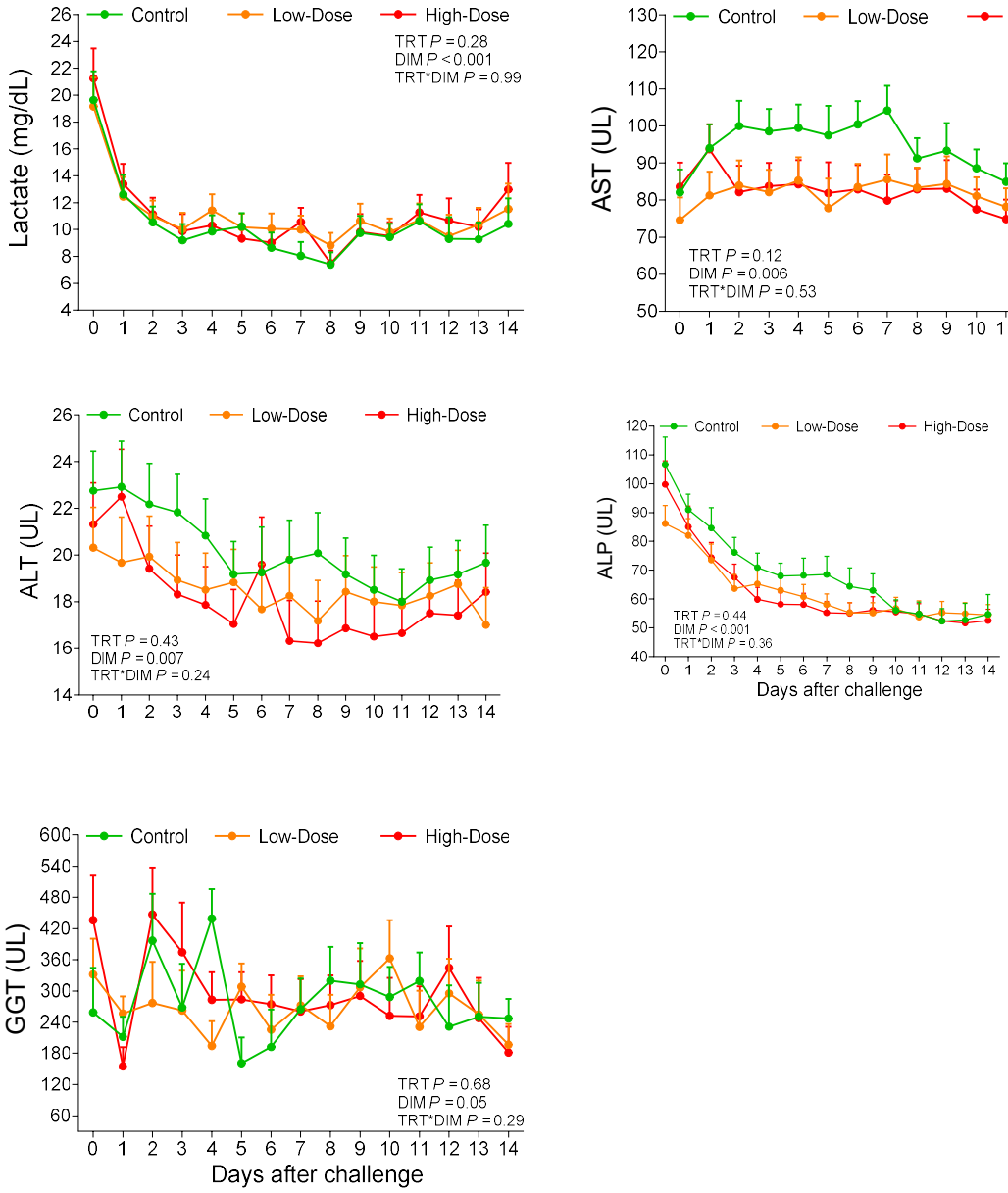


Figure 3.10. Lactate, ALT, AST, ALP, and GGT levels during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^3 cfu (n = 12), or 10^6 cfu (n = 11) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 12).

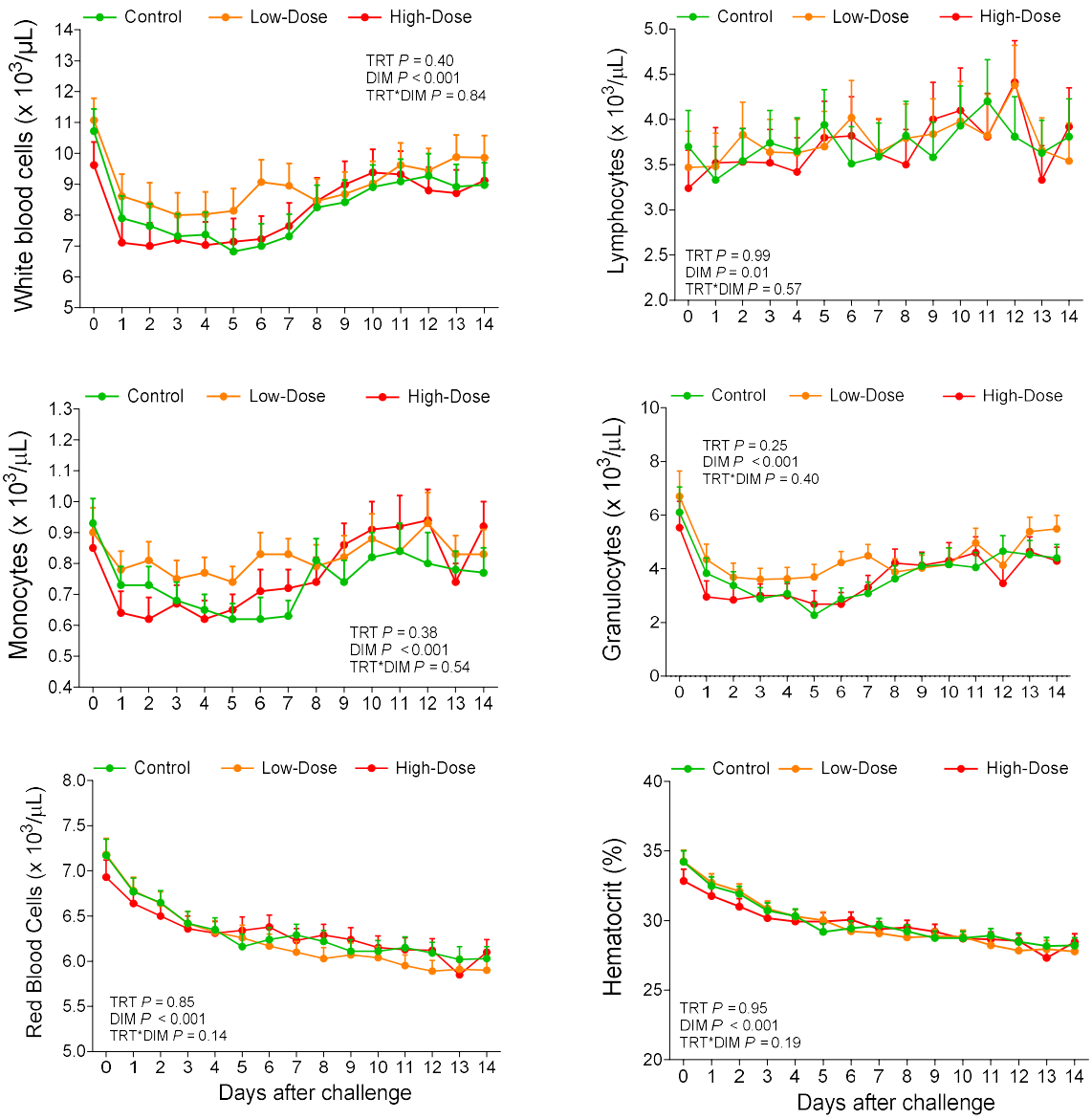
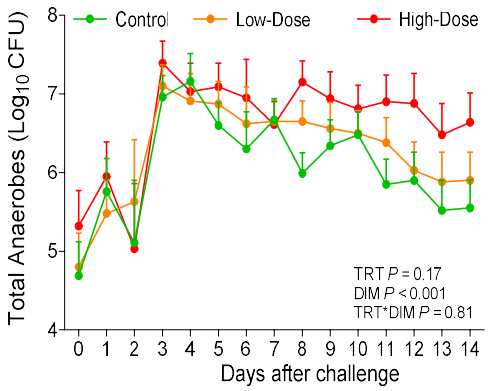


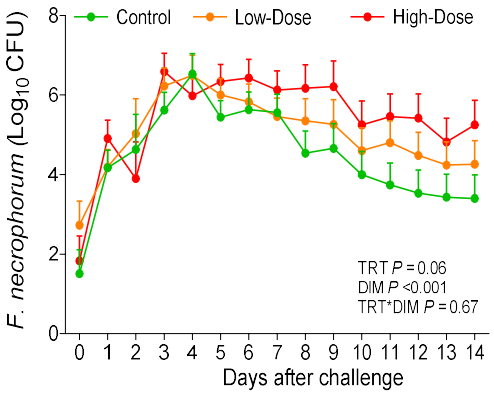
Figure 3.11. White blood cells, lymphocytes, monocytes, granulocytes, red blood cells, and percent hematocrit during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^3 cfu (n = 12), or 10^6 cfu (n = 11) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 12).

Challenge administration altered the uterine microbiome.

We observed that bacterial challenge marginally altered the cfu counts for *F. necrophorum* (*P* = 0.06). Furthermore, we did not observe an effect of challenge on cfu count of total anaerobes (*P* =

0.17; total anaerobes: control = 6.06 ± 0.20, low-dose = 6.27 ± 0.20, high-dose = 6.61 ± 0.21 Log10 cfu;) or the relative abundance of the genus *Fusobacterium* (P = 0.32) (Figure 3.12). We did observe an effect of challenge on the relative abundance of the genus *Escherichia* (P = 0.02), but no differences in cfu of total aerobes (P = 0.24), *E. coli*, (P = 0.23), (Figure 3.13) or cfu of total facultative anaerobes (P = 0.47), *T. pyogenes* (P = 0.18), or the relative abundance of the genus *Trueperella* (P = 0.20) among groups (Figure 3.14).





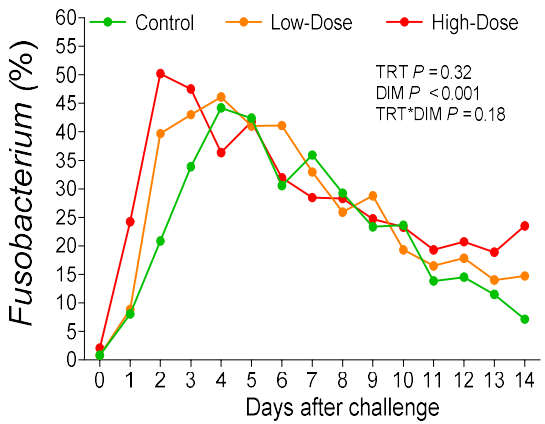
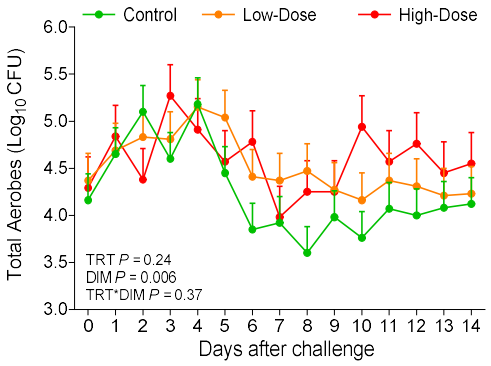
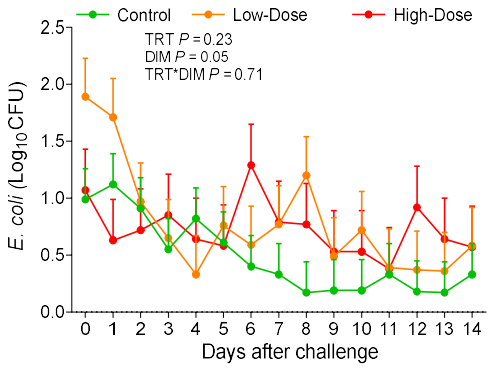


Figure 3.12. Bacterial cultures (total anaerobes and *F. necrophorum*) and microbiome (genus *Fusobacterium*) from vaginal swabs during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^3 CFU (n = 12), or 10^6 CFU (n = 11) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 12).





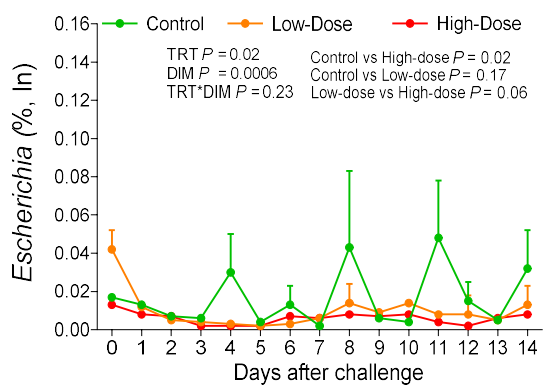


Figure 3.13. Bacterial cultures (total aerobes and *E. Coli*) and microbiome (genus *Escherichia*) from vaginal swabs during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^3 cfu (n = 12), or 10^6 cfu (n = 11) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 12). ln = natural log.

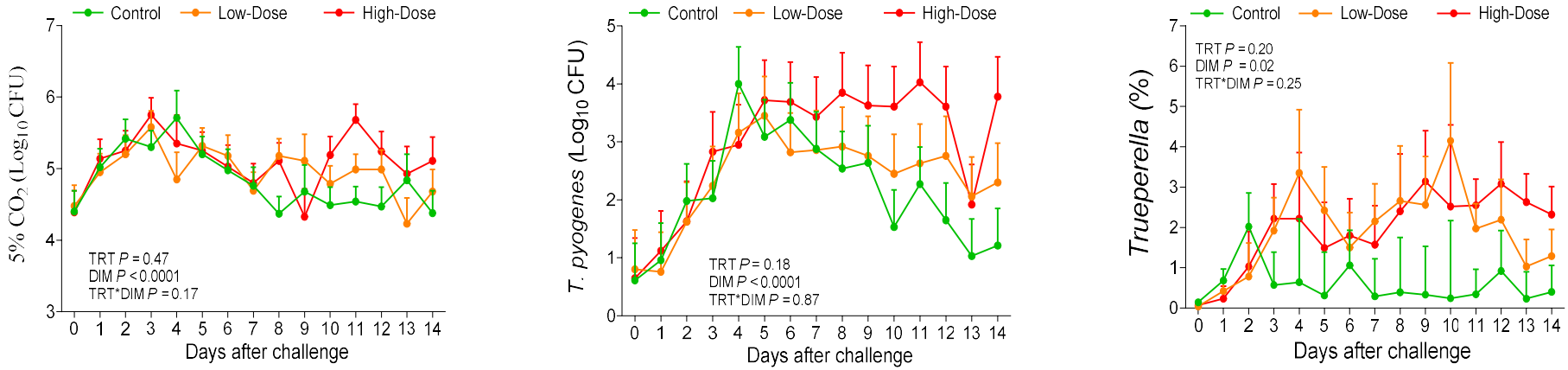


Figure 3.14. Bacterial cultures (total facultative anaerobes and *T. pyogenes*) and microbiome (genus *Trueperella*) from vaginal swabs during the first 14 days of lactation of cows challenged intrauterine with a bacterial inoculum containing 10^3 cfu (n = 12), or 10^6cfu (n = 11) of *E. coli*, *T. pyogenes*, and *F. necrophorum*, and controls (n = 12).

We then further compared cows diagnosed with metritis, independently of the challenge assigned, to cows not diagnosed with metritis. We observed that metritic cows presented greater bacterial count of *F. necrophorum* and *T. pyogenes* and greater relative abundance of the genera *Fusobacterium* and *Trueperella*. On the other hand, no difference was observed between both groups for bacterial count for *E. coli* or for the relative abundance of the genus *Escherichia*. Data are provided in Figure 3.15 –3.17.

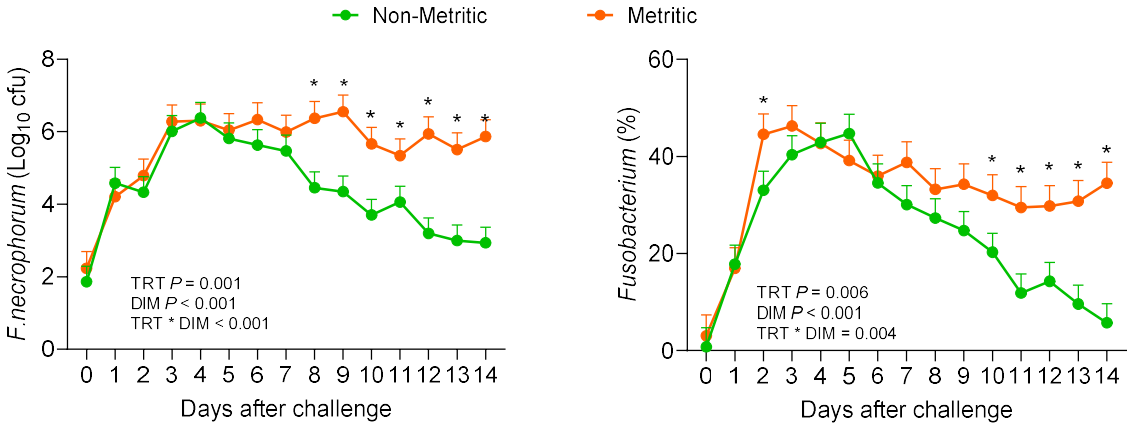


Figure 3.15. Bacterial cultures (*F. necrophorum*) and microbiome (genus *Fusobacterium*) from vaginal swabs during the first 14 days of lactation of cows diagnosed with metritis (n = 16) and cows diagnosed as healthy (n = 19).

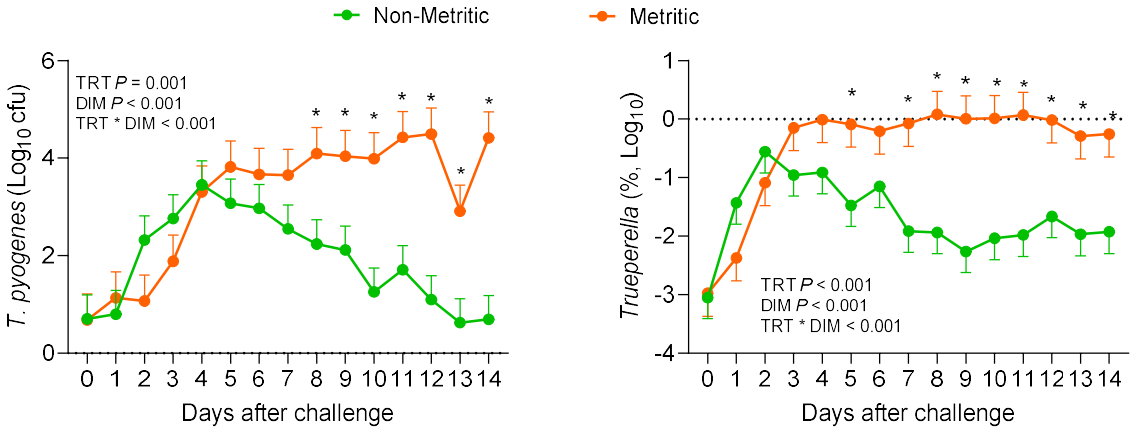


Figure3.16. Bacterial cultures (*E. coli*) and microbiome (genus *Escherichia*) from vaginal swabs during the first 14 days of lactation of cows diagnosed with metritis (n = 16) and cows diagnosed as healthy (n = 19).

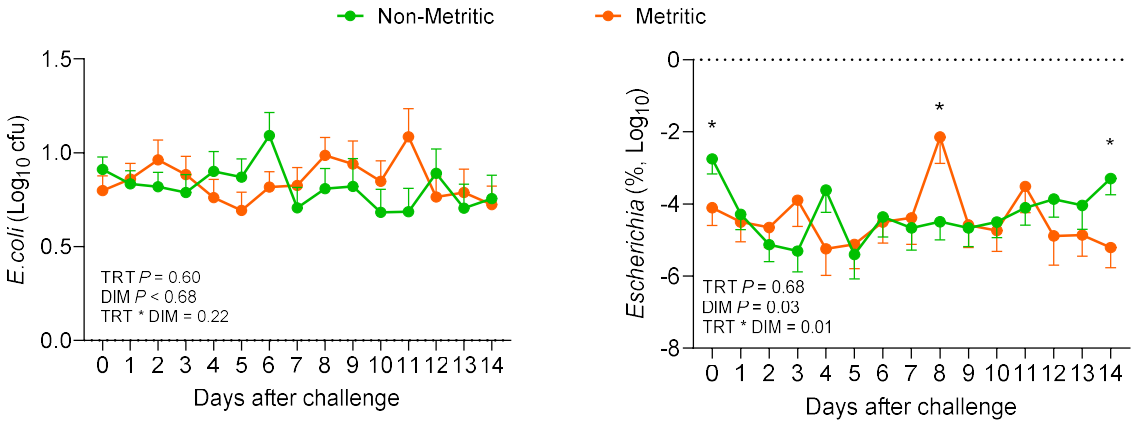


Figure 3.17. Bacterial cultures (*T. pyogenes*) and microbiome (genus *Trueperella*) from vaginal swabs during the first 14 days of lactation of cows diagnosed with metritis (n = 16) and cows diagnosed as healthy (n = 19).

DISCUSSION

Our group has recently developed a novel metritis challenge model in post-partum multiparous dairy cows using intrauterine inoculations of bacterial cocktails containing either 10^6 cfu or 10^9 cfu of *E. coli*, *T. pyogenes* and *F. necrophorum*. The model successfully induced metritis in 83% of cows receiving the lower dose, 10^6 cfu, of each bacterial species. Moreover, the same challenge group presented a decrease in dry matter intake, lower milk production, and an increased level of systemic inflammatory biomarkers (JDS to provide reference). As the uterus of primiparous cows is smaller when compared to multiparous, in the present study we used the same combination of microorganisms (*E. coli*, *T. pyogenes* and *F. necrophorum*) in two different doses,10^3 and 10^6 cfu, administered intrauterine right after parturition. We did not observe a

significant increase in the incidence of metritis among groups, but cows challenged with 10^6 cfu (high-dose group) of inoculum had a higher hazard of being diagnosed with metritis when compared with controls, and presented clinical signs as reduced milk production, and marginal reduction in dry matter intake.

The concept about resilient cows can support the findings in this study, helping to understand why we saw interesting results in our in vivo model of metritis in multiparous cows (JDS to provide reference) that were not reproduced here. According to Sheldon et al. (2020) although most cows were exposed to pathogens during parturition, those who were able to employ strategies as avoidance, tolerance, and resistance, become resilient animals and remain healthy. According to the author, avoidance is the result of an intrinsic behavior, with cows naturally avoiding pathogens, reducing the risk of diseases; tolerance is the ability of cows to limit the damage caused by bacteria; and resistance is a mechanism that limits infection by pathogenic bacteria, activating the innate and adaptative immune system. Based on results observed in this report, we suggest that primiparous cows are tolerant to pathogenic bacteria. For instance, we expected activation of the immune system with the administration of challenge to post-partum cows, increasing serum levels of chemokines, including IL-8 and pro-inflammatory cytokines as TNF-α, IL-6 and IL-1 (Chapwanya et al., 2009, Galvao et al., 2011). Intriguingly, our findings seem to contradict this theory, as we were able to measure serum levels of only IL-6 and IL-8, observing that the challenge administration did not affect those parameters. Based on this, we hypothesize that primiparous cows are somehow able to control the invasion of microorganisms without activation of the immune system. In agreement with Sheldon et al. (2020) theory, we consider primiparous cows tolerant animals.

During the post-partum period, cows usually experience a negative energy balance (NEB) as feed intake is not sufficient to meet the demands for growth, maintenance, and milk production (Butler et al., 1981, Doepel et al., 2002). During NEB, polymorphonuclear (PMN) activity is

impaired, as their phagocytic and killing capacity are reduced (Gilbert et al., 1993, Cai et al., 1994), favoring the development of diseases such as metritis. Primiparous cows experience a less pronounced NEB (Wathes et al., 2007), and a less severe post-partum neutrophil impairment (Gilbert et al., 1993). In agreement with this, we observed that the group challenged with the higher dose of the bacterial cocktail had increased incidence of disease, but when compared to controls, this difference was not significant, suggesting that the innate immune system in primiparous cows is more prompt to combat invading microorganisms and preventing disease.

Although social behavior or stressor levels were not part of the aims of this study, we believe that stress can be a factor associated with the development of metritis (Nordlund, 2009) and that would provide an explanation for the similar incidence of disease demonstrated by challenged and control cows. The evidence for this contention is mixed: regrouping cows does not affect incidence of retained placenta or metritis (Silva et al., 2013), but others have defined regrouping as a stress factor that negatively affects the interaction among cows, causing alteration in behavior and competition (Schirmann et al., 2011, Crossley et al., 2017). These behaviors result in decrease in feed time, and consequently feed intake and rumination time, later causing decrease in milk production, and increasing NEB, increasing chances for the development of diseases (Schirmann et al., 2011, Crossley et al., 2017). In the present study, all enrolled cows were purchased from the same commercial farm, being raised in pens according to age. After parturition, these cows were transported to the farm where the study was conducted, being housed in the tie-stall barn as a group, not interacting with other animals from that farm. We therefore consider that the stress of regrouping was reduced. Also, as the farm where the study was conducted was a small teaching facility, the farm provided more comfort for those cows when compared to a commercial environment. Taking all this in consideration, we hypothesize that as stress was reduced, enrolled cows were more effectively tolerating the pathogenic bacteria.

An interesting point of discussion in this study is the importance of genomic traits to predict

the development of diseases as metritis, as demonstrated in a recent study (McNeel et al., 2016). In that study, cows were ranked in quartiles based on the standardized transmitting ability to develop a disease. Referring to metritis, animals from the bottom 25 percentile had an incidence of metritis of 23.64 % whereas animals from the top 25 percentile had an incidence of metritis of 12.86 %. These results highlight the role of genetics in predisposing an animal to develop a disease, in this example, clinical metritis. Although no significant differences were detected in genomic traits between treatment groups in the present study, we observed that challenged cows had numerically higher genomic traits associated with uterine diseases (Z_MET, $P = 0.24$; Z_RP, $P = 0.09$) when compared to control cows. Thus, it is possible that control cows were more susceptible to clinical metritis when compared with low and high dose groups. Moreover, the DWP index was almost 1.5 times higher ($P = 0.10$) in high-dose animals when compared with control and low-dose cows. The DWP index combines all wellness Zoetis traits, as fertility, longevity, and calving ability. Therefore, it is possible that high-dose animals could have been more resistant to develop postpartum diseases, such as clinical metritis. Taken together, although significant differences on health genomic traits were not observed among treatment groups, these results demonstrate the importance of using genomic testing for *in vivo* studies, as the genomic potential of study animals to develop a disease could have affected the outcome of the study, in this case clinical metritis.

Although the present study was carefully designed to control for confounders, we are aware that our research has limitations and flaws that deserve a detailed discussion. We associated the data obtained for bacterial growth with the data obtained by 16S rRNA gene sequencing to describe the dynamics of microorganisms from challenge to the development of metritis. We observed a 1.5-fold difference ($P = 0.06$) on *Fusobacterium necrophorum* on selective media (LKV agar plates) for the high-dose group compared to control cows, but we did not observe an effect of challenge when analyzing relative abundance of the genus *Fusobacterium* with the 16S rRNA

sequencing. A possible explanation for this is that, although the medium used for culture was selective for *Fusobacterium necrophorum*, other microorganisms such as *Bacteroides* and *Prevotella* also grew on this medium. It is therefore possible that the information obtained from bacterial culture on selective medium was not accurate.

Another possible flaw of this study is that we used lower numbers of bacteria than did our previous model with multiparous cows (JDS to provide reference), which used 10^6 and 10^9 cfu of the same bacterial cocktail. Considering that the uterus of primiparous is smaller when compared to multiparous cows, and the fact that 10^6 cfu was a successful dose for induction of metritis in multiparous cows, we designed the present study to use two different challenge doses: 10^6 cfu as the higher dose and 10^3 cfu as the lower dose. It may therefore be necessary to use a higher dose, such as 10^9 cfu, to overwhelm the immune system of these cows and cause disease.

Another limiting factor was the group size. The study design was based on the induction of metritis in multiparous cows, with three groups of 12 cows each, in which two were challenged with the bacterial cocktail, producing a significant difference in metritis induction when compared to controls (JDS to provide reference). Unfortunately, this study design lacked the power to detect differences between the high-dose group and control group; a total of 23 cows would be necessary per group to generate a significant difference based on the incidence of metritis observed in control cows. Such a study with more animals is, however, limited by economic resources and the detrimental effect on animal welfare.

With the present report we propose a change in the way that analysis of microbiome data is performed. With our model of metritis induction in multiparous cows (unpublished data), we observed that the genus Fusobacterium was the most relevant for the development of metritis. We did not, however, see differences in this genus among groups in primiparous cows. From this finding, we speculate that the growth dynamics of microorganisms for the development of metritis in primiparous cows are different than those observed in multiparous cows. Reports that discuss

the microbiome in metritic uterus do no stratify or compare primiparous and multiparous cows, as the focus is on the comparison of microorganisms present in the uterus of metritic and non-metritic cows (Jeon et al., 2017, Moore et al., 2017, Jeon et al., 2021). We therefore suggest that further research could focus on the dynamics of microorganism growth for the development of metritis, stratifying primiparous and multiparous cows. We also propose that further research should be conducted to better understand the differences in metritis induction between primiparous and multiparous cows, and to better understand the dynamics of microorganisms, the environmental and physiological factors that can be associated with the development of the disease in cows with differing numbers of parturitions.

CONCLUSION

We observed that a model that successfully induced metritis in multiparous Holstein cows did not significantly induce metritis in primiparous cows but, when accounting for time, increased the hazard ratio of metritis induction to 2.7 when comparing cows challenged with the inoculum containing 10^6 cfu of *E. coli*, *T. pyogenes* and *F. necrophorum* (high-dose group) to control cows that received an intrauterine infusion of sterile saline solution. We observed that the model marginally altered the DMI and significantly affected the milk production in cows enrolled in the high-dose group, whereas we did not observe differences in rectal temperature, plasma cytokines levels, blood metabolites or hepatic enzymes. Further research needs to be conducted to elucidate the difference in metritis induction in multiparous and primiparous cows.

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CHAPTER FOUR: Exploratory study of the effect of the rbIL-8 treatment in multiple lactations on markers of physiologic status and uterine microbiome.

ABSTRACT

During the lactation of dairy cows, the transition period is crucial for their health, productivity, and profitability. Health disorders occur most often during this period, as a consequence of reduced activity of the immune system. The first defense against pathogens is the neutrophil, which is attracted to the site of infection by interleukin 8, a chemokine that attracts and activates neutrophils to phagocytize and kill pathogens. Many studies have shown that neutrophil activity is reduced during the transition period, and that metritis and retained placenta are common diseases associated with it. Our group has produced bovine interleukin-8 recombinantly, characterized its activity, and administered it to postpartum cows to improve overall health and consequently increase milk production. Based on those results, we aimed to determine if a subsequent administration in cows that had received the same treatment two lactations before would have any effect in rectal temperature, liver enzymes levels, milk production or uterine microbiome. We therefore performed a convenience sampling using cows that were enrolled in a previous study and remained in the herd; 20 cows that had previously received intrauterine recombinant bovine IL-8 **(rbIL-8)** and 20 cows that had received a placebo were given these same intrauterine treatments. Cows were intrauterine infused with the treatment withing 18 hours after parturition and followed for 28 days. At timepoints throughout the study, we evaluated rectal temperature and alteration in serum metabolites levels, as a measure of immune system activation; milk production; and the uterine microbiome. The second administration of rbIL-8 did not induce an immune response sufficient to alter the rectal temperature, increase levels of acute phase proteins or liver enzymes. Although milk production was not significantly increased, we observed an increase of 2 kg/week in cows infused with rbIL-8. The uterine microbiome was altered, with cows treated with rbIL-8 having a lower bacterial load, and lower numbers of the genus *Fusobacterium*, the genus associated with metritis development. These findings thus demonstrate that a second infusion of rbil-8 did not elicit an immune response and modulated the uterine

microbiome, controlling the overgrowth of pathogenic microorganisms and therefore reducing the bacterial load.

Keywords: rbIL-8, metritis, microbiome, chemokine, recombinant protein.

INTRODUCTION

The transition period, which extends from 3 weeks before until 3 weeks after parturition, is very important to dairy production, as most health disorders happens during this time and consequently define the cow's profitability during the lactation (Drackley, 1999). One of the challenges that dairy cows experience after parturition is a decline in immune activity, specifically a reduction in neutrophil phagocytic and killing activity. A cow that fails to maintain a strong immune system will not be able to control the overwhelming invasion of infectious microorganisms and therefore develop infectious diseases (Goff, 2008).

Neutrophils are the first line of defense in the uterus, migrating from the blood stream to the site of infection, where they ingest pathogens, and release enzymes and free radical components to eliminate it (Goff, 2008). Neutrophils in the blood are attracted by interleukin 8 (**IL-8**), a chemokine secreted by monocytes, activated neutrophils, endothelial and epithelial cells (Baggiolini et al., 1989, Caswell et al., 1999). Many studies reported reduction in neutrophil activity after parturition, including decreases in chemotaxis, phagocytosis, and killing ability in high-producing dairy cows, particularly in those that develop uterine diseases (Kimura et al., 2002, Hammon et al., 2006, Goff, 2008). Among the factors that contribute to the decrease in neutrophil activity are the elevation of blood estradiol and cortisol near the time of parturition, and deficiencies in vitamin A, E, calcium, and selenium (Goff and Horst, 1997, Kimura et al., 2002, Hammon et al., 2006).

Cows affected with retained fetal membranes have decreased neutrophil chemotactic activity, impaired myeloperoxidase activity and low plasma levels of IL-8 (Kimura et al., 2002). Hammon et al. (2006) showed that cows with metritis have neutrophils less able to kill bacteria on the day of calving. On the other hand, cows with an early influx of neutrophils and enhanced neutrophil activity have reduced incidence of uterine diseases and better clearance of mastitis pathogens (Paape et al., 2002).

Given the importance of proper secretion of IL-8 and the necessity of a responsive and active immune system, especially through the activity of neutrophils, our group developed a recombinant bovine interleukin 8 (**rbIL-8**) molecule with the objective of improving uterine health (Bicalho et al., 2019). Extensive work was performed to assess the biological activity and safety of the molecule, followed by field trials to evaluate its effect on prevention of uterine diseases, overall health, milk production and metabolism (Zinicola et al., 2019b). We observed a reduction in the incidence of puerperal metritis in multiparous cows, improved overall postpartum health, increased milk yield, fat-corrected milk, and energy corrected milk in the long term, and increased dry matter intake (**DMI**) during the first 4 weeks post-partum (Zinicola et al., 2019a, Zinicola et al., 2019b, Zinicola et al., 2019c).

As the protein administered to enrolled cows is recombinant and produced in *Escherichia coli* cells, there is a concern that multiple administrations could induce an immune response that trigger anaphylaxis and autoimmunity to the endogenous form of the protein. Therefore, this descriptive study assessed whether the subsequent administration of this molecule would have effects on rectal temperature, acute phase proteins or liver enzymes, as markers of immune activation; and assess the effects on milk production and uterine microbiome.

MATERIALS AND METHODS

Ethics Statement

The research protocol was reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (2016-0065). All procedures carried out with cows were in accordance with approved protocols.

Animals, Facilities, Management

This study was conducted between August 2019 and February 2020, at the Cornell University Ruminant Center (CURC) located in Harford, NY. The farm is structured to house approximately 600 lactating cows in naturally ventilated free stall barns with concrete flooring, deep sanding bedding, self-locking head gates and sprinklers over the feeding line. Water and feed were offered ad libitum, with feed delivered once daily. Cows were milked three times daily at approximately 8 hours intervals. Milk yield was recorded at every milking using individual milking stall milk meters and transferred to the dairy herd management software (Dairy Comp 305, Valley Ag Software, Tulare, CA).

Experimental Design

Multiparous Holstein cows (n = 40) enrolled in this study were a convenience sample selected from a cohort of 75 cows that were still present at the farm, from a group of 140 cows that were enrolled in a previous study, that happened two lactations before, in which cows were randomly allocated to receive rbIL-8 or vehicle control. Cows enrolled in the present study had a wash out period of two full lactations before enrollment (i.e., one lactation in which they received the original treatments and a subsequent lactation). In the present study all cows were multiparous (3-6 lactations), but when enrolled in the previous study they were between 1 and 4 lactations. Thus, the cohort of cows available for enrollment represented 53.6% (75/140) of the cows enrolled in the previous experiments. Of the cows available for enrollment, 39 received rbIL-8 and 36 received the control treatment previously. The only criteria for eligibility for the current study was presence in the herd at the time that the study was planned and an expected calving date later than August 2019. In the present study, cows received the same treatment received in the previous randomized controlled experiments. Cows allocated to rbIL-8 treatments received a second

administration of rbIL-8 and cows that previously received control received a second administration of the control treatment. Cows in the control treatment group (n = 20) received a single intrauterine infusion of 1 mL of vehicle control (serial number K0519JM05) added to 250 mL of diluent (serial number K0419JM28) before administration. Cows in the rbIL-8 group (n = 20) received a single intrauterine infusion of 1 mL of rbIL-8 immunomodulator (Product Code 9250.R0, unlicensed) containing 500 μg of rbIL-8 (serial number K0519JM04) added to 250 mL of diluent (serial number K0419JM28). The treatment was intrauterine infused within no more than 18 hours after parturition in a maternity pen where cows were moved at the first signs of delivery. For treatment administration, cows were restrained in headlocks, the perineal area and vulva cleaned with a paper towel followed by disinfection with 70% ethanol. Intrauterine infusions were administered using a sterile gilt foam tip catheter (QC supply, Schuyler, NE) attached to a syringe (Air-tite products Co., Inc., Virginia Beach, VA). The catheter was introduced into the cranial vagina and manipulated through the cervix to access the uterine lumen. Three syringes of 100 mL were used for administration of treatment or control. After treatment administration cows remained locked in headlocks for at least 10 min to ensure that the infused content was not expelled out the vagina.

All cows were commingled during the entire study in free stall barns. The study was masked, with one member of the research team assigned to treatment administration and a second member assigned to perform clinical observation and sample collection throughout the study period. From 40 cows selected to be enrolled in this study, two did not complete the follow up period of 28 days after treatment administration. One cow was sold before calving due to repeated cases of lameness, whereas the other cow was euthanized on study-day 6 due to diagnosis of subcutaneous emphysema associated with metritis and dehydration.

Animal sampling

Rectal temperature was measured at the time of enrollment, 6 hours after treatment administration and on study days 1, 4, 7, 14, and 28 using a digital thermometer (GLA M750, GLA Agriculture Electronics, San Luis Obispo, CA). Blood samples for serum separation were collected on the day of enrollment, and on study days 4, 7, 14 and 28 from coccygeal vessels using 10 mL vacutainer tubes with spray-coated silica (BD Vacutainer, Franklin Lakes, NJ). Serum was obtained after sample centrifugation at 2,000 x *g* for 15 min at room temperature and frozen at -80 °C until assayed. Serum concentrations of total protein, bovine serum albumin, alkaline phosphatase **(ALP)**, gamma glutamyl transferase **(GGT)**, aspartate aminotransferase **(AST)**, alanine aminotransferase **(ALT)** were measured using an automated clinical chemistry analyzer (Daytona, Randox Laboratories Ltd., Kerneysville, WV). Uterine swabs were collected at the time of enrollment and on study-day 7. Briefly, cows were restrained on head locks and the perineal area cleaned and disinfected with 70% ethanol. A sterile swab (Equine Uterine Culture Swab Sterile 27", Performance Vet Supply, Aiken, SC) covered by a plastic sheath was introduced into the cranial vagina and manipulated trough the cervix into the uterus. Once in the uterus the sheet was ruptured, the swab exposed to the uterine lumen and retracted inside the pipet for removal from the vaginal cavity. The swab was placed in a sterile conical tube and stored in ice during transport to the laboratory, where it was transferred to 2 mL sterile microtubes and stored at -80°C until processing.

DNA extraction and microbiome

A total of 1 mL of UltraPure^{TM} distilled water (DNAse and RNAse free, Invitrogen, Grand Island, NY) was added to the conic tubes containing the uterine swabs, followed by 10 min of homogenization in vortex mixing (Fisher Scientific, Hampton, NH). Swabs were discarded and

the remaining liquid was centrifuged for 5 minutes at 15.7 × *g* in room temperature. The pellet obtained was used for DNA extraction using the DNeasy PowerFood Microbial Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Prior the DNA sequencing, a polymerase chain reactions **(PCRs)** was done using primers 515F and 806R to amplify the V4 hypervariable region of the bacterial/archaeal 16S rDNA gene; following a previously optimized method for the platform Illumina MiSeq (Caporaso et al., 2012). Amplification of extracted DNA from uterine swabs was performed using different barcodes for 16S rRNA gene PCR with 12-bp error-correcting Golay (http://www.earthmicrobiome.org). For PCR, a total of 10 µM of primer (one for each sample), EconoTaq Plus Green 1x Master Mix (Lucigen®, Middleton, WI), 10 ng–100 ng of DNA and UltraPure™ distilled water (DNAse and RNAse free, Invitrogen, Grand Island, NY) were added a final volume of 50 µL of reaction. The steps for the PCR were: initial denaturing in 94 °C for 3 min; 35 cycles of 45 s at 94 °C, 1 min at 50 °C, 90 s at 72 °C; and final elongation for 10 min at 72 °C. Samples of amplified DNA were loaded in agarose gel (1.2%, wt/vol) containing 0.5 mg/ml ethidium bromide for verification of amplicon presence. Purification was carried out in amplified DNA using the Mag-Bind® Total Pure NGS (Omega Bio-Tek Inc., Norcross, Georgia) following the manufacturer's instructions. The DNA concentration was estimated using spectrophotometric estimation (A_{260} of 1.0 = 50µg/ml pure dsDNA). Dilution was carried in the quantified DNA to standardize it in similar concentrations and then pooled to prepare the library, for sequencing with the MiSeq platform (Illumina Inc., San Diego, CA) using the Reagent MiSeq V2 300 cycles. The PCR, purification and pooling were automated using the OT-2 robot pipetting (Opentrons, New York, NY).

After sequencing, the MiSeq Reporter Metagenomics Workflow was extracted to generate the Operational Taxonomic Unit **(OTU)** tables based on the Greengenes database (http://greengenes.lbl.gov/). The output used from this workflow has multiple levels for classification of reads (phylum, genus, and species).

Extracted DNA was also used to determine the number of 16S gene copies by real-time PCR, following the method previously described (Nonnenmacher et al., 2004, Boutin et al., 2015). Briefly, the PCR reaction was composed of 1x Power Sybr Green PCR Master Mix (Thermo Fisher Scientific, Applied Biosystems™), 300 nM of primer (forward 5′ TGG AGC ATG TGG TTT AAT TCG A 3′ and reverse 5′ TGC GGG ACT TAA CCC AAC A 3′) and 5 pg-50 ng of genomic DNA (or plasmid DNA standards), and UltraPure™ distilled water (DNAse and RNAse free, Invitrogen, Grand Island, NY) to bring the final reaction volume to 10 μL. Thermal cycling parameters applied followed the PCR master mix manufacturer's instructions. The quantitative reactions were carried using QuantStudio™ 5 real-time PCR System 384 wells (Thermo Fisher Scientific, Applied Biosystems™).

The data obtained with the real-time PCR was combined with the 16S rDNA sequencing for determination of genus-specific bacterial absolute abundance, according to calculation previously described by Bicalho et al. (2017).

Data Analysis

Analysis of descriptive statistics were performed using SAS (SAS Institute Inc., Cary, NC). Continuous data with repeated measurements (e.g., RT, milk production, genus relative abundance, bacterial load, and blood parameters) were analyzed with general linear mixed models using the MIXED procedure of SAS version 9.4 (SAS Institute Inc.). Residual plots were used to assess normality and homoscedasticity of residuals. Models included the fixed effects of treatment (control, rbIL-8), day after challenge, lactation number, season of parturition, sex of the calf born, and the interaction term between treatment and days after challenge. Animal was considered a random effect and a subject of the repeated measurement. Continuous data that did not follow normal distribution were log10-transformed. Based on Akaike information criterion, the covariance structure with the lowest AIC was selected for each variable, in this case the default

covariance structure of SAS (DIAG) was chosen.

For all models, variables were considered statistically significant when a *P*-value ≤ 0.05 was observed, and a tendency when the *P*-value was between 0.05 and 0.10. In all models, Fisher's Protected LSD was used for multiple comparisons such that pairwise treatment comparisons were performed only if the treatment effect or treatment by DIM effect (only for repeated measures models) was significant at 0.05 level.

RESULTS

Descriptive data

Cows enrolled in each group were not different in regards to parity (control: 3.26 ± 0.45, rbIL-8: 3.42± 0.50, $P = 0.15$), gestation length in days (control: 279.52 ± 6.96, rbIL-8: 278.6 ± 5.27, $P = 0.64$), projected milk production (M305) for the previous lactation (control: 26407.9 ± 3438.6, rbIL-8: 25768 ± 3355.8, $P = 0.56$), days open (control 80.63 ± 18.9 days, treated: 81.25 ± 71, $P = 0.91$), or days dry (control: 61.47 ± 7.03, rbIL-8: 62.75 ± 13.85, $P = 0.72$). None of the cows were assisted at calving and all calves were born alive. Out of the 19 cows infused with control, 9 calves were males and 10 females. From the 20 cows receiving rbIL-8, 11 had a female calf, 7 had a male calf, and 2 cows had twins.

Rectal temperature, liver enzymes and milk production

Administration of rbIL-8 did not affect rectal temperature ($P = 0.43$, Figure 4.1) but there was a tendency ($P = 0.09$) for an effect of days postpartum as rectal temperature declined after calving. For milk production there was no effect of treatment ($P = 0.28$, Figure 4.2) or an interaction between treatment and week ($P = 0.31$) but there was an effect of week ($P < 0.001$) as milk production increased from week 1 to 4 after parturition. Serum levels of the acute phase protein albumin ($P = 0.19$) and serum levels of total protein ($P = 0.12$) did not differ by treatment (Figure

4.3) and there was no treatment by day interaction ($P > 0.10$), but there was an effect of day ($P < 0.001$) as concentrations changed dynamically after parturition. Serum level of the liver enzymes ALT ($P = 0.56$), ALP ($P = 0.42$), AST ($P = 0.70$), and GGT ($P = 0.73$) did not differ by treatment (Figure 4.4) but there was an effect of day ($P < 0.001$) for all except ALT, as concentrations changed dynamically after parturition. There was no interaction between treatment and day for any of the enzymes ($P > 0.10$).

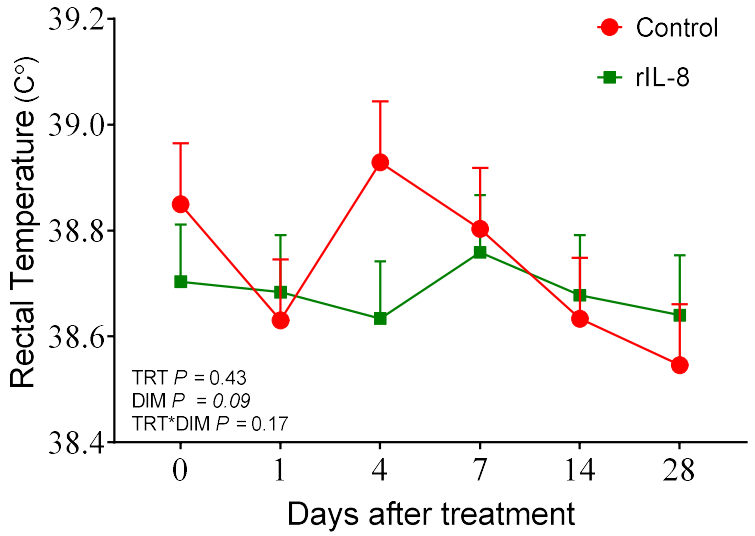

Figure 4.1. Rectal temperature at the time of intrauterine administration of 500 µg of rbIL-8 (n = 19) or control (n = 19), and study - days 0, 1, 4, 7, 14 and 28.

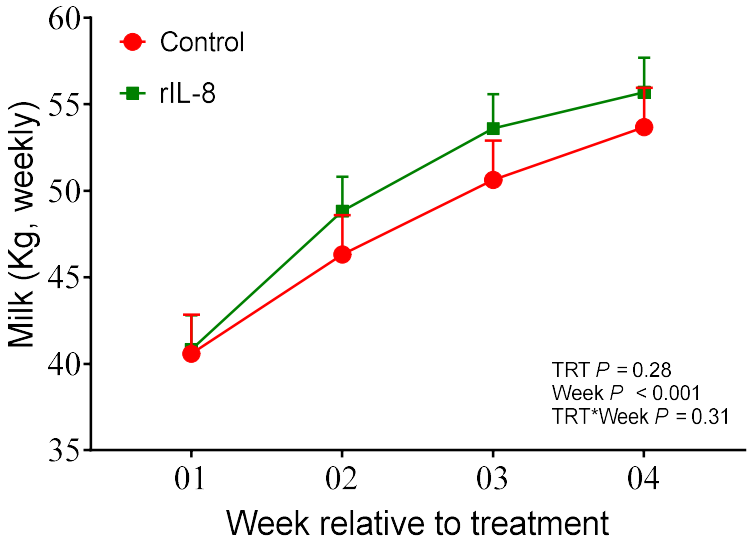

Figure 4.2. Weekly milk production (Kg) of cows receiving intrauterine administration of 500 µg of rbIL-8 (n = 19) or control (n = 19).

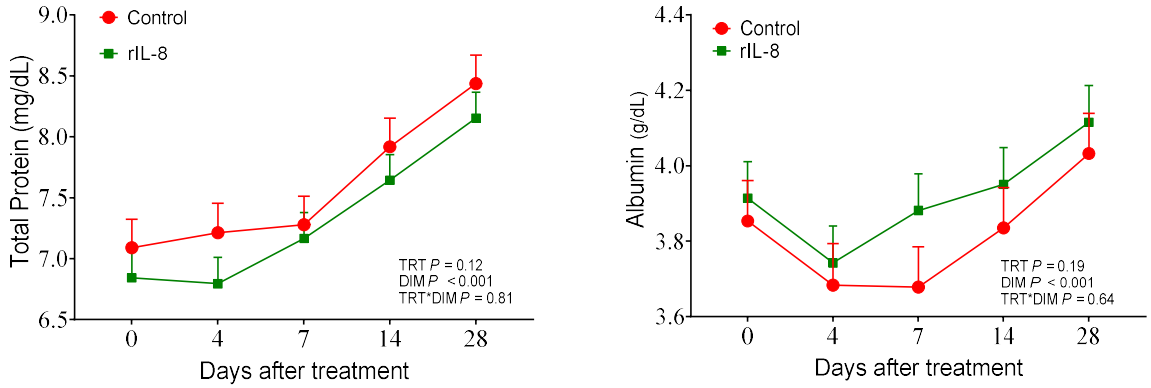

Figure 4.3. Serum levels of total protein and albumin at the time of intrauterine administration of 500 µg of rbIL-8 (n = 19) or control (n = 19), and study days 0, 4, 7, 14 and 28.

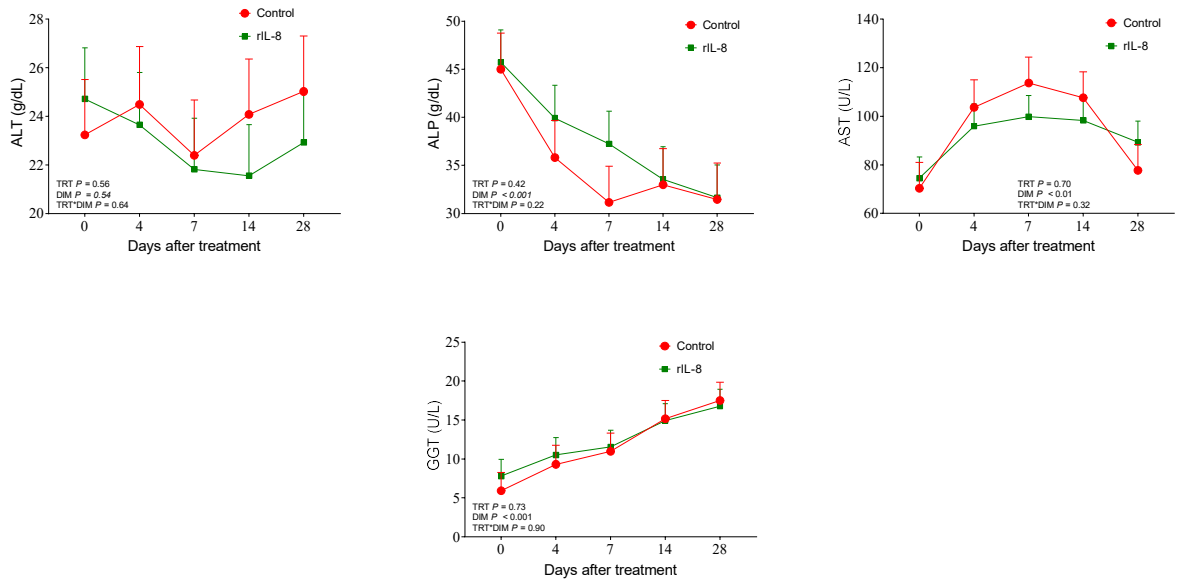

Figure 4.4. Serum levels of ALT, ALP, AST and GGT at the time of intrauterine administration of 500 µg of rbIL-8 (n = 19) or control (n = 19), and study - days 0, 1, 4, 7, 14 and 28.

Effect of treatments on the uterine microbiome

Both groups had similar bacterial load on study-day 0, but on study-day 7, control cows had a higher bacterial load ($P = 0.04$) compared with cows that received rbIL-8 infusion. Interestingly, we observed that cows receiving rbIL-8 did not have alteration in the bacteria load from day 0 to day 7, but control cows had an increase of approximately 1.5 log10 copy number showing that the effect of treatment is dependent on time ($P = 0.01$, Figure 4.5). No effect of treatment was observed on the relative abundance of the genus *Fusobacterium* ($P = 0.43$) or *Trueperella* ($P = 0.40$). In contrast, for the genus *Escherichia*, both groups had similar relative abundance at enrollment, but on study-day 7 control cows presented even lower abundance, while cows receiving rbIL-8 had no alteration in the relative abundance of the genus (Figure 4.6). There was no effect of treatment ($P = 0.81$), day ($P = 0.15$), or treatment by day interaction ($P = 0.99$) for the total number of the genus *Trueperella*. Conversely, there was a treatment by day interaction for the total numbers of the genus *Escherichia* ($P = 0.003$) and *Fusobacterium* ($P = 0.03$). For both

genus we did not observe difference among groups on study-day 0, but on study day 7, cows enrolled in the rbIL-8 group had lower total numbers of the genus *Fusobacterium* and greater total numbers of the genus *Escherichia* (Figure 4.7).

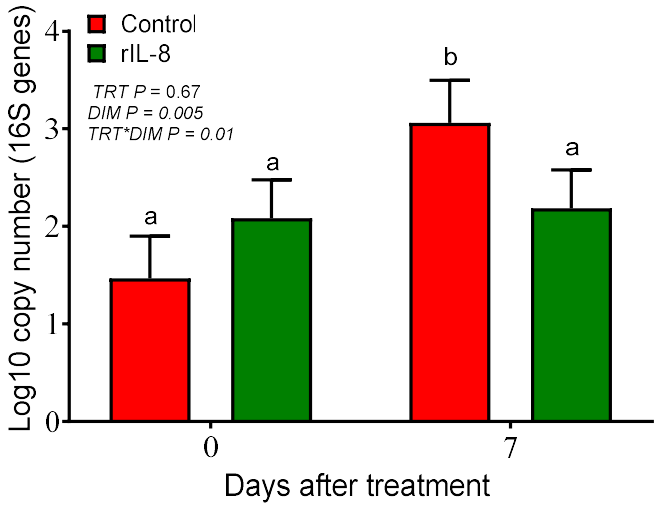

Figure 4.5. Log10 copy numbers of the gene 16S at the time of intrauterine administration of 500 µg of rbIL-8 (n = 19) or control (n = 19), and study - days 4, 7, 14 and 28.

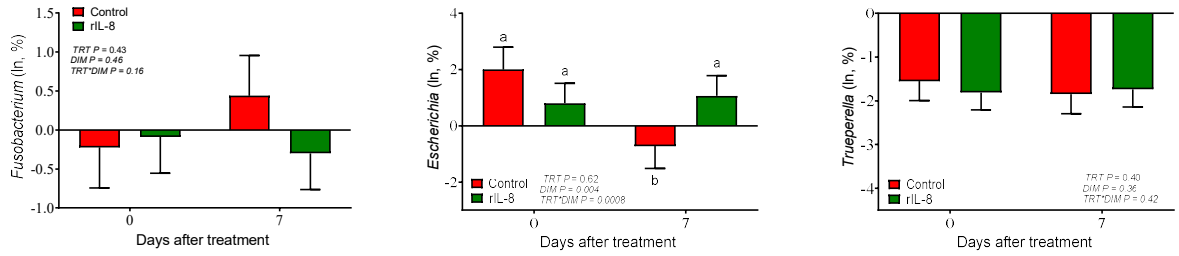

Figure 4.6. Relative abundance of the genus *Fusobacterium*, *Escherichia* and *Trueperella* at the time of intrauterine administration of 500 µg of rbIL-8 (n = 19) or control (n = 19), and study day 7.

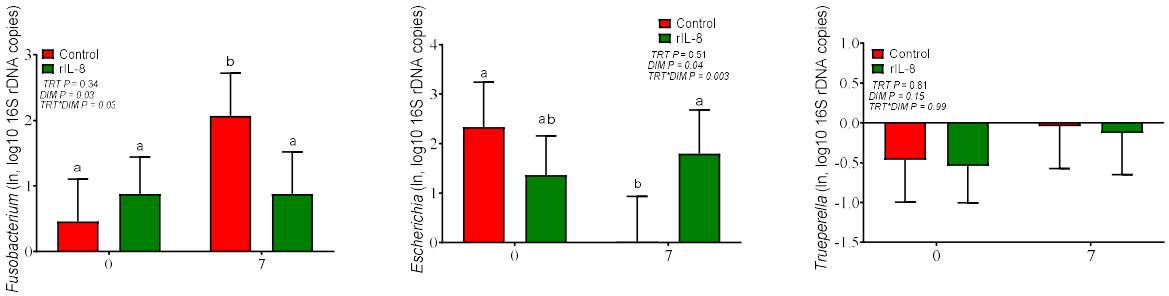

Figure 4.7. Absolute abundance of the genus *Fusobacterium*, *Escherichia* and *Trueperella* at the time of intrauterine administration of 500 µg of rbIL-8 (n = 19) or control (n = 19), and study – day 7.

DISCUSSION

In the present descriptive study, we found that cows treated with rbIL-8 did not have adverse responses to the rbIL-8, based on rectal temperature and serum levels of acute phase proteins and liver enzymes. When comparing the uterine microbiome of both groups, we found that the bacterial load in both groups was different on study day 7, with cows treated with rbIL-8 presenting lower bacterial load then control cows; and the absolute abundance of the genus *Fusobacterium* was reduced in cows receiving rbIl-8.

The rbIL-8 used in this study was produced in *Escherichia coli* host cells. *E. coli* is the first choice for production of recombinant protein to the biopharmaceutical industry, due to its advantages of easy culture, rapid expression, high yields, and low cost (Demain and Vaishnav, 2009). This organism is used for production of approximately half of all approved recombinant proteins commercially available (Ferrer-Miralles and Villaverde, 2013, Mamat et al., 2015). However, as the microorganism is Gram-negative, the outer membrane contains the endotoxin lipopolysaccharide **(LPS)**, that can contaminate the recovered protein, causing a pyrogenic response and ultimately trigger septic shock (Ding and Ho, 2001, Heumann and Roger, 2002, Mamat et al., 2015). Therefore, strict regulations are imposed for purification of commercially

available recombinant proteins to assure that the levels of LPS contamination does not exceed the limits stablished by the FDA (< 5 EU/kg) (FDA, 1985). For this study, we found that the endotoxin contamination of the recombinant protein administered to the cows was 0.2 EU/mL, thus eliminating the possibility of adverse effects due to LPS.

Besides the concerns related to LPS contamination and the effects that it can causes to the animal organism, there is a concern that multiple administrations of a recombinant protein could induce an immune response that creates anaphylaxis and autoimmunity to the endogenous form of the protein (Porter, 2001). Therefore, safety trials are important to assure that cows receiving repeated administration of the same recombinant protein do not develop an immune response to the protein. Unfortunately, in the present study we were not able to perform assays to monitor antibody production. Instead, we used rectal temperature, serum levels of the acute phase proteins albumin and levels of total protein, and serum levels of liver enzymes to evaluate if the immune system was activated due to administration of the recombinant protein. Of note, treated cows did not present fever, a response for the integrated action of physiological and neuronal signs to promote survival during activation of immune response (Leon, 2002, Evans et al., 2015).

Cows have a greater incidence of metabolic and infectious diseases immediately after calving (Drackley, 1999, Wankhade et al., 2017). The occurrence of health issues during this period has detrimental effects on productive and reproductive performance of dairy cows (Wankhade et al., 2017). Previous studies have demonstrated that cows treated with rbIl-8 had increased overall health, and multiparous cows had decreased odds for the development of metritis when compared to control cows (Zinicola et al., 2019b). Here, we were interested in exploring effects of rbIL-8 on the uterine microbiome, specially focusing on the effect of this protein in the main microorganisms associated with metritis (Galvao et al., 2019).

Metritis is caused by polymicrobial infections of the genital tract immediately after parturition (Sheldon et al., 2009, Galvao et al., 2019). Culture-dependent studies have associated

the disease with the presence of pathogenic *Trueperella pyogenes*, *Escherichia coli*, and *Fusobacterium necrophorum*, having virulence factors such as Pyolisin, type 1 pilus adhesin FimH and leukotoxin (Noakes et al., 1991, Dohmen et al., 2000, Bicalho et al., 2012b). With the development of microbiome techniques, it is now known that the uterine microbiome of healthy cows is more complex than that of metritic cows, in which a greater abundance of anaerobic bacteria belonging to the genus *Fusobacterium* and *Bacteroidetes* reduces microbial diversity (Sheldon et al., 2009, Jeon et al., 2015, Galvao et al., 2019). We also have developed an *in vivo* model of metritis induction in post-partum multiparous cows and observed that cows that developed metritis had an increased relative abundance of the genus *Fusobacterium* (unpublished data). In the present study, intrauterine infusion of rbIL-8 modulated the uterine microbiome, controlling the growth of bacteria in the uterus. This was evident based on the bacterial load in the uterus of untreated cows, that increased during the post-partum period, while the bacterial load of cows receiving rbIL-8 did not. Although we did not see significant differences in the relative abundance of genus *Escherichia*, *Trueperella* or *Fusobacterium*, when combining the data of bacterial load with 16S rDNA sequencing to obtain the absolute abundance of each genus, we observed a constant population for the genus *Fusobacterium* from study day 0 to 7 in treated cows, whereas for control cows we observed increase in the population of this microorganism. Previous reports have associated the increase in *Fusobacterium* population after parturition with the development of metritis (Jeon et al., 2015, Galvao et al., 2019), we therefore hypothesize that this growth of *Fusobacterium* in control cows would be responsible for overwhelming the immune system, leading to the development of metritis. Although this hypothesis seems plausible, more research needs to be conducted to address the mechanism of rbIL-8 to control the growth of bacteria in the uterus.

Although cows treated with rbIL-8 did not present a statistically significant increase in milk production, we observed an increase in 2 kg/week when compared to control cows. Previous

reports have also shown that cows treated with rbIL-8 also produced more milk when compared to control cows (Zinicola and Bicalho, 2019). In the preset study we enrolled 20 cows per groups, and we were not able to detect the difference in milk production between both. Despite the lack of statistical significance, an increase in milk production of 2 kg/week would be important for dairy producers and thus this finding deserves discussion. Extensive work has been done by our group to understand the effects of an intrauterine infusion of rbIL-8 on postpartum cows. Among the different effects observed, we have repeatedly observed increased milk production (Zinicola et al., 2019b, Zinicola et al., 2019c), and have postulated that this effect stems from an increase in dry matter intake (**DMI**) and overall health. Unfortunately, in this experiment we were not able to measure DMI or incidence of uterine diseases, but we would like to explore the idea that rbIL-8 treated cows had a modulation of uterine microbiome, which would reflect in reduced incidence of metritis, a disease that occurs in up to 40% of dairy cows within one week after parturition (Sheldon et al., 2009). Because cows were able to maintain a stable microbiome, without developing uterine diseases, they were able to maintain the DMI and express its capability to produce milk. Inflammatory mediators play a role in metabolism, and it is proven that the administration of recombinant bovine TNFα daily for 7 days mimics a chronic low-grade inflammatory state (Yuan et al., 2013). The group also illustrated that cows in an inflammatory state had 18% reduction in feed intake and 15% reduction in milk production (Yuan et al., 2013). Also, it has been reported that metritis, which was previously reported to have reduced incidence in cows receiving rbIL-8, decrease milk production from 2 to 13 kg/day during the period of 2 to 20 weeks post parturition (Rajala and Grohn, 1998, Dubuc et al., 2011, Wittrock et al., 2011, Zinicola et al., 2019b). Based on this, we hypothesize that cows infused with rbIL-8 had a robust response to bacteria infecting the uterus post-calving, therefore preventing the development of uterine disease. Cows were thus able to express their full capacity of milk production, without diseases that would lead to its reduction.

A limitation of this study is that we tested cows using a convenience sample. As we aimed to evaluate the effect of the administration of rbIL-8 in a subsequent lactation, and the study was conducted two lactations after the first administration, not all cows enrolled in the previous experiments remained in the herd and thus, were not eligible to be enrolled in the current study. As cows had a two lactation wash out period it is unlikely that the effects observed after the second administration of rbIL-8 were due to the prior administration. However, it would be interesting to evaluate the effects of two administrations of rbIL-8 in two subsequent lactations. Also, the inability to detected significant effects of the treatment on milk production, as had been observed in previous studies, may have been due to a lack of power, as a result of small group sizes.

CONCLUSION

We observed that the intrauterine administration of rbIL-8 modulated the uterine microbiome by reducing overgrowth of bacteria in the uterus, shown by the reduction in bacterial load when comparing control to treated cows. Although we did not see a significant effect of treatment on the relative abundance of the genus *Escherichia*, *Trueperella* or *Fusobacterium*, we observed a reduction in the absolute abundance of the genus *Fusobacterium,* a microorganism known to be associated with uterine diseases.

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CHAPTER FIVE: Future work and final remarks

The aim of the present dissertation was to present *in vivo* models for metritis induction in multiparous and primiparous cows that did not present with known risk factors for the development of the disease, via the intra-uterine infusion of a bacterial mix containing *E. coli*, *T. pyogenes* and *F. necrophorum*, isolated from the uterine content of cows previously diagnosed with metritis. The bacterial challenge was administered in two different dose combinations: 10^6 and 10^9 cfu of each microorganism for multiparous, or 10^3 and 10^6 cfu for primiparous cows. The successful models would reveal the pathogens, and the clinical and metabolic changes associates with the disease, also clarifying whether disease development would be similar in primiparous and multiparous cows. The rational for the development of *in vivo* models of metritis is that they can be useful for testing the development of preventive tools, the benefits of treatments that are already available in the market, and to reduce the number of animals enrolled in field trials to test efficacy of treatments or preventions. The last chapter of this dissertation discusses a novel strategy for prevention of metritis that will soon be available on the market.

Based on our model of metritis induction for multiparous cows, we discovered that the disease develops in a dose-dependent manner (10^6 cfu of *E. coli*, *T. pyogenes* and *F. necrophorum*) and that *F. necrophorum* plays an important role in the pathophysiology of the disease. Cows that developed the disease had an increase in rectal temperature, a decrease in dry matter intake, and trends of acute phase proteins and cytokines that are related to the clinical signs of disease. Although the study brought clarification about the disease development, some questions remained unanswered. We will discuss strategies to test two of these questions in this dissertation: (A) As *Fusobacterium* was the main microorganism associated with the development of the disease, can metritis be induced via the intrauterine administration of only *F. necrophorum*, or are the presence of *E. coli* and *T. pyogenes* necessary to make the uterine environment suitable for the colonization of *F. necrophorum*? (B) Why did the higher dose of bacterial infusion (10^9 cfu) not induce disease in challenged cows? Did the bacteria infused die or did not express virulence factors, therefore

not being pathogenic, or did a localized immune reaction kill the infused bacteria without the need of a systemic immune activation? To answer the first question a randomized clinical trial could be conducted with three different groups: (1) a group intrauterine infused with 10^6 cfu of *F. necrophorum*; (2) a group receiving 10^6 cfu of *E. coli*, *T. pyogenes* and *F. necrophorum*, and (3) a control group receiving sterile saline solution. Enrolled animals would not present risk factors for the development of metritis, and after enrollment they would be followed daily from enrolment to study day 14. During the study period, daily sample collection of blood samples for measurement of acute phase proteins and pro-inflammatory cytokines, vaginal swabs for assessment of microorganism's dynamics via 16S rDNA sequencing, measurement of rectal temperature, milk production and dry matter intake, and diagnosis of metritis with the use of the Metricheck device would be performed. This study would determine whether infusion of *F. necrophorum* alone is sufficient to produce metritis and create clinical signs of disease, also helping to determine the dynamics of microorganisms in the uterus. To address the second question, it would be interesting to conduct a clinical trial infusing cows with 10^6 cfu and 10^9 cfu of the bacterial mix, with a follow-up at 14 days. Daily collection of vaginal swabs for measurement of bacterial load and expression of virulence factors of each bacterial species infused via qPCR techniques, and collection of uterine content to analyze the presence of cells belonging to the innate immune response as a result of a localized defense would be performed. The experiment would clarify if the high concentration of microorganisms allows the bacteria to express virulence factors, and to assess whether the uterus possesses a localized immune response that neutralizes pathogens without activation of a systemic immune response.

Based on results obtained in our metritis induction model in multiparous cows, we decided to assess the effect of challenge on primiparous cows, but as the uterus of these animals is smaller and the cows were not pre-exposed to pathogens, the challenge doses were reduced. For that experiment, we used 10^6 and 10^3 cfu of each bacterial species, and, unfortunately, we were not

able to achieve a significant difference in the incidence of metritis. Although cows showed a reduction in milk production, we did not see clinical signs of disease in challenged animals. With these findings, it would be interesting to test whether a higher dose of bacterial challenge, for example 10^9 cfu, would induce disease in this animal category. This idea comes from the principle that a challenge can cause disease when it overwhelms the immune system, and based on our results, we did not see any sign of immune response in challenged cows. To test this hypothesis, a randomized clinical trial would be conducted, with cows being challenged with a high dose (10^9 cfu) and a low dose (10^6 cfu) and compared with cows infused with sterile saline solution. To assess the disease incidence and clinical signs of diseases, blood samples and vaginal swabs would be collected to measure acute phase proteins and pro-inflammatory cytokines production, measurement of DMI, rectal temperature and milk yield, and diagnosis of metritis with the use of Metricheck to assess the vaginal discharge would be useful to diagnose clinical signs of disease.

Finally, in the third chapter of this dissertation, we tested whether a novel strategy to prevent uterine diseases could be used in subsequent lactations. The results obtained revealed reproducibility of the results obtained with the first administration, but the study had a big limitation that consisted in enrolling cows two lactations after the first administration and enrolling cows via convenience sampling, as few cows enrolled in the previous trial remained in the herd. A randomized clinical trial could be used to test whether the results obtained in that study were accurate, using multiparous cows that have never received the treatment before, performing the intrauterine infusion of treatment (rbIL-8) or placebo after parturition and following cows through the lactation to assess effects on milk production and incidence of diseases. To assess the results of the treatment in a subsequent lactation, the same group of enrolled cows would be enrolled in a field trial in the next lactation, receiving the same treatment, and would be followed through the lactation to assess results of the treatment on milk production and incidence of diseases. With this experiment, we would be able to assess whether the treatment has results that are reproduced in

subsequent lactations and whether the repeated administration of this recombinant protein would create any deleterious outcomes in treated cows.

Final remarks

The findings obtained with the metritis induction models for multiparous and primiparous cows elucidate that little is known about the mechanism that cause diseases in primiparous cows, making this group different from cows with multiple parturitions. Our findings suggest that disease induction can be caused by a different combination of microorganisms, or that the factors associated with disease in this animal category can be different compared to multiparous cows. Also, our research consolidated the concept that primiparous cows are more tolerant of bacterial challenge while multiparous cows are more resistant to bacterial challenge, and reasons for this need to be further explored.