A NOVEL BIOACTIVE EXTRACT FROM *OLEA EUROPEA* PROTECTS NEWLY WEANED HEIFERS AGAINST EXPERIMENTALLY-INDUCED CHRONIC INFLAMMATION AND MODULATES RUMINAL FERMENTATION PARAMETERS IN IN VITRO INCUBATIONS

By

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

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To my life partner, Macarena
ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

ADG  Average daily gain
BW   Body weight
Ca   Calcium
CD   Clusters of differentiation
CP   Crude protein
CV   Coefficient of variation
d    Day
DM   Dry matter
DMI  Dry matter intake
EE   Ether extract
g    grams
GNB  Gram-negative bacteria
GIT  Gastrointestinal tract
h    Hours
i.v. intravenous
i.p. intraperitoneal
IL   Interleukin
kg   Kilogram
L    Liters
LPS  Lipopolysaccharide
LSM  Least squares mean
Mcal Megacalorie
mg   Milligrams
mM   Millimolar
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<tr>
<td>MP</td>
<td>Metabolizable protein</td>
</tr>
<tr>
<td>NDF</td>
<td>Neutral detergent fiber</td>
</tr>
<tr>
<td>NE</td>
<td>Net energy</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
</tr>
<tr>
<td>NEm</td>
<td>Net energy of maintenance</td>
</tr>
<tr>
<td>NF kb</td>
<td>Nuclear factor kappa beta</td>
</tr>
<tr>
<td>NRC</td>
<td>National Research Council</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OM</td>
<td>Organic matter</td>
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<tr>
<td>P-value</td>
<td>Probability value</td>
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<tr>
<td>SAA</td>
<td>Serum amyloid A</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<td>TLR-4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>TMR</td>
<td>Total mixed ration</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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Abstract of Thesis Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Master of Science

A NOVEL BIOACTIVE EXTRACT FROM OLEA EUROPEA PROTECTS NEWLY WEANED HEIFERS AGAINST EXPERIMENTALLY-INDUCED CHRONIC INFLAMMATION AND MODULATES RUMINAL FERMENTATION PARAMETERS IN IN VITRO INCUBATIONS

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Weaning is one of the most stressful periods in the life of a ruminant. There are several factors embedded within our management practices that pose a challenge to the calf gastrointestinal health. Weaning is associated with losses in body weight and voluntary feed intake. Followed weaning, transportation and commingling with other animals is also associated with transient restriction to food and water. Ultimately, upon arrival to a new operation there is a change of diet, usually composed of highly fermentable carbohydrates and low physically effective fiber that predispose the development of rumen acidosis, increase concentration of endotoxin in rumen fluid and increased permeability of the lower gut to luminal contents. Endotoxin translocation into systemic circulation can elicit immune activation shifting the metabolic priorities towards the immune system, that if sustain over time can hinder animal health and performance. It is fundamental to find ways to mitigate this condition to increase the overall efficiency of feeding, while improving animal health and wellbeing. Strategic supplementation with additives with anti-inflammatory capacity could decrease systemic inflammation,
restoring barrier function to luminal contents. Bioactive extracts from *Olea Europea* have anti-inflammatory, antioxidant and antimicrobial activity. We showed that dietary supplementation of an olive oil bioactive extract to newly weaned heifers exposed to sequential increasing doses of lipopolysaccharide contributed to reducing some of the negative effects of sustained immune activation. This was reflected on an improvement in dry matter intake, and a decrease in intravaginal temperature, inflammatory marker concentration in plasma and immune cell activation and migration.
CHAPTER 1
LITERATURE REVIEW

Introduction

Mammals have several mechanisms to maintain a tightly regulated metabolism, to ensure homeostasis. However, when an external factor disrupts that equilibrium, an imbalance of the interior milieu might occur (Zebeli and Metzler-Zebeli, 2012).

Weaning is one of the most stressful periods in the life of a ruminant. Abrupt weaning is associated with short periods of feed restriction, which results in loss of body weight (BW) 3 to 5 d after separation from their dams (Stookey and Watts, 2007). Usually, this is followed by transportation to another operation and commingling with other animals. This is characterized by a period of restricted access to food and water, followed by the introduction of the animal into a new environment, and a different diet, in most cases composed of highly fermentable carbohydrates and low physically effective fiber (Zhang et al., 2013; Gifford et al., 2015). Newly arrived calves into the feedlot, in general, consume 0.5 to 1.5% of their BW during the first week, and then intake increases to 1.5 to 2.5% of their BW in the second week, and normal intakes (2.5 to 3.5% of BW) are only restored between the second and fourth week after arrival (Hutcheson and Cole, 1986). Understandably, this generates an imbalance of the internal environment, which activates several mechanisms to try to reestablish it. Disruption of the ruminant digestive physiology is observed when it cannot cope with intakes in the high-grain diet recently introduced (Zebeli and Metzler-Zebeli, 2012). This generates a decrease in ruminal pH, which may be maintained for a prolonged time, resulting in a metabolic disorder referred to as sub-acute ruminal acidosis (SARA). The development of SARA is often a causative effect of increased permeability of the rumen
epithelia, alongside with feed restriction during transportation, and low feed intakes for the first couple of days after arriving into the feedlot. This scenario combine different challenges to the newly weaned calf, and has been described in the literature to alter intestinal barrier function, and increase translocation of endotoxin into systemic circulation which can result in the development of an inflammatory process, extensively linked in the literature with negative consequences for the host (Zhang et al., 2013; Pederzolli et al., 2018).

Development of SARA is characterized by the inability of the animal to cope with the increasing acid load generated by the consumption of highly fermentable carbohydrates. Acids are not cleared fast enough by absorption through the rumen wall, or neutralized by rumen buffers. Short chain fatty acid accumulation in the rumen leads to the development of a detrimental environment for the ruminant and with profound effects on bacterial populations, and on the absorptive capacity as well as the barrier function of the rumen epithelia (Owens et al., 1998; Penner et al., 2014; Pederzolli et al., 2018). Low pH is the major factor leading to disruption of barrier function. Short term hyperosmolarity, a consequence of the rapid fermentation of carbohydrates is also linked to an increased permeability to luminal contents (Gäbel et al., 1987; Penner et al., 2010). The introduction of young animals to high-grain (HG) diets when they arrive at the feedlot is intended to maximize the efficiency of conversion of feed to gain. However, HG diets shift microbial populations in the rumen towards more gram-negative bacteria (GNB). This increases the concentration of lipopolysaccharide (LPS) in the ruminal fluid that is shed from the cell wall of GNB. Lipopolysaccharide is a highly conserved microbial molecular pattern capable of eliciting immune activation, leading to
the induction of a local inflammatory response (Penner et al., 2011). When LPS is translocated into blood circulation, a more systemic response takes place, with the rapid increase of pro-inflammatory cytokines, and the development of an acute phase response in an attempt to clear the potential infection. This process shifts the metabolic priorities towards the immune system, with a negative effect on animal performance (Kvidera et al., 2017).

It is fundamental to find ways to mitigate the previously described scenario of multiple nutritional and environmental challenges faced by newly received feedlot cattle, to increase the overall efficiency of feeding, while improving animal health and wellbeing. One reasonable solution to tackle this problem is by decreasing systemic inflammation and therefore decreasing permeability of the vasculature and epithelial cell lining, restoring barrier function to luminal contents, and blocking the entry of antigens into systemic circulation. One possible way of achieving this goal is by strategic supplementation with additives with the ability to modulate the immune response and with anti-inflammatory capacity.

Virgin olive oil has been described to have bioactive properties such as anti-inflammatory, antioxidant and to have antimicrobial activity. These effects are attributed to the presence of bioactive molecules, mainly triterpenes, and polyphenols. They are part of the unsaponifiable fraction of virgin olive oil and are present in the skin of the fruit and leaves of *Olea europea*. The bioactivity exerted by these molecules has promising potential to be used as nutraceuticals. Dietary supplementation of an olive oil bioactive extract could contribute to reducing the negative effects of subclinical chronic inflammation on animal growth and overall performance (Liehr et al., 2017).
Lipopolysaccharide, Structure, and Recognition by the Host

The cell wall of gram-negative bacteria (GNB) is composed of two phospholipid membranes, an internal membrane, and an outer membrane. The external membrane contains a highly conserved glycolipid known as lipopolysaccharide (LPS), which has protective functions for the bacteria, acting as a defense mechanism, providing a barrier against external harmful factors (Steimle et al., 2016; Kvidera, 2017). Because LPS is a highly conserved molecule within GNB, it makes it an important pathogen-associated molecular pattern, which is recognized by the innate immune system which in turn elicits a series of rapid changes to mount an immune response and clear the invading pathogen (Steimle et al., 2016). LPS structure consist of three distinct domains, the lipid A, a phosphorylated glucosamine anchored to the bacterial outermost membrane, a core oligosaccharide portion, and an O-polysaccharide. The lipid A, sugar portion is usually acetylated, and the length and position of the acyl groups vary within species, and this is the structure recognized by the immune system (Steimle et al., 2016; Kvidera, 2017). The structure of the lipid A portion will dictate the overall toxicity of the LPS molecule.

Lipopolysaccharides are anchored to the cell surface of bacteria. In order to be recognized by the host immune system, the LPS has to be detached from the cell membrane. For the LPS to be in its free form, the cell has to die and be lysed or be detached by a host-mediated mechanism. There are soluble proteins such as LPS binding protein (LBP), that can remove LPS from bacterial cell membranes in blood, but also can be secreted into the gut lumen, acting as a scavenger of potential pathogenic bacteria and communicate it back to the gut immune system, which will act accordingly (Miyake, 2006; Steimle et al., 2016; Kvidera, 2017). Lipopolysaccharide binding protein
transports the lipid A portion, and presents it to the cluster of differentiation 14 (CD14), an LPS sensing receptor that can be membrane-bound, or secreted as a soluble protein by immune cells (Wright et al., 1990; Steimle et al., 2016). CD14 transports LPS to another receptor, MD-2, which can also be found anchored to the cell surface or free as a soluble protein. MD-2 binds to LPS, leading to the formation of a protein complex between MD-2 and the toll-like receptor 4 (TLR4), a member of the family of pathogen-sensing receptors. This event initiates the dimerization of the TLR4 receptor, which will induce the dimerization of its intracellular portion, TIR. Dimerization of the intracellular domain leads to the activation of the myeloid differentiation primary response gene 88 (MyD88), or TIR-domain-containing adapter-inducing interferon-B (TRIF) downstream signaling (Kim et al., 2007; Steimle et al., 2016; Figure 1-1). This result in the activation of the transcription factor NF-κB, by phosphorylation of the repressor subunit, liberating the transcription factor to translocate into the nucleus and bind to its promoter region. This, in turn, will lead to the activation, and transcription of an array of genes involved in the inflammatory response, such as pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and IL-1β; chemokines, and adhesion molecules like L-selectin (Zebeli and Metzler-Zebeli, 2012; Guo et al., 2017).

**Endotoxin Tolerance**

The conformation of the lipid A structure in LPS will determine its immunogenicity. Thus, the structure of the LPS, especially the lipid A motif, will dictate the strength of TLR4 signaling of host cells. For example, a lipid A that has more acyl chains will induce a greater immune response, with the hexaacylated forms being the most immunogenic (Munford and Varley, 2006; Steimle et al., 2016). The relationship between structure and affinity is due to the fact that five acyl chains are inside the
pocket of the MD-2 receptor, while the sixth chain can interact with TLR4. This sixth chain is the one that mediates the intracellular signaling of TLR4 (Park et al., 2009; Meng et al., 2010). Therefore, TLR4 signaling is usually decreased if the sixth acyl chain is missing. In order to be able to regulate the magnitude of the inflammatory response, the host relies on several mechanisms to control overexpression of the immune response, and tolerate a sustained presence of endotoxin in circulation, generating endotoxin tolerance over time. By this mechanism, the host system tries to maintain a balance between the inflammatory response, without overloading the system with inflammatory mediators, when the triggering signal persists. Acyloxyacyl hydrolase (AOAH) is an enzyme that removes acyl chains from the lipid A disaccharide backbone, and is present in antigen-presenting cells and neutrophils (Munford and Hall, 1986), therefore decreasing the overall immunogenicity of the LPS molecule, and TLR4 signaling activation.

Source of Endotoxin in Ruminants and Sites of Translocation

Cattle newly arrived at the feedlot usually undergo a period of transient low feed intake, coupled with the introduction to highly fermentable diets. This intake reduction overlapped with a rapid dietary change is often characterized by the occurrence of ruminal acidosis. Because low feed intake is accompanied by a reduction in ruminal short-chain fatty acid absorption when highly-fermentable diets are introduced, morphological and functional changes take place in order to adjust to the new acid load. These changes have negative consequences on gastrointestinal function and gut barrier function. Penner et al. (2010) described that rumen acidosis is characterized by low pH and increased luminal osmolarity generated by a rapid accumulation of organic acids that are not absorbed fast enough. Both the reduction in pH and increased
osmolarity negatively impacts the rumen epithelia. An ex vivo experiment was conducted to isolate the individual effects of each component of these series of changes. Initially, hyperosmolarity increase permeability of the ruminal epithelium, but the outcome is a sustained increase in permeability of the ruminal epithelium due to low pH in the presence of short chain fatty acids (SCFA). The authors concluded that low pH conditions coupled with hyperosmolarity generated by the presence of SCFA increase permeability across ruminal epithelia. Zhang et al. (2013) exposed animals to several levels of feed restriction. Animals exposed to the greatest feed restriction, of 25% of the ad libitum intake for 5 days, had compromised gut barrier function, which corresponded with a reduction of SCFA absorption. Inducing ruminal acidosis does not just affect rumen permeability, but it also affected more distal regions of the GI tract, such as the cecum and proximal colon (Pederzolli et al., 2018).

Highly fermentable carbohydrates in the diet, coupled with low ruminal pH, favor the development of gram-negative bacteria (GNB), which are lysed when they cannot survive the low pH, liberating large amounts of LPS (Andersen et al., 1994; Gozho et al., 2006). This highly conserved molecule from the cell wall of GNB can trigger immune activation and development of local inflammation, which further increases permeability, disruption of barrier function and translocation of LPS into circulation (Emmanuel et al., 2008; Plaizier et al., 2012).

The lower gut serves both as a nutrient absorbing tissue and prevents the entry of microbes and microbial components present in the lumen. Collectively this is one of the largest organs in the body and is in direct contact with the digesta and its natural microbiota. Under normal physiological conditions, the epithelia selectively enable the
transit of nutrients while restricting access of harmful molecules and pathogens. Understandably, any alteration on the barrier function capacity can result in increased translocation of luminal contents. These are highly conserved molecules that are recognized by the local immune cells, and elicit a quick response (Gozho et al., 2006; Plaizier et al., 2012).

When endotoxin reaches portal circulation, it is recognized by the immune system, which will respond by developing an acute phase response in an attempt to clear the recognized threat to the internal aseptic environment. This acute phase response increases the nutrient requirements, resulting in a decreased efficiency of feed utilization used by the animal, which is shift from anabolic purposes to support the immune activation (Kvidera et al., 2017).

In conclusion, these nutritional challenges related to sudden changes in diet composition, not only change the supply of nutrients but also alter ruminal pH and osmolarity, which impacts epithelial permeability in the rumen and in more distal regions in the lower gut. This is accompanied by increased proliferation of GNB, which release large amounts of LPS and other pathogen-associated molecular patterns, which can also increase permeability. Upon translocation of endotoxin into blood circulation, the immune system recognizes and triggers an immune response in a costly energetic process in detriment of animal performance.

**Inflammatory Response, and Impact of Sustained Inflammation in Animal Performance**

Inflammation is an evolutionarily conserved response underlying many physiological and pathological processes. Common livestock management practices such as weaning, transportation and the receiving phase upon feedlot entry are some of
the most stressful events in the life of beef cattle. Collectively, it has been reported that this scenario can be related to endotoxin translocation from the lumen of the gut into circulation. Feed restriction can increase gut permeability, possibly by a reduction in the amount of nutrients arriving at the gut that in turn increase endothelial cell death without rapid turnout, and internalization of tight junction proteins allowing passive diffusion of lumen contents. This condition can be aggravated if the animals experience SARA during the adaptation period in the feedlot.

Inflammation can be classified into acute and subacute. In response to acute inflammatory stimuli, the body increases the expression and release of inflammatory mediators including cytokines, chemokines, adhesion molecules, eicosanoids, and complement proteins (Newton and Dixit, 2012). These molecules form complex regulatory networks to promote increased blood flow to the infected tissue, immune cell infiltration and activation, and systemic responses, including increased body temperature, increased heart rate, and decreased appetite (Dantzer and Kelley, 2007). A dramatic elevation of inflammatory signals can induce what is known as a “cytokine storm” (Canna and Behrens, 2012) leading to tissue damage.

Conversely, subacute inflammation causes a less abrupt increase in inflammatory mediators, generating a more chronic and progressive alteration in tissue function (figure 1-2). Low-grade chronic inflammation occurs in a wide variety of diseases, including obesity and Type 2 diabetes in humans. Acute inflammation can be induced by infection and injury, generates a quick and severe increase in pro-inflammatory mediators, which typically resolves in a relatively short period of time. Subacute inflammation is associated with tissue malfunction, and a shift in nutrient
partitioning, changing the priority of nutrients, away from anabolic processes and into supporting the immune activation, and inflammatory response. These symptoms have been reported during cases of SARA (Guo et al., 2017).

Upon immune activation due to the recognition of antigenic molecules that translocate from the lumen into the interstitial space or circulation, the innate immune system initiates an inflammatory response. Pro-inflammatory cytokines like TNF-α, IL-1, and IL-6 are highly pleiotropic, decreasing circulating concentrations of Insulin-like growth factor (IGF-1), reducing hepatic cell sensitivity to growth hormone (Broussard et al., 2001; Arthington, 2012), and promoting skeletal muscle catabolism to supply amino acids and energy substrates for immune tissues (Johnson, 1997), in a process that is called the acute phase response (APR). During this early immune response, the liver changes its metabolic priorities to the production of acute phase proteins (APP) for use in host defense. Protein metabolism in the liver is modified to increase the production of APP. Production of α-1-acid glycoprotein (α-GP), C-reactive protein, fibrinogen, haptoglobin (Hp), and serum amyloid-A (SAA) are increased during the APR, whereas albumin and transferrin are decreased. These changes in protein synthesis that occur in the liver, likely modify the amino acids requirements by the host animal.

Together, these shifts in systemic metabolism are of great importance for pathogen clearance, but also may explain the detrimental effects on performance and carcass traits commonly associated with inflammatory processes in weaned calves and feedlot cattle (Gifford et al., 2015). During an APR, there is an alteration in the carbohydrates and lipids metabolism due to a hypermetabolic state which is
characterized by a hyperglycemia in the early development of the response, followed by a hypoglycemic state that can be prolonged for up to 10 hours (Werling et al., 1996).

**Bioactive Compounds Present in Olive Products**

Bioactive compounds present in *Olea europea* have been shown to have suppressive effects on inflammation, modulated at a gene expression and protein synthesis level. Additionally, studies have shown promising anti-microbial potential, providing an alternative to conventional antibiotics and coccidiostats. These effects are mainly exerted by triterpenes and phenolic compounds found in olive products. They differ in structure and synthetic pathways but share similarities in their mode of action.

**Pentacyclic Triterpenes**

Triterpenes encompass one of the largest classes of plant natural products, with more than 20,000 known molecules. In the plant, their main function is to exert protection against pathogens and pests (Thimmappa et al., 2014). They are biosynthesized from 5-carbon isoprene units joined by head-to-tail bonds, that bind to each other to form larger structures classified as hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterpenes (C25), triterpenes (C30), and tetraterpenes. Isoprene units are the building blocks for the formation of the different terpene molecules (Bramley, 1997). For the synthesis of Isoprene, mevalonic acid (MVA) formation is needed, and this process is catalyzed by the enzyme hydroxymethylglutaryl-CoA (HMG-CoA) reductase, an enzyme which has been extensively studied in animals due to its role in the regulation of cholesterol formation (Bach, 1988). In a 3-step process, MVA is converted into Isopentenyl pyrophosphate, where each step requires a phosphate group donated by an ATP. Isopentenyl pyrophosphate is the active Isoprene form (Bramley, 1997). Several IPP units come
together to form farnesyl acid, a 15-carbon compound, and then 2 farnesyl molecules bind to form squalene acid, a 30-carbon compound, which then undergoes a series of cyclizations to give rise to different triterpenic forms (Oldfield and Lin, 2012). Squalene undergoes a series of cyclizations via 2,3-oxidosqualene, a reaction catalyzed by squalene epoxidase, requiring O2 and NADPH as cofactors. An extensive series of cyclizations and rearrangements can give rise to the final products (e.g., lanosterol, β-amyring and his isomer α-amyrin). Further modifications by oxidation processes are frequently encountered, producing oleanolic acid, frequently found in olives, in which β-amyrin has been oxidized to a carboxylic acid, and the corresponding derivative of α-amyrin, ursolic acid. Oleanolic acid (OA) and its isomer, ursolic acid (UA), widely occur in nature in free acid form or as an aglycone precursor for triterpenoid saponins. These triterpenoid acids frequently occur simultaneously because they share similar structural features. These compounds have shown similar pharmacological activities, such as hepatoprotective, anti-inflammatory, antioxidant, and anticancer effects, which may be attributable to the different substructures in A, C, and E rings positions (Dewick, 2009; Jesus et al., 2015).

**Oleanolic Acid**

Oleanolic acid (OA) is a pentacyclic triterpenoid compound found in the non-glyceride fraction of olive pomace oil (3β-hydroxyolean-12-en-28-oic acid). It has widespread occurrence throughout the plant kingdom in the form of free acid or aglycones for triterpenoid saponins (Liu, 1995). It is derived from B-Amyrin, by a series of modifications on the scaffold of B-amyrin by P450 enzymes (CYP85 clan), that converts this molecule into OA (Thimmappa et al., 2014; Figure 1-3). It has been isolated from almost 2000 plant species, and the main source of this compound
includes plants belonging to the Oleaceae family, such as Olea europaea (the olive) (Liu, 1995; Fukushima et al., 2011). In plants, the biological roles of this compound are often associated with the formation of a barrier against water loss and pathogens.

This compound and its derivatives possess several interesting pharmacological activities, such as anti-inflammatory, antioxidant, and antimicrobial (Reisman et al., 2009; Jesus et al., 2015).

**Ursolic Acid**

Ursolic acid (UA) (3β-hydroxyurs-12-en-28-oic acid) is a pentacyclic triterpenoid compound that shares a common co-occurrence with OA in several plant species; however, it features a more restricted distribution when compared to OA (Liu, 1995; Yu et al., 2009). Similar to what is observed with OA, the biological role of UA in plants seems to be associated with protection against herbivores and pathogens. Different reports have shown that OA and UA exhibit antimycotic, antitumoral, antibacterial, antiviral, and anti-parasitic properties, suggesting that these compounds are important classes of prototypical natural antibiotic molecules (Jesus et al., 2015).

**Maslinic Acid**

Maslinic acid is an oleanane-type triterpenoid compound abundantly in *Olea europea*. It has been reported to attenuate intracellular oxidative stress via inhibition of nitric oxide (NO) and hydrogen peroxide (H2O2) production and reduction of pro-inflammatory cytokine generation in murine macrophages (Marquez-Martin et al., 2006).

**Phenolic Compounds: Hydroxytyrosol**

Hydroxytyrosol (HT) is an o-diphenolic compound present in virgin olive oil as a secoiridoid derivative (Reyes et al., 2013; Figure 1-4). It has reported that the byproducts from olive oil production constitute a major source of hydroxytyrosol
It is described in the literature as an antioxidant and anti-inflammatory molecule. The mechanisms of action through which hydroxytyrosol exerts its effects have yet to be fully elucidated. This compound affects the expression of various components of the inflammatory response, possibly through the modulation of the nuclear factor-kappa β (NF-κβ) pathway (Richard et al., 2011). Hydroxytyrosol has been shown to effectively decrease the production of pro-inflammatory cytokines, such as the inhibition of interleukin-1alpha (IL-1α), IL-1β, IL-6, IL-12, and tumor necrosis factor-alpha (TNF-α), chemokines (CXCL10/IP-10, CCL2/MCP-1), and reduced the expression of inducible nitric oxide synthase (iNOS), and prostaglandin E2 synthase (PGES) (Richard et al., 2011). Overall the anti-inflammatory and antioxidant modes of action are related to directly interfering with the signaling molecules involved in the transcription of pro-inflammatory molecules, rather than radical scavenging, as shown in a study conducted by Wu et al. (2017). Hydroxytyrosol decreased the production of pro-inflammatory cytokines, as well as its expression, and changed the phosphorylation of pathway intermediates of the NF-κB pathway in an in vivo mice model and in an in vitro mammary epithelial cell culture.

**Antibacterial Effects of Olive Oil Bioactive Extracts**

**Antibacterial Properties of Oleanolic and Ursolic Acid**

Different studies have demonstrated the antibacterial, and anti-parasitic properties of OA and UA in vitro (Jiménez-Arellanes et al., 2007; Passero et al., 2011; Zhou et al., 2012), suggesting that these compounds have promising potential as natural antibiotic molecules (Jesus et al., 2015). Both of these triterpenes inhibit the synthesis of insoluble glucan, catalyzed by crude glucosyltransferase (GTase) from cariogenic Streptococcus mutans (Kozai et al., 1987; Jesus et al., 2015). In vitro studies
conducted with E. coli demonstrated that OA can moderately affect the efflux of pumps, posing this mechanism as one of the potential modes of actions by which this molecule exerts its antimicrobial properties (Martins et al., 2011). In another study, both OA and UA inhibited peptidoglycan turnover in Listeria monocytogenes, affecting the amount of muropeptides and, ultimately, the integrity of the cellular wall of bacteria (Kurek et al., 2010).

The Antiprotozoal Properties of OA and UA

Oleanolic acid and ursolic acid have been shown to display anti-parasitic effects against different parasite strains. Plasmodium, the etiological agent responsible for malaria, has generated resistance against some traditional drugs; and in this regard, the search for novel antimalarial compounds has driven the finding that OA and UA can be effective anti-parasitic agents (Jesus, 2015).

Triterpenes have been proved effective against antibiotic-resistant strains of Plasmodium falciparum (Steele et al., 1999). In a study conducted by Jesus et al., (2015), they demonstrated through ultrastructural studies, that Leishmania amazonensis promastigote forms treated with UA presented irreversible morphological changes after 18 hours of incubation. Control parasites presented with normal membrane morphology, cytoplasm, nucleus, mitochondrion, and flagellum, whereas treated parasites presented with rounded-shape morphology, and the intracellular environment presented with vacuoles, suggesting organelle degradation. The group concluded that UA induced apoptosis on the parasitic strain in the study.
Olive Oil Bioactive Extracts, Anti-Inflammatory Effects, and Protection of Epithelial Tissues

Anti-inflammatory Effects of OBE

Hydroxytyrosol, a phenolic compound present in olive products, has anti-inflammatory effects, decreasing inflammatory mediators by suppressing the signaling of NF-κB. In a study conducted by Zhang et al. (2009) with human monocyte cells (THP-1), the authors were able to show that HT reduced the secretion of nitric oxide from cells exposed to LPS, and decreased the expression of iNOS and COX-2 a key enzyme required for the formation of prostaglandin E2 from arachidonic acid. This was further confirmed by Richard et al. (2011) when they stimulated murine macrophages with LPS and with or without HT. They found that HT decreased pro-inflammatory cytokines secretion, and inhibited the production of prostaglandin E2 (PGE2) and nitric oxide. Furthermore, it has been recently shown that HT protected against tissue injury in a mastitis model performed in mice, and that HT suppressed the activity of myeloperoxidase, which relates to a reduction of neutrophil infiltration, and a decrease in extravasation of immune cells from circulation into the inflicted tissue (Wu et al., 2017).

Lee et al. (2013) investigated the possible anti-inflammatory effect of OA. Oleanolic acid is one of the main triterpenic molecules present in OBE. For this study, they used a mice model and human umbilical vein endothelial cells (HUVECs) challenged with LPS and reported changes in the associated signaling pathways. Lee et al. (2013) showed that OA had the ability to down-regulate the inflammatory reaction triggered by LPS. Oleanolic acid suppressed the LPS-mediated inflammatory response in vitro and decreased the acetic acid-induced vascular permeability in the in vivo
model. In the HUVECs model, LPS induced transcription of some inflammatory genes (VCAM-1, IVCAM-1) and the adhesion molecule E-selectin. Oleanolic acid impaired both the adhesion of monocytic cells and leukocytes to the LPS activated endothelium, and also inhibited migration though the endothelium. Oleanolic acid inhibited the activation of NF-κB and the production of TNF-α in HUVECs. From the study by Lee et al. (2013), it seems that OA can significantly reduce some inflammatory pathway mediators such as NF-κB, therefore decreasing some inflammatory molecules associated with those mediators. A reduction in the overall inflammatory signal can decrease the expression of molecules involved in permeability induction and adhesion.

The main mode of action of oleanolic acid is through the nuclear stabilization of the transcription factor Nrf2, facilitating its nuclear accumulation, causing induction of Nrf2-dependent genes, which protects the liver from oxidative stress. In a study conducted by Reisman et al. (2009), it was hypothesized that OA is an activator of the Nrf2–Keap1 pathway. To test this hypothesis the authors induced hepatotoxicity with acetaminophen to Nrf2-null mice and Nrf2 in wild-type, and dosed OA (90 mg/kg, i.p.), once daily for 3 d. They observed that Nrf2 nuclear accumulation was increased in the wild-type mice, but not in the null mice. The increased nuclear accumulation derived in an increased mRNA of Nrf2, as well as Nrf2-target genes involved in oxidative stress response (Nqo1, Gclc, and Ho-1). Nrf2-null mice had enhanced susceptibility to acetaminophen. Dosing OA decreased hepatotoxicity in the wild type mice, but surprisingly, it also did, to a lesser extent in Nrf2-null mice. This indicates that there are Nrf2 independent hepatoprotective mechanisms against hepatic toxicity. The authors pointed out the possibility that OA might induce MT expression in Nrf2-null mice. MT is
a low-molecular-weight, cysteine-rich, metal-binding protein that is suggested to be capable of trapping reactive oxygen and nitrogen species, as well as electrophiles (Reisman et al., 2009; Klaassen and Reisman, 2010).

**Olive oil Bioactive Extracts as Agonists of Bile Acids**

Another possible mechanism of OA is through the activation of the bile acid receptor Tgr5. This receptor is a member of the G-protein-coupled receptors that, a cell surface receptor that when activated by secondary bile acids like lithocholic acid and Tauro-lithocholic acid, can suppress liver macrophage pro-inflammatory cytokine production (Keitel et al., 2008). It has been found that OA is an agonist of the TGR5 receptor, and can modulate similar responses (Sato et al., 2007). Induction of IL-1 and TNF-α can lead to induction of iNos (Busse and Mülsch, 1990; Kilbourn et al., 1990). iNos contributes to increased production of nitric oxide and subsequent peroxynitrite which can have hepatotoxic effects upon formation (Klaassen and Reisman, 2010). Thus, iNos suppression, possibly mediated by OA, may also contribute to the observed hepatoprotection from acetaminophen in Nrf2-null mice (Reisman et al., 2009).

Bile acids play an essential role in integrating multiple homeostatic functions in the liver and gastrointestinal tract (Cipriani et al., 2011). They are steroid molecules that, until not so long ago, were only thought as emulsifiers that aid in the formation of micelles for better lipid absorption and digestion. It has been shown that these molecules have pleiotropic effects acting through activation of a variety of specific nuclear and cell surface receptors, in an endocrine fashion (Cipriani et al., 2011).

The activation of a cell surface receptor named TGR5 is expressed in brown adipose tissue, spleen, macrophages/monocytes, gallbladder and intestine (Maruyama et al., 2002). In the small and large intestine, TGR5 has been detected in the mucosa, in
enterocytes of the crypts and villi, while in the cecum and colon the receptor is expressed in muscle layers and the mucosa (Poole et al., 2010). The activation of TGR5 by bile acids increases the intracellular concentrations of cAMP and causes the receptor internalization (Kawamata et al., 2003). In intestinal endocrine L-cells that are highly enriched in receptor expression, its activation by bile acids stimulates the secretion of glucagon-like peptide (GLP)-1 from the L cells of the enterocyte, an insulinotropic hormone that regulates insulin secretion, gastrointestinal motility, and appetite (Katsuma et al., 2005). This incretin hormone has been also found to increase under pathophysiological conditions, where gut barrier integrity is compromised and LPS is able to enter in contact with enterocytes, specifically L cells (Lebrun et al., 2017). Lebrun et al. (2017) observed that LPS can stimulate itself the secretion of GLP-1 through its interaction with the TLR4 receptor, which in turn stimulates the secretion of GLP-1 into the lumen of the gut, and into circulation. GLP-1 may act as an anti-inflammatory peptide, modulating intestinal barrier integrity, and local inflammation (Lebrun et al., 2017). L cells co-secrete GLP-1 along with GLP-2, which also promotes gut barrier function, and reduce intestinal permeability through modulation of gut barrier function (Drucker and Yusta, 2014). The production of this peptide is regulated by nutrient availability, and it can increase nutrient absorption. It has been shown that GLP-2 stimulates cell proliferation of the intestinal epithelium (Cani et al., 2013), increases tight junction proteins expression, regulates innate immune cells by controlling the expression of antimicrobial peptides produced by Paneth cells (Cani et al., 2013). Antimicrobial peptides have an intricate function of regulating the host microbiota and therefore maintaining gut barrier functions (Lee et al., 2012).
The TGR5 receptor is also expressed in monocytes, peripheral blood derived macrophages and liver macrophages, modulating the immune function of this innate immune cells (Fiorucci et al., 2010). The activation of the receptor can reduce effector functions, reduce LPS mediated immune cell activation and consequent decrease the production of pro-inflammatory cytokines (TNF-α, IL-1α, IL-1β, IL-6), and decreased macrophage phagocytic activity (Fiorucci et al., 2010).

Several compounds of the oleanane triterpenic family have been described as an agonist of bile acid and can interact with a similar affinity with its receptors, both nuclear and cell surface receptors. One of the most prominent and most studied ones is OA. In a study conducted by Cipriani et al. (2011) it was demonstrated that OA is an agonist of TGR5 and aids in the reduction of epithelial damage in the colon. Using null TGR5 mice and wild-type counterparts, that were experimentally induced to colitis, and administered with OA orally at a dose of 0.1, 1, and 10 mg/kg/day. OA decreased the damage of barrier function on wild type mice but not in null mice, and reduced IL-10, TNF-α, and IL-6. This suggests that the anti-inflammatory effect of OA were exerted through activation of the TGR5 receptor, confirming that this compound is a bile acid agonist, and its protective effect is not only carried out by interaction with nuclear and cytoplasmic transcription factors but also through specific modulation of cell surface receptors. Which are highly expressed in L cells of the enterocytes and in monocytes and macrophages.

**Olive Oil Bioactive Extracts Isolation**

Oleanolic acid along with hydroxytyrosol and maslinic acid are the bioactive molecules present in OBE and are isolated from pomace oil, which is the residue left after olive oil extrusion. After centrifugation and separation of solids from liquid fractions,
these compounds can be isolated with purified ethanol from the solid fraction (CITA). After that, the compounds can be quantified through HPLC by dissolving them in methanol, and knowing the concentration of each compound, specific formulations can be made.

**Olive Oil Bioactive Compounds Supplementation to Young Farm Animals**

In a study conducted by Liehr et al. (2017) weaned piglets were induced to subclinical chronic inflammation to model a scenario typically presented in weaned piglets in intensive production systems. They used sequential increasing doses of LPS to generate the subclinical chronic inflammation condition and another group was also supplemented with olive oil bioactive extracts (OBE) at 0.05% of the diet. The OBE contained 10% maslinic acid, 2% hydroxytyrosol, and 4% oleanolic acid. They observed that the animals exposed to LPS and supplemented with OBE had a decrease in inflammatory markers such as IL-1β, and pigMAP (a common APP measured in pigs). They also showed a tendency for better feed intake and better body weight gain. Feeding OBE was able to increase the expression of tight junction proteins of the ileum and decreased gut permeability compared to piglets not supplemented with OBE Liehr et al. (2017).

Another study with OBE supplementation on the effects of gut permeability and animal performance was conducted by Morrison et al. (2017). In this study, the researchers supplemented OBE at 30 and at 60 mg/kg of BW. With the greatest dose of OBE, the researchers observed an increase in plasma GLP-2 concentrations, but this was not reflected in an improvement of gut barrier function. The authors attributed this to a high incidence of diarrhea on the calves during the trial that severely compromised
intestinal integrity, to the point that even if GLP-2 concentrations were elevated, it did not translate in a reduction in permeability.
Figure 1-1. Molecular mechanisms of extracellular and intracellular sensing of bacterial LPS. (A) Extracellular LPS sensing. The host lipid binding protein (LBP) samples LPS from the outer membrane of Gram-negative bacteria and mediates its transport to host CD14. CD14 can either be soluble (sCD14) or anchored in the membrane of TLR4 expressing cells. CD14 shuttles LPS to the TLR4 co-receptor protein MD-2 which initiates a conformational change in MD-2 and TLR4, resulting in dimerization of the extracellular and subsequently of the intracellular TIR domains. TIR domain dimerization mediates downstream signaling leading to enhanced expression of more than 1000 different genes. Extracted from Steimle et al. (2016).
Figure 1-2. Summary of metabolic responses to exogenous inflammatory agents in lactating dairy cows. (A) Single acute inflammatory events are often resolved quickly, and there is little evidence that systemic metabolism is disrupted in these scenarios. (B) Chronic administration of very low doses of inflammatory agents can result in an anti-inflammatory response to prevent a cytokine storm and maintain homeostasis. (C) Repeated inflammatory insults, even at low doses, can alter metabolic homeostasis. Extracted from Bradford et al. (2015)
Figure 1-3. Sterol pathway and heterologous biosynthesis of oleanolic acid and its derivatives. Dashed lines indicate multiple biosynthetic steps. tHMGR = N-terminal truncated 3-hydroxy-3-methylglutaryl-CoA reductase, BAS = β-amyrin synthase, LAS = lanosterol synthase, CPR = cytochrome P450 reductase. Extracted from Pollier and Goossens (2012).
Figure 1-4. Molecular structure of HT.
CHAPTER 2
ANTIMICROBIAL EFFECTS OF BIOACTIVE COMPOUNDS FROM OLEA EUROPEA ON IN VITRO FERMENTATION

Introductory Remarks

Ruminal fermentation, among other functions, allows the ruminant host to degrade fiber and utilize nutrients and microbial protein as an energy and protein supply. This process is not 100% efficient, and energy can be lost contributing to reduced animal performance, and to the release of greenhouse gases to the environment. Scientists in the field of ruminant nutrition are continuously in the search for novel additives with the ability to modulate ruminal fermentation to increase the efficiency of substrate utilization into available sources of energy and protein for the animal, while reducing pollutant emissions (Calsamiglia et al., 2007). Changes in dietary formulation and inclusion of additives can enhance or inhibit specific bacterial populations. This is the case of ionophores, which have been extensively used to increase animal performance for the last couple of decades. The use of ionophores is facing increasing social opposition, and they have already been banned for non-medical purposes in Europe since 2006 (Directive 1831/2003/EC, 2003), although no connection has been made between the use of ionophores and antimicrobial resistance.

Several additives have been evaluated in the two decades as alternatives to modulate ruminal fermentation, including the use of tannins, essential oils, yeasts, organic acids, plant extracts, probiotics, and antibodies (Calsamiglia et al., 2006). Bioactive compounds present in *Olea europea* have shown promising anti-microbial potential, as alternative to conventional antibiotics and coccidiostats. These effects are mainly exerted by triterpenes and phenolic compounds found in olive products, which differ in structure and synthetic pathways but share similarities in their mode of action.
Triterpenes encompass one of the largest classes of plant natural products, with more than 20,000 known molecules. In the plant they exert protection against pathogens and pests (Thimmappa et al., 2014). The structural diversity of triterpenes is highly associated with its pharmacological effects. In Asian countries, triterpenes are traditionally used as antimicrobial agents (Hill and Connolly, 2017). Oleanolic acid (OA), ursolic acid (UA), and maslinic acid (MA), are the triterpenic acids with more abundance in the olive plant.

Different studies have demonstrated the antibacterial and antiparasitic properties of OA and UA in vitro (Jiménez-Arellanes et al., 2007; Passero et al., 2011; Zhou et al., 2012), suggesting that these compounds have promising potential as natural antibiotic molecules (Jesus et al., 2015). Both of these triterpenes inhibit the synthesis of insoluble glucan, catalyzed by crude glucosyltransferase (GTase) from cariogenic Streptococcus mutans (Kozai et al., 1987; Jesus et al., 2015). In vitro studies conducted with E. coli demonstrated that OA can moderately affect the efflux of pumps, posing this mechanism as one of the potential mode of actions by which this molecule exerts its antimicrobial properties (Martins et al., 2011). In another study both OA and UA inhibited peptidoglycan turnover in Listeria monocytogenes, affecting the amount of muropeptides and, ultimately, the cellular wall of bacteria (Kurek et al., 2010). We hypothesized that these compounds could have modulatory effects on ruminal fermentation parameters, reducing total gas production, changing the VFA profile, with a potentially concomitant reduction in enteric CH₄ production. The objective of this study was to determine the effects of olive oil bioactive compounds namely, oleanolic acid,
ursolic acid and malsinic acid on ruminal fermentation parameters in in vitro batch culture fermentation of bahiagrass hay and a high-grain substrate.

Materials and Methods

In vitro Batch Culture

The study was conducted as a randomized complete block design using bahiagrass hay, or a high-concentrate as basal substrate. In Exp. 1, a batch culture incubation was performed with increasing doses of three triterpene types: oleanolic acid, ursolic acid or maslinic acid, each at 0, 4, 25, 40, and 100 mg/L of incubation fluid. For Exp. 2, oleanolic acid was chosen to conduct the second series of batch cultures based on the effects shown on the previous study. It was used in two chemical forms, a sodium salt or a phytosome with the following doses: 0, 4, 40, 100, and 200 mg/L of incubation fluid. The doses selected on both batch cultures were based on previous in vitro studies were effects were reported at similar concentrations. In both experiments, the dose 0 was used as a control (CTL). Sodium monensin at 4 mg/L of incubation fluid was used as a positive control (MON). Substrates were sent to a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY) for nutrient composition analyses, which are presented in Table 1.

In vitro Incubations

Ruminal fluid was collected from four ruminally cannulated crossbred steers (512.5 ± 34.6 kg of BW). Two steers consumed either a high concentrate diet comprised of (DM basis): 43% soybean hulls pellets, 42% corn gluten feed pellets, and 15% peanut hulls, while the other two were fed a forage-based diet comprised of ad libitum amounts of bahiagrass (*Paspalum notatum* Flügge) for at least 2 wk before the collection of ruminal fluid for inoculum. A representative sample of digesta was collected
through the ruminal cannula, and ruminal fluid was strained through 4 layers of cheesecloth, placed in a pre-warmed thermos container, and transported to the laboratory within 30 min of collection. A 4:1 McDougall’s buffer:ruminal fluid mixture was utilized as inoculum for the forage incubation, and a 2:1 McDougall’s buffer:ruminal fluid mixture for the high concentrate substrate. Substrates were incubated in 125-mL serum bottles. Bottles containing their respective treatments and 50 mL of inoculum were incubated for 48 h for bahiagrass hay and for 24 h in the case of the high concentrate substrate. Bottles were maintained at 39°C under constant agitation (60 rpm). Each bottle or tube was flushed with CO$_2$ while the inoculum and treatments were added. Serum bottles were crimp-sealed with butyl rubber stoppers, and polycarbonate centrifuge tubes were stoppered with a rubber stopper fitted with a 16-gauge needle for gas release. Each treatment was dissolved in 0.2 mL DMSO in Exp. 1, and in ethanol in Exp. 2. This solution delivering the treatments was then pipetted into each of the bottles. The negative CTL received the same amount of ethanol without any treatment and blank bottles received the same volume but of distilled water. This was done to ensure that every tube/bottle received the same amount of volume (0.2 mL ethanol or DI water in addition to the 50 mL of incubation fluid), therefore every treatment had equal headspace volume. Total gas production was measured at the end of the incubation period along with VFA concentrations, NH$_3$-N concentrations, final pH, H$_2$S, and CH$_4$ production. At the end of incubation, 1 mL of a 20% H$_2$SO$_4$ solution was added to each bottle to stop fermentation. After that, a 10-mL sample was taken and frozen for subsequent VFA and NH$_3$-N analyses.
End of the Incubation

At the end of the 24-h and 48-h incubation periods, serum bottles were placed for 15 min in an ice bath to stop the fermentation and then allowed to reach room temperature for a minimum of 15 min before the beginning of the gas production measurements. The 100-mL polycarbonate tubes used for IVOMD were removed from the incubator and then a pepsin-HCl solution was added immediately to continue with the determination of IVOMD.

Total Gas, NH3-N, VFA, CH4, and H2S Production Analyses

Total gas produced was determined using a manometer. The manometer measures gas pressure in psi, and the pressure values in psi were converted mL of gas produced in 24 or 48 h. The conversion from psi to mL of total gas was calculated as follows: The 125-mL serum bottles sealed with the blue rubber stoppers had an incubation volume of 50 mL of inoculum plus 0.7 g of substrate. Total bottle volume to the bottom of the stopper is 152.5 mL. So, the volume of the headspace was 102.5 mL. The atmospheric pressure (Pa), which for our location was 14.6399, divides this value, which is then multiplied by the recorded value in psi from the manometer (Pt). Thus the formula for 125-mL serum bottles is:

\[ G \text{ (in mL)} = \left[ \frac{102.5 \text{ mL}}{(Pa)} \right] \times Pt \]

A 10mL sample of gas from the bottle headspace was collected to determine methane concentrations and analyzed by gas chromatography (Agilent 7820A GC, Agilent Technologies, Palo Alto, CA) using flame ionization and a capillary column (Plot Fused Silica 25 m × 0.32 mm, Coating Molsieve 5A, Varian CP7536). Injector, column, and detector temperatures were 80°C, 160°C, and 200°C, respectively, and N₂ was
used as the carrier gas with a flow rate of 3.3 mL/min. The split ratio for the injected CH$_4$ sample was 100:1. To determine H$_2$S production, 5 mL of gas from the serum bottle headspace was collected, and were bubbled slowly into 10-mL evacuated tubes (BD Vacutainer, Franklin Lakes, NJ) containing 5 mL of alkaline water (pH 8.5 to 9.0) prepared as described by Smith et al. (2010). The tubes were shaken vigorously to ensure proper dispersion of the gas in the alkaline water. An injection of 0.5 mL of $N$, $N$-dimethyl-$p$-phenylenediamine sulfate was made into the tubes, followed by 0.5 mL of ferric chloride. Tubes were again shaken vigorously and allowed to stand at room temperature for 30 min for the reaction to occur (Smith et al., 2010). Absorbance was read at 665 nm in flat-bottom 96-well plates using a plate reader (DU-500, Beckman Coulter Inc.). Concentrations of VFA in the inoculum samples were determined in a water-based solution using ethyl acetate extraction. Samples were centrifuged for 10 min at 10,000 × g. Inoculum supernatant was mixed with a metaphosphoric acid:crotonic acid (internal standard) solution at a 5:1 ratio, and samples were frozen overnight, thawed, and centrifuged at 4°C for 10 min at 10,000 × g. Supernatant was transferred into glass tubes and mixed with ethyl acetate in a 2:1 ratio of ethyl acetate to supernatant. After shaking tubes vigorously, the ethyl acetate fraction (top layer) was transferred to vials. Samples were analyzed by gas chromatography (Agilent 7820A GC, Agilent Technologies, Palo Alto, CA) using a flame ionization detector and a capillary column (CP-WAX 58 FFAP 25 m 0.53 mm, Varian CP7767, Varian Analytical Instruments, Walnut Creek, CA). Column temperature was maintained at 110°C, and injector and detector temperatures were 200°C and 220°C, respectively. Concentrations of NH$_3$-N were measured after centrifuging inoculum samples at 10,000 × g for 15 min
at 4°C (Avanti J-E, Beckman Coulter Inc., Palo Alto, CA) following the phenol-hypochlorite technique described by Broderick and Kang (1980) with the following modification: absorbance was read at 620 nm in flat-bottom 96-well plates using a plate reader (DU-500, Beckman Coulter Inc.).

**In Vitro Organic Matter Digestibility (IVOMD)**

For initial DM and OM of the bahiagrass hay and the high concentrate substrate, 0.5 g samples of each substrate were weighed into ceramic crucibles and dried at 100°C for 24 h. After weighing the dried samples, the crucibles were placed in an ashing oven at 550°C for 3 h. Crucibles were then placed in a 100°C forced-air oven for 24 h to determine ash content and, subsequently, OM of the samples. For the IVOMD, 0.5 g of each substrate were weighed into 100-mL centrifuge tubes along with 50 mL of the same inoculum used for in vitro incubations. Two tubes per treatment and two blank tubes (inoculum without any substrate) were incubated in each of three separate replicate days. The ruminal fluid was collected from the same cannulated steers on the same day as the in vitro gas production experiment in an attempt to prevent any differences in ruminal fluid. The tubes with sample and inoculum were purged with CO2 to ensure an anaerobic environment. Tubes were topped with a rubber stopper fitted with a Bunsen valve and placed in a forced-air oven at 39°C for 24 h for the high concentrate and for 48 h for the bahiagrass substrate. Tubes were maintained under constant agitation (60 rpm). Following the incubation time, 6 mL of 20% (vol/vol) HCl were added to each tube to decrease pH to between 1 and 2. Subsequently, 2 mL of 5% (wt/vol) pepsin (1:3,000; Amresco Inc., Solon, OH) solution were added to the tubes, which were returned to the oven for 48 h under constant agitation (60 rpm).
Samples were then filtered (Whatman No. 541 ashless; Whatman International Ltd., Maidstone, UK), rinsed with water, dried at 100 C, and then ashed to determine IVOMD.

Statistical Analyses

Total gas production, CH\textsubscript{4}, H\textsubscript{2}S, IVOMD, NH\textsubscript{3}-N, final pH, and VFA data were analyzed as a randomized complete block design using the MIXED procedure of SAS, with the fixed effect of treatment and random effect of day (block). An average of 2 bottles or tubes within day was considered the experimental unit with 3 replicates (days). In addition, for the second batch culture orthogonal contrasts were used to determine the linear effects of increasing doses of oleanolic acid on its sodium or phytosome form on in vitro fermentation parameters, and comparing both forms of oleanolic acid versus monensin, and CTL.

Results

Exp. 1

Total gas production, CH\textsubscript{4} and H\textsubscript{2}S

Increasing concentrations of the different triterpene types (TT-type; OA, MA, and UA) had no effect on total gas production ($P > 0.11$) in both the HC (Table 2), and the forage substrate (Table 2-3). Monensin inclusion at 4 mg/mL of incubation fluid decreased total gas production in the forage substrate ($P < 0.01$; Table 2-3), but not in the HC substrate ($P = 0.08$; Table 2-2). Both monensin and the different TT-types were able to decrease CH\textsubscript{4} (expressed as mmol per g of incubated substrate) in the HC substrate when compared to CTL ($P < 0.04$; Table 2-2). However, for the forage substrate (Table 2-3), only monensin decreased CH\textsubscript{4} production ($P < 0.01$). Monensin increased H\textsubscript{2}S production in the HC substrates ($P < 0.01$; Table 2-2). Increasing
concentrations of different TT-types, increased H$_2$S production ($P < 0.01$) in the HC substrate (Table 2-2), with no effect in the forage substrate ($P = 0.31$; Table 2-3).

**IVOMD**

In vitro organic matter digestibility was decreased by monensin inclusion in the fermentation fluid ($P \leq 0.05$) in both substrates (Table 2-2, and 2-3). None of the triterpene acids decreased IVOMD in the forage or HC substrate (Table 2-2, and 2-3 respectively).

**pH and ammonia-nitrogen**

No treatment affected pH or ammonia nitrogen concentration ($P > 0.58$) in the HC substrate (Table 2-2). In the forage substrate, monensin decreased NH$_3$-N concentration (Table 2-3) compared with CTL from 4.8 mM to 3.1 mM ($P < 0.01$).

**VFA concentration and profile**

The total concentration of VFA was not modified by any of the treatments ($P > 0.45$) in the incubations containing the HC (Table 2-4), or forage substrate (Table 2-5). Acetate and propionate concentrations where not modified by any of the triterpenic acids tested in the batch culture incubations ($P > 0.09$), regardless of substrate (Table 2-4, and 2-5). The acetate to propionate ratio (A:P), was decreased by MON ($P < 0.01$) in both substrates (Table 2-4, and 2-5). The different triterpenes had no effect on butyrate, branched-chain VFA (BCVFA), or valerate, in both HC (Table 2-4) and forage substrates (Table 2-5). On the contrary, MON decreased all of the later mentioned VFA ($P < 0.03$) on the forage substrate (Table 2-5).

**Exp. 2**

For exp. 2, two chemical forms of oleanolic acid were used. A sodium salt oleanolic acid (OA-NA), and a phytosome oleanolic acid (OA-PHYT). The goal of these
chemical modifications were to increase activity and solubility of the compounds in the incubation fluid.

**Total gas production, CH4 and H2S**

Monensin decreased total gas production in both HC (Table 2-6), and forage substrate \( (P < 0.01; \text{Table 2-7}) \). Increasing concentrations of both forms of OA linearly decreased total gas production in the HC substrate \( (P < 0.01; \text{Table 2-6}) \). In the forage OA-NA linearly decreased total gas production \( (P = 0.02; \text{Table 2-7}) \). In the HC substrate (Table 2-6), only monensin decreased CH4 production \( (P < 0.01) \). Both monensin and OA-NA decreased CH4 production in the forage substrate (Table 2-7) when compared to CTL \( (P \leq 0.01) \). Monensin did not affect H2S production in the HC (Table 2-6), or forage substrate (Table 2-7). On the contrary, the 4 mg/L dose of OA increased H2S production in the HC substrate \( (P < 0.05) \). Increasing production of OA-Phyt decreased linearly H2S production \( (P = 0.03) \), on the HC substrate (Table 2-6). On the forage substrate (Table 2-7), none of the treatments affected H2S production \( (P > 0.34) \).

**IVOMD**

There was a treatment by dose interaction for IVOMD \( (P < 0.05) \), for OA in Exp. 2. Increasing concentrations of OA-Phyt linearly decreased IVOMD in both the HC (Table 2-6), and forage substrate \( (P < 0.01; \text{Table 2-7}) \). The OA-NA only decreased IVOMD in the HC substrate \( (P < 0.01; \text{Table 2-6}) \). In the case of the forage substrate (Table 2-7), both OA forms linearly decreased IVOMD \( (P < 0.01) \). Monensin inclusion decreased IVOMD with both of the substrates used \( (P < 0.01; \text{Tables 2-6 and 2-7}) \).
pH

The final pH of the incubation was increased with MON inclusion in the forage diet ($P < 0.01$; Table 2-7). The OA treatment also increased the final pH ($P < 0.01$), within the forage substrate (Table 7). Increasing concentrations of OA-Phyt had a linear effect on the final pH of the incubation ($P = 0.02$) on the forage substrate (Table 2-7).

**VFA concentration and profile**

For the Exp. 2, total concentration of VFA was not modified by any of the treatments ($P > 0.51$), in both the HC (Table 2-8), and forage substrate (Table 2-9). Acetate and propionate concentrations where not modified by any of the treatments ($P > 0.16$), regardless of OA form or substrate (Table 2-8, and 2-9). Monensin decreased the A:P ratio in both substrates ($P \leq 0.01$; Table 2-8, and 2-9). Both forms of OA linearly decreased the A:P ratio in the forage substrate ($P = 0.01$; Table 2-9). Neither monensin nor OA had an effect on butyrate, branched-chain VFA (BCVFA), or isovalerate ($P > 0.19$) in both substrates (Table 2-8, and 2-9). Both OA-NA and OA-PHYT linearly decreased valerate concentrations ($P = 0.04$) in the HC substrate (Table 2-8).

**Discussion**

To the best of our knowledge, this is the first time that pentacyclic triterpenes and the sodium and phytosome chemical modifications of oleanolic acid are reported in batch culture fermentation systems. The effect of these compounds were evaluated using two different substrates, and ruminal fluid from steers consuming two different diets. A high concentrate diet and a forage diet. The purpose of this approach was to observe if the effect of these compounds was substrate dependent, and to understand and characterize its effect on ruminal fermentation parameters. In Exp. 1, generating the dilution of the pure acids was difficult given the characteristics of these molecules that
are lipophilic in nature. For this reason, we used DMSO as a solvent for the pentacyclic triterpenes in the Exp. 1. In Exp. 2 the chemical modifications of OA increased the solubility of the treatments and ethanol was used as a solvent. The abnormally great concentrations of sulfur in a seemingly low S diet in both substrates in Exp. 1 are attributed to the solvent used to solubilize the treatments before the inclusion in the inoculum (DMSO). Dimethyl sulfoxide (DMSO) is an organosulfur compound with the formula $(\text{CH}_3)_2\text{SO}$, and it has low toxicity, with a median lethal dose greater than ethanol (Wadhwani and Desai, 2009). In the forage substrate, the $\text{H}_2\text{S}$ concentrations were even greater because along with the DMSO effect, the animals were foraging a pasture recently fertilized with sulfur. To ensure that DMSO had no effect in any fermentation parameter ethanol was included as an internal control (data not shown), and compare with the DMSO control to test for potential effects of DMSO on the fermentation parameters measured. Except for $\text{H}_2\text{S}$ concentration ($P < 0.01$) due to the high levels of sulfur at the beginning of the incubation, no other effects were observed.

Total gas production has been previously reported to decrease with monensin inclusion on in vitro batch culture incubations, both with high concentrate and forage substrates. Henry et al. (2015) reported a reduction in gas production with the inclusion of 4 mg/L of incubation fluid, similar to the dose used in this study. The authors observed a reduction of the asymptotic maximal gas production in both a high-concentrate and a low-concentrate substrate. Similar to those results, we observed that monensin decreased total gas production in both experiments, and in both substrates used. As for the different pentacyclic triterpenes, in the first study no effect was observed, whereas by increasing solubility of oleanolic acid in the second experiment,
total gas production was decreased linearly by the sodium salt form of oleanolic acid with both substrates. In vitro organic matter digestibility (IVOMD) was decreased by monensin across the different experiments and regardless of substrate. However, it seemed to have a more prominent reduction in digestibility for the forage-based substrate. This is in agreement with previous studies were it has been shown that monensin has a depressive effect on digestibility due to the impact on cellulolytic bacteria, reducing the rate of digestion and the amount of product that is fermented (Bogaert et al., 1990; Anassori et al., 2012), ultimately having a greater impact in diets with greater forage content. In a study conducted by Simpson (1980), it was showed that monensin reduced total in vitro DM degradation. The antimicrobial activity of these molecules have a special toxic effect on gram-positive bacteria, which are a large pool of cellulolytic agents found in the rumen (Chen and Wolin, 1979).

The effect of triterpenes on IVOMD was similar to that on gas production. Increasing the solubility of oleanolic acid increased the depressing effect of OA on IVOMD, with a more prominent effect on the forage substrate.

It has been extensively reported that monensin inclusion consistently decreased methane concentrations both on in vitro and in vivo models (Smith et al., 2010; Ranga Niroshan Appuhamy et al., 2013). Henry et al., (2015) reported a substrate dependent effect of monensin on methane concentration. They observed no effect on a high-concentrate substrate, while on the low-concentrate substrate, monensin inclusion reduced CH$_4$ concentrations by 16% when compared with control. Similarly, Smith et al. (2010) reported a dose-dependent effect of monensin on HC substrates, where CH$_4$ production was only successfully reduced when monensin was included at 6 mg/L. In
In our study, monensin had a depressive effect on CH₄ only in the forage substrate. In addition, OA-NA was able to decrease methane concentration only in the forage substrate as well. In the case of monensin, this substrate-dependent effect can be explained by the selective effect of the ionophore on cellulolytic bacteria. Understandably, this reduction in fiber digestion is more evident with diets with more fibrous ingredients, like in the case of our forage substrate. The acetate to propionate ratio (A:P) was also reduced by monensin. This is explained by the inhibitory effect of monensin on hydrogen and ammonia producing bacteria (Callaway et al., 1997; Smith et al., 2010). Propionate captures more H₂ when a hexose molecule is fermented into this short chain volatile fatty acid. Less H₂ means less substrate available to methanogenic archea to reduce CO₂ to CH₄, ultimately decreasing methane production. In a similar fashion, the sodium form of oleanolic acid decreased the A:P ratio, which in part might help to explain the reduction in CH₄ concentration that was also observed with OA-NA inclusion with a forage substrate.

Different studies have demonstrated the antibacterial properties of OA in vitro (Jiménez-Arellanes et al., 2007; Passero et al., 2011; Zhou et al., 2012), suggesting that this compound is a natural antibiotic (Jesus et al., 2015). It has been reported that OA inhibited the synthesis of insoluble glucan of Streptococcus mutans (Kozai et al., 1987; Jesus et al., 2015). In vitro studies conducted with E. coli demonstrated that OA can moderately affect the efflux of pumps, which could directly interfere with the viability of this species (Martins et al., 2011). OA inhibited peptidoglycan turnover in Listeria monocytogenes, affecting the amount of muropeptides and, ultimately, the cellular wall of bacteria (Kurek et al., 2010).
When considering all the results from both experiment, we can conclude that OA was able to modulate some ruminal fermentation parameters in an in vitro batch culture system. Furthermore, as with monensin, the effects observed appears to be more prominent in incubations with forage-based substrate.
Table 2-1. Composition and analyzed nutrient content (DM basis) of the substrates used for in vitro incubations

<table>
<thead>
<tr>
<th>Item</th>
<th>High-concentrate&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Forage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient, % of DM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry-rolled corn</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Bahiagrass hay</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Analyzed nutrient content, % of DM&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>94.3</td>
<td></td>
</tr>
<tr>
<td>CP</td>
<td>13.36</td>
<td>9.3</td>
</tr>
<tr>
<td>Neutral detergent fiber</td>
<td>14.63</td>
<td>69.9</td>
</tr>
<tr>
<td>Acid detergent fiber</td>
<td>6.83</td>
<td>38.9</td>
</tr>
<tr>
<td>Ether extract</td>
<td>0.18</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>1</sup>All ingredients were ground to pass a 2-mm screen with the exception of urea, which was ground using a mortar and pestle.

<sup>2</sup>Dairy One Forage Laboratory, Ithaca, NY.
Table 2-2. Effects of oleanolic, malsinic and ursolic acid on batch culture in vitro ruminal fermentation using a high-concentrate substrate.

<table>
<thead>
<tr>
<th>Item</th>
<th>TT type¹</th>
<th>Dose, mg/L</th>
<th>P-value³</th>
<th>Type × dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OLE</td>
<td>MAL</td>
<td>URS</td>
<td>0</td>
</tr>
<tr>
<td>Total Gas, mL 24 h</td>
<td></td>
<td></td>
<td></td>
<td>188.0³</td>
</tr>
<tr>
<td>IVOMD⁴, %</td>
<td></td>
<td></td>
<td></td>
<td>67.0³</td>
</tr>
<tr>
<td>pH</td>
<td>6.2a</td>
<td>6.2a</td>
<td>6.2a</td>
<td>6.3a</td>
</tr>
<tr>
<td>CH₄/ g incubated</td>
<td>0.8b</td>
<td>0.9a</td>
<td>0.9a</td>
<td>1.0a</td>
</tr>
<tr>
<td>CH₄ mmol/g of DM fermented</td>
<td>1.2a</td>
<td>1.4a</td>
<td>1.3a</td>
<td>1.5a</td>
</tr>
<tr>
<td>NH₃-N, mM</td>
<td>4.0a</td>
<td>3.7a</td>
<td>3.7a</td>
<td>3.2a</td>
</tr>
<tr>
<td>H₂S µmol/g of ferm. OM</td>
<td>9.6b</td>
<td>9.4b</td>
<td>9.4b</td>
<td>8.1a</td>
</tr>
</tbody>
</table>

¹Pentacyclic triterpene type: OLE = Oleanolic acid; MAL = Malsinic acid; URS = Ursolic acid.
²MON = monensin sodium at 4 mg/L of incubation fluid.
³P-values for the effect of pentacyclic triterpene (TT) type, dose and their interaction when analyzed as a 5 × 3 factorial arrangement (excluding the effect of monensin).
⁴IVOMD = In vitro organic matter digestibility.
Table 2-3. Effects of oleanolic, malsinic and ursolic acid on batch culture in vitro ruminal fermentation using a bahiagrass substrate.

<table>
<thead>
<tr>
<th>Item</th>
<th>TT type</th>
<th>Dose, mg/L</th>
<th>P-value&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Type × dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OLE</td>
<td>MAL</td>
<td>URS</td>
<td>Trt</td>
</tr>
<tr>
<td>Total gas, mL 48 h</td>
<td>157.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>164.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>162.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>177.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IVOMD&lt;sup&gt;4&lt;/sup&gt;, %</td>
<td>54.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pH</td>
<td>6.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CH&lt;sub&gt;4&lt;/sub&gt;/ g incubated</td>
<td>1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CH&lt;sub&gt;4&lt;/sub&gt; mmol/g of DM fermented</td>
<td>1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>NH&lt;sub&gt;3&lt;/sub&gt;-N, mM</td>
<td>5.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;S µmol/g of ferm. OM</td>
<td>41.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Pentacyclic triterpene type: OLE = Oleanolic acid; MAL = Malsinic acid; URS = Ursolic acid.

<sup>2</sup>MON = monensin sodium at 4 mg/L of incubation fluid.

<sup>3</sup>P-values for the effect of pentacyclic triterpene (TT) type, dose and their interaction when analyzed as a 5 × 3 factorial arrangement (excluding the effect of monensin).

<sup>4</sup>IVOMD = In vitro organic matter digestibility.
Table 2-4. Effects of oleanolic, malsinic and ursolic acid on total volatile fatty acids, and volatile fatty acids profile using a high-concentrate substrate.

<table>
<thead>
<tr>
<th>Item</th>
<th>TT type</th>
<th>Dose, mg/L</th>
<th>P-value</th>
<th>Trt</th>
<th>TT type</th>
<th>dose</th>
<th>TT x dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLE</td>
<td>MAL</td>
<td>URS</td>
<td>0</td>
<td>4</td>
<td>25</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>Total VFA conc., mM</td>
<td>103.3ᵃ</td>
<td>97.8ᵃ</td>
<td>102.0ᵃ</td>
<td>77.4ᵃ</td>
<td>106.8ᵃ</td>
<td>106.9ᵃ</td>
<td>105.5ᵃ</td>
</tr>
<tr>
<td>Acetate conc., mM</td>
<td>58.1ᵃ</td>
<td>55.2ᵃ</td>
<td>57.2ᵃ</td>
<td>44.8ᵃ</td>
<td>59.9ᵃ</td>
<td>59.6ᵃ</td>
<td>59.0ᵃ</td>
</tr>
<tr>
<td>Propionate conc., mM</td>
<td>32.8ᵃ</td>
<td>31.0ᵃ</td>
<td>32.7ᵃ</td>
<td>24.3ᵃ</td>
<td>32.1ᵃ</td>
<td>34.2ᵃ</td>
<td>33.7ᵃ</td>
</tr>
<tr>
<td>Butyrate conc., mM</td>
<td>10.1ᵃ</td>
<td>9.5ᵃ</td>
<td>9.9ᵃ</td>
<td>6.7ᵃ</td>
<td>10.4ᵃ</td>
<td>10.7ᵃ</td>
<td>10.5ᵃ</td>
</tr>
<tr>
<td>BCVFA⁴</td>
<td>1.2ᵃ</td>
<td>1.2ᵃ</td>
<td>1.2ᵃ</td>
<td>0.9ᵃ</td>
<td>1.3ᵃ</td>
<td>1.3ᵃ</td>
<td>1.3ᵃ</td>
</tr>
<tr>
<td>Valerate</td>
<td>1.0ᵃ</td>
<td>1.0ᵃ</td>
<td>1.0ᵃ</td>
<td>0.6ᵃ</td>
<td>1.1ᵃ</td>
<td>1.1ᵃ</td>
<td>1.1ᵃ</td>
</tr>
<tr>
<td>A:P⁵</td>
<td>1.8ᵃ</td>
<td>1.9ᵃ</td>
<td>1.8ᵃ</td>
<td>1.8ᵃ</td>
<td>1.8ᵃ</td>
<td>1.8ᵃ</td>
<td>1.8ᵃ</td>
</tr>
</tbody>
</table>

¹Pentacyclic triterpene type: OLE = Oleanolic acid; MAL = Malsinic acid; URS = Ursolic acid.
²MON = monensin sodium at 4 mg/L of incubation fluid.
³P-values for the effect of pentacyclic triterpene (TT) type, dose and their interaction when analyzed as a 5 × 3 factorial arrangement (excluding the effect of monensin).
⁴BCVFA=Branch chain volatile fatty acids
⁵A:P = acetate to propionate ratio.
Table 2-5. Effects of oleanolic, malsinic and ursolic acid on total volatile fatty acids, and volatile fatty acids profile using a bahiagrass substrate

<table>
<thead>
<tr>
<th>Item</th>
<th>TT type¹</th>
<th>Dose, mg/L</th>
<th>P-value³</th>
<th>Type × dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OLE</td>
<td>MAL</td>
<td>URS</td>
<td>0</td>
</tr>
<tr>
<td>Total VFA conc., mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate conc., mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionate conc., mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyrate conc., mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCVFA⁴</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valerate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A:P⁵</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Pentacyclic triterpene type: OLE = Oleanolic acid; MAL = Malsinic acid; URS = Ursolic acid.
²MON = monensin sodium at 4 mg/L of incubation fluid.
³P-values for the effect of pentacyclic triterpene (TT) type, dose and their interaction when analyzed as a 5 × 3 factorial arrangement (excluding the effect of monensin).
⁴BCVFA=Branch chain volatile fatty acids
⁵A:P = acetate to propionate ratio.
Table 2-6. Effects of increasing concentrations of two chemical forms of oleanoic acid: sodium salt, or phytosome on in vitro batch culture fermentation, using high-concentrate substrate

<table>
<thead>
<tr>
<th>Item</th>
<th>mg/L</th>
<th>Control (CTL)</th>
<th>MON</th>
<th>OA-Na</th>
<th>OA-Phyt</th>
<th>SE</th>
<th>Trt OA DOSE</th>
<th>Trt x DOSE</th>
<th>OA-Na</th>
<th>OA-Phyt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Gas, mL 24 h</td>
<td></td>
<td>155.0</td>
<td>144.6</td>
<td>155.8</td>
<td>152.0</td>
<td>147.8</td>
<td>146.2</td>
<td>146.8</td>
<td>148.1</td>
<td>150.6</td>
</tr>
<tr>
<td>IVOMD, %</td>
<td></td>
<td>80.4</td>
<td>77.4</td>
<td>77.8</td>
<td>81.4</td>
<td>78.4</td>
<td>76.7</td>
<td>80.1</td>
<td>79.2</td>
<td>77.3</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>5.7</td>
<td>5.7</td>
<td>5.7</td>
<td>5.7</td>
<td>5.7</td>
<td>5.7</td>
<td>5.7</td>
<td>5.7</td>
<td>5.7</td>
</tr>
<tr>
<td>CH4 total</td>
<td></td>
<td>6.5</td>
<td>4.8</td>
<td>6.5</td>
<td>6.2</td>
<td>6.7</td>
<td>6.2</td>
<td>6.5</td>
<td>6.7</td>
<td>6.7</td>
</tr>
<tr>
<td>CH4 incubated</td>
<td></td>
<td>1.1</td>
<td>0.7</td>
<td>1.1</td>
<td>1.0</td>
<td>1.1</td>
<td>1.0</td>
<td>1.0</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>CH4 mmol/g of DM fermented</td>
<td></td>
<td>1.3</td>
<td>0.9</td>
<td>1.4</td>
<td>1.2</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>H2S µmol/g of ferm. OM</td>
<td></td>
<td>7.5</td>
<td>7.8</td>
<td>8.3</td>
<td>7.8</td>
<td>7.8</td>
<td>7.9</td>
<td>7.3</td>
<td>6.8</td>
<td>7.1</td>
</tr>
</tbody>
</table>

1 Control = distilled water. 2 MON = monensin sodium at 4 mg/L of inoculum. 3 OA-Na = Oleanolic acid, Sodium salt chemical form; OA-Phyt = Oleanolic, Phytosome form. 4 P-values for the effect of oleanolic acid, dose and their interaction when analyzed as a 5 x 2 factorial arrangement (excluding the effect of monensin). 5 Linear effects = P-values of linear effect of OA-Na, OA-Phyt; 6 IVOMD = In vitro organic matter digestibility.
Table 2-7. Effects of increasing concentrations of two chemical forms of oleanoic acid: sodium salt, or phytosome on in vitro batch culture fermentation, using bahiagrass hay substrate

<table>
<thead>
<tr>
<th>Item</th>
<th>mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL¹ MON² OA-Na³ OA-Phyt³</td>
</tr>
<tr>
<td>Total Gas, mL 48 h</td>
<td>151.6⁰ 102.5⁰ 151.4⁰ 153.9⁰ 141.0⁰ 134.6⁰ 151.7⁰ 150.3⁰ 149.8⁰ 156.6⁰</td>
</tr>
<tr>
<td>IVOMD⁶, %</td>
<td>50.4⁰ 46.3⁰ 48.8⁰ 49.9⁰ 49.7⁰ 45.6⁰ 50.2⁰ 48.4⁰ 50.4⁰ 46.2⁰ 0.58 &lt;0.01 &lt;0.01 &lt;0.01</td>
</tr>
<tr>
<td>pH</td>
<td>6.3⁰ 6.5⁰ 6.3⁰ 6.3⁰ 6.4⁰ 6.4⁰ 6.3⁰ 6.3⁰ 6.3⁰ 6.3⁰ 0.02 &lt;0.01 &lt;0.01 0.82</td>
</tr>
<tr>
<td>CH₄ total</td>
<td>7.4⁰ 4.5⁰ 7.5⁰ 7.6⁰ 7.2⁰ 6.9⁰ 7.5⁰ 7.5⁰ 7.5⁰ 7.5⁰ 1.10 &lt;0.01 0.03 0.63</td>
</tr>
<tr>
<td>CH₄/ g incubated</td>
<td>1.0⁰ 0.4⁰ 1.1⁰ 1.1⁰ 0.9⁰ 0.9⁰ 1.1⁰ 1.1⁰ 1.0⁰ 1.1⁰ 0.19 &lt;0.01 0.01 0.24</td>
</tr>
<tr>
<td>CH₄ mmol/g of DM fermented</td>
<td>2.1⁰ 0.9⁰ 2.2⁰ 2.2⁰ 1.9⁰ 1.9⁰ 2.1⁰ 2.2⁰ 2.1⁰ 2.5⁰ 0.40 &lt;0.01 0.02 0.11</td>
</tr>
<tr>
<td>H₂S µmol/g of ferm. OM</td>
<td>10.8⁰ 6.9⁰ 11.5⁰ 12.4⁰ 10.1⁰ 10.0⁰ 10.8⁰ 10.5⁰ 10.4⁰ 10.0⁰ 2.3 0.47 0.57 0.70</td>
</tr>
</tbody>
</table>

¹Control = distilled water.  
²MON = monensin sodium at 4 mg/L of inoculum.  
³OA-Na= Oleanolic acid, Sodium salt chemical form; OA-Phyt = Oleanolic, Phytosome form  
⁴P-values for the effect of oleanolic acid, dose and their interaction when analyzed as a 5 × 2 factorial arrangement (excluding the effect of monensin).  
⁵Linear effects = P-values of linear effect of OA-Na, OA-Phyt;  
⁶IVOMD = In vitro organic matter digestibility.
Table 2-8. Effects of increasing concentrations of two chemical forms of oleanoic acid: sodium salt, or phytosome on total volatile fatty acids, and volatile fatty acids profile, using a high concentrate substrate

<table>
<thead>
<tr>
<th>Item</th>
<th>mg/L</th>
<th>CTL(^1)</th>
<th>MON(^2)</th>
<th>OA-Na(^3)</th>
<th>OA-Phyt(^3)</th>
<th>P-values(^4)</th>
<th>Linear effect(^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>4</td>
<td>4</td>
<td>40</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>Total VFA conc., mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate conc., mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionate conc., mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyrate conc., mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCVFA conc., mM(^6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valerate conc., mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A:P(^7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Control = distilled water.

\(^2\) MON = monensin sodium at 4 mg/L of inoculum.

\(^3\) OA-Na = Oleanolic acid, Sodium salt chemical form; OA-Phyt = Oleanolic, Phytosome form

\(^4\) P-values for the effect of oleanolic acid, dose and their interaction when analyzed as a 5 x 2 factorial arrangement (excluding the effect of monensin).

\(^5\) Linear effects = P-values of linear effect of OA-Na, OA-Phyt

\(^6\) BCVFA = Branch chain volatile fatty acids

\(^7\) A:P = acetate to propionate ratio.
Table 2-9. Effects of increasing concentrations of two chemical forms of oleanic acid: sodium salt, or phytosome on total volatile fatty acids, and volatile fatty acids profile, using bahiagrass hay substrate

<table>
<thead>
<tr>
<th>Item</th>
<th>mg/L</th>
<th>P-values⁴</th>
<th>Linear effect⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL¹ MON² OA-Na³ OA-Phyt⁶</td>
<td>Trt OA DOSE Trt x DOSE OA-Na OA-Phyt</td>
<td></td>
</tr>
<tr>
<td>Total VFA, mM</td>
<td>51.4 38.0 46.0 47.3 45.2 56.7 38.9 51.8 51.3 49.8 6.36 0.51 0.85 0.34 0.55 0.88 0.875</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate, mM</td>
<td>37.3 24.5 33.4 34.4 32.8 40.9 28.4 33.1 37.3 36.3 4.61 0.30 0.61 0.38 0.68 0.96 0.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propionate, mM</td>
<td>8.9  10.2  8.1  8.1  10.3  6.6  8.0  9.0  8.4  1.19 0.30 0.39 0.28 0.51 0.22 0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyrate, mM</td>
<td>3.6  2.1  3.2  3.3  3.1  3.9  2.7  3.2  3.6  3.5  0.49 0.19 0.67 0.40 0.69 0.80 0.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCVFA⁶, mM</td>
<td>1.1  0.6  0.9  1.1  0.9  1.2  0.8  1.0  1.0  1.1  0.19 0.64 0.87 0.42 0.89 0.87 0.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valerate, mM</td>
<td>0.4  0.3  0.4  0.4  0.4  0.3  0.4  0.4  0.4  0.4  0.07 0.77 0.98 0.51 0.76 0.80 0.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A:P⁷</td>
<td>4.2  2.4  4.2  4.3  4.0  4.0  4.3  4.3  4.2  4.2  4.3  0.2  &lt;0.01 0.13 0.45 0.25 0.01 0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ Control = distilled water.
²MON = monensin sodium at 4 mg/L of inoculum.
³OA-Na= Oleanic acid, Sodium salt chemical form; OA-Phyt = Oleanic, Phytosome form
⁴P-values for the effect of oleanolic acid, dose and their interaction when analyzed as a $5 \times 2$ factorial arrangement (excluding the effect of monensin).
⁵Linear effects = P-values of linear effect of OA-Na, OA-Phyt
⁶BCVFA=Branch chain volatile fatty acids
⁷A:P = acetate to propionate ratio.
CHAPTER 3
EFFECTS OF OLIVE OIL BIOACTIVE EXTRACTS ON INFLAMMATORY AND IMMUNE RESPONSE IN LIPOPOLYSACCHARIDE CHALLENGED WEANED HEIFERS

Introductory Remarks

Mammals has several mechanisms to maintain a tightly regulated metabolism, to ensure homeostasis. But, when an external factor disrupt that equilibrium, an imbalance of the inner milieu might occur (Zebeli and Metzler-Zebeli, 2012).

Weaning is one of the most stressful periods on the life of a ruminant. Separation from their dams is usually followed by transportation to another operation and commingling with other animals. This is accompanied by a reduction in intake overlapped with a rapid dietary change, often characterized by the occurrence of ruminal acidosis (Penner et al., 2010). Understandably, this has negative consequences to gastrointestinal and gut barrier function. Highly fermentable carbohydrates coupled with low ruminal pH favor the development of gram-negative bacteria (GNB), that can liberate large amounts of lipopolysaccharides (LPS; Andersen et al., 1994; Gozho et al., 2006). Nutritional insults not only change the supply of nutrients, it also changes pH and osmolarity, which impacts epithelial permeability in the rumen and in more distal regions in the lower gut (Zhang et al., 2013; Pederzolli et al., 2018). This is accompanied by increased proliferation of GNB, which release large amounts of LPS. This highly conserved molecule can trigger immune activation and development of local inflammation, which further increases permeability, disruption of barrier function and translocation of LPS into circulation (Emmanuel et al., 2008; Plaizier et al., 2012). When endotoxin reaches portal circulation the immune system recognize and trigger an immune response in an energetically costly process in detriment of animal performance (Kvidera et al., 2017).
It is fundamental to find ways to mitigate the negative effects of nutritional and environmental challenges to increase performance, health and wellbeing. Decreasing systemic inflammation can be an effective strategy, as this should reduce the permeability of the epithelial cell lining, restoring barrier function to luminal contents, and blocking the entry of antigenic molecules. Supplementation of nutraceuticals with anti-inflammatory capacity is a possibility worth testing to ameliorate this condition. Bioactive compounds present in virgin olive oil and olive byproducts, such as triterpenes and polyphenols, have been described to have anti-inflammatory and antioxidant bioactivity. Dietary supplementation with olive oil bioactive extracts has been showed to decrease the negative effect of subclinical chronic inflammation (Liehr et al., 2017; Morrison et al., 2017), and could contribute to reducing the detrimental effect of translocation of endotoxin.

We hypothesized that feeding olive oil bioactive extracts (OBE) could ameliorate the detrimental effects of LPS challenge through modulation of the immune response and reduction of systemic inflammation. Our objective was to evaluate the impact of feeding OBE to newly weaned Angus crossbred heifers injected intravenously with increasing doses of lipopolysaccharide every other day over a 10-d period on immune status and inflammatory state.

**Materials and Methods**

**Experimental Design, Animals, and Treatments**

The experiment was conducted at the University of Florida, North Florida Research and Education Center (NFREC) in Marianna, FL, from June 2017 to October 2017. All procedures were carried out according to the University of Florida’s Institutional Animal Care and Use Committee.
A generalized randomized block design was used to evaluate the impact of feeding OBE to newly weaned Angus crossbred heifers (210 ± 19 kg of BW) injected intravenously with sequential increasing doses of lipopolysaccharide (LPS; 0.10, 0.25, 0.50, 0.75, 1.00, and 1.25 μg/kg of BW). A total of 36 newly weaned heifers were used, distributed across 3 experimental periods consisting of at least 21 d of adaptation, followed by the LPS injections every 48 h for the last 10 d of each period. The LPS used was E. coli 0111:B4 (Sigma-Aldrich, Saint Louis, MO) and was infused into jugular vein using a winged butterfly needle.

Heifers were blocked by BW and then randomly assigned to 1 of 4 treatments: negative control (receiving saline, CTL-); positive control (receiving LPS, CTL+); and positive control plus a low (0.04% of diet dry matter [DM], OBE-L) or high (0.16% of diet DM, OBE-H) dose of OBE. The OBE was fed for at least 21 d before the first LPS challenge. The rationale for the OBE inclusion rates was based on a target dose of 1 mg/kg of BW and 4 mg/kg of BW of the bioactive compound oleanolic acid. These target doses were obtained via allometric scaling using as reference the OBE dose tested by Liehr et al. (2017) in pigs and adapting the equation described by Wojcikowski and Gobe (2014). Based on the predicted dry matter intake (DMI) and BW of the heifers, the target dose was converted into percentage of inclusion in the diet DM (0.04% and 0.16% for OBE-L and OBE-H correspondently). The OBE product contained 10% oleanolic acid, 4% malsinic acid, and 2% hydroxytyrosol. For the extraction of this molecules, pomace oil was filtrated, and the bioactive compounds were extracted with purified ethanol. From the resulting product a sample was taken and dissolved in methanol followed by quantification by HPLC. The final OBE product was provided by Pro Nutra (Pro Nutra., Madrid, Spain), and was standardized to contain 4% oleanolic acid, 10% malsinic acid,
and 2% of hydroxytyrosol. The OBE was mixed with the mineral premix before mixing with the rest of ingredients of the total mixed ration (TMR), to facilitate an even distribution in the TMR. Molasses was included in the diet to add condition to the diet and minimize small particle separation in the feed bunk. Heifers assigned to the control treatments received the same diet without OBE in the mineral premix (Table 3-1).

Immediately after weaning, heifers were allocated to pens (108 m²/pen; 4 pens of 9 heifers each) at the NFREC Feed Efficiency Facility (FEF). Individual daily DMI was measured using the GrowSafe system (GrowSafe Systems Ltd., Airdrie, AB, Canada). Each pen in the FEF was equipped with two GrowSafe feed bunks. Heifers were blocked by BW and then randomly assigned to pens to receive treatments. A 21-d period of adaptation to facilities and diets preceded the sequential LPS challenge.

Heifers were assigned to receive a growing diet fed as a TMR diet (0.97 Mcal/kg of NEm, and 16.7% of CP). The diet consisted of 61% gluten feed, 32% cottonseed hulls, 4% molasses, and 3% of a mineral premix, and the OBE additives were mixed with the mineral premix before adding to the TMR (Table 3-1). The diet was formulated using the 2016 beef NRC, and to allow 600 g of BW gain per day. Before allocation to the different treatments, heifers were weighed, and tagged with radio frequency identification. Additionally, heifers were weighted immediately before every LPS challenge to adjust the dose of LPS. Average daily gain (ADG) was calculated from day -21 to day -1 before challenge, and during challenge from day 0 to day 12, calculated as final minus initial.

**Feed Sample Collection and Analyses**

Representative samples of each treatment TMR diet were taken 3 times during each period, before feeding. All samples were bagged and frozen immediately after collection until drying. All feed samples analyzed for nutritive values were dried at 55°C.
for 48 h in a forced-air oven. At the conclusion of the drying period, all samples were ground in a Wiley mill (Arthur H. Thomas Co., Philadelphia, PA) using a 1.0-mm screen. After grinding, samples were composited for analysis on an equal weight basis. The composited samples were analyzed for DM, CP, TDN, ADF, ash free NDF (aNDF), Ca, and P in duplicate by a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY).

**Calf Health Assessment**

After every LPS challenge, heifers were observed every 2 h for 8 h, for nasal or eye discharge, cough, respiratory score, and fecal consistency. Intravaginal temperature was measured every 5 min during the entire period of repeated LPS injections (d 0 to d 12; Fig. 3-1). A temperature logging button (iButton, DS1921H-F5#, Whitewater, WI) was placed intravaginally with a blank control internal drug release (CIDR) insert, and was used to record intravaginal temperature. Temperature recordings were average every 30 min for the first 12 h post challenge, then every hour up to 24 h and until 6 h before the next challenge every 12 h. Heifers with a rectal temperature ≥ 39.5°C were categorized as febrile. One calf had to be treated with an anti-inflammatory drug containing fluoxacin, and had to be removed from the experiment.

**Sample Collection and Laboratory Analyses**

Blood samples from each calf were collected from the jugular vein into a 10-mL evacuated glass vial containing 143 IU of sodium heparin (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) inverted carefully several times, and immediately placed on ice. Within 60 min of collection, chilled samples were centrifuged at 1,500 × g for 15 min at 4°C (Avanti J-E, Beckman Coulter Inc., Palo Alto, CA), plasma was harvested, and multiple aliquots of 1.5 mL were frozen at -20°C until analysis. Blood samples were
collected 1 day before the first LPS challenge, and every day from day 0 up to day 12 (Fig. 3-1). On the first day of challenge on day 0 and on the last one on day 10, blood samples were collected immediately before LPS injection and at hour 1, 2, 4, and 8 (Fig. 3-1). Plasma samples were run in duplicate and samples from the four treatments were included in each plate/run along with a plasma sample of known composition. Plasma concentrations of glucose, NEFA, and haptoglobin were measured every day from day 0 to 12. Additionally on day 0 and day 10 glucose and IL-6 were measured on h 0, 1, 2, 4, and 8. Glucose was measured using a quantitative colorimetric kit (G7521-1L; Pointe Scientific Inc., Canton, MI).

Intra- and inter-assay CV were 2.9% and 3.6% for glucose. Concentrations of non esterified fatty acids (NEFA-C kit; Wako Diagnostics Inc., Richmond, VA; as modified by Johnson and Peters, 1993) was determined using enzyme assays in duplicate. Intra- and inter-assay CV was 2.1 and 5.7%. Plasma concentrations of haptoglobin were measured using a colorimetric procedure that measures haptoglobin-hemoglobin complexing by estimating differences in peroxidase activity (Makimura and Suzuki, 1982). The absolute absorbance values at 450 nm were multiplied by 100 and used for statistical analysis. Intra- and inter-assay CV were 5.1 and 9.7%, respectively. Interleukin 6 was measured using a commercially available ELISA kit (DuoSet ELISA; R&D Systems, Inc., Minneapolis, MN). A 7-step serially diluted recombinant bovine IL-6 standard curve was used. Samples were run in duplicate. The inter- and intra-assay coefficients of variation for IL-6 were 9.7% and 3.4%, respectively.

Flow Cytometry

A fraction (200 μL) of the heparinized whole blood was pipetted into a 15 mL conical centrifuge tubes and suspended in 2 mL of cold hypotonic buffer (10.6 mM of Na2HPO4 and 2.7 mM of NaH2PO4; pH 7.2) for 1 min. This was followed by addition of
1 mL of restore solution (10.6 mM of Na2HPO4, 2.7 mM of NaH2PO4, and 462 mM of NaCl; pH 7.2). Tubes then were centrifuged at 650 × g for 5 min at 4°C, and the supernatant was discarded. This process was repeated until erythrocytes were removed. Next, samples were incubated on ice with an antibody cocktail of 0.25 uL phycoerythrin (PE, Abcam Cambridge, MA)-conjugated anti CD62L, 0.25 uL allophycocyanin (APC) -conjugated anti-CD14 (Tük4 clone, Life Technologies) and 0.25 uL fluorescein isothiocyanate (FITC)-conjugated mouse anti-bovine CD11b (CC126 clone, AbD Serotec; Raleigh, NC) in 25 μL PBS containing 0.5% BSA (Fisher Scientific; Fair Lawn, NJ) and 2mM EDTA (Fisher Scientific). After incubation, 1 mL of PBS was added, vortexed, and centrifuged at 650 × g for 5 min at 4°C. Supernatant was discarded, and cells resuspended in 200 μL of PBS. Samples were analyzed using an Accuri C6 digital analyzer flow cytometer (Becton Dickinson Biosciences, SanJose, CA). Lymphocytes, monocytes and neutrophils were analyzed on the basis of their size and granularity in the density cytogram. Data acquisitions of the total amount of cells per sample were analyzed using Flowjo software v10.1 (Single Cell Analysis Software, LLC, OR). Data analyzed were total cell counts of lymphocytes, monocytes and neutrophils. Likewise, histogram analysis for median fluorescence intensity (MFI) of cluster of differentiation 14 (CD14), CD62L, and CD11b were used as an indication of the intensity of receptor expression on monocytes and neutrophils populations.

**Statistical Analyses**

Of the 36 heifers enrolled in the experiment, one animal was excluded due to treatment with an anti-inflammatory drug, and only 35 were included in the data set and considered in the statistical analysis.

Experiment was a randomized block design. Every period was the blocking factor, and calf was the experimental unit and was nested within treatment, and
considered random. Data were analyzed using the MIXED procedure of SAS version 9.4 (SAS/STAT, SAS Institute Inc., Cary, NC). The REPEATED statement was used for dependent variables measured over time. Model included the fixed effects of treatment, time, and treatment by time interaction. Calf was nested within treatment and was the error term for testing the effects of treatment. Contrast statements to test treatment effects on heifers were the following: 1) effect of LPS (heifers challenged with LPS vs. those that only received saline; CTL- vs. CTL+), 2) supplementation with OBE exposed to LPS plus supplementation of OBE-L and OBE-H vs. heifers only exposed to LPS; OBE vs. CTL+), and 3) Dose effect of OBE (OBE-L vs. OBE-H). The covariance structure with the lowest Akaike’s information criterion was selected for each variable. Most analyses used the first-order autoregressive structure for equally spaced measurements or spatial power for unequally spaced measurements. When an interaction of treatment with time was detected as significant, treatment means at time points of measure were partitioned using the diff command of SAS. For data collected at single time points only, the MIXED procedure of SAS was used without the REPEATED statement. The Kenward-Roger method was used to calculate the approximate denominator degrees of freedom for the $F$ tests in the statistical models. Continuous data were tested for the distribution of the residuals after fitting the statistical models using Shapiro-Wilk and homogeneity of variance by plotting residuals against predicted values. Non-normally distributed data were subjected to Box-Cox transformation using the TRANS-REG procedure of SAS to achieve normality before analyses. The LSMeans were back-transformed and SEM calculated as outlined by Jørgensen and Pedersen (1998). Statistical significance was considered at $P \leq 0.05$ and tendency was considered at $0.05 < P \leq 0.10$. 
Results

DMI and ADG

Intake was measured for 14 d prior the first LPS challenge, and during the 12 d period of challenge. No treatment difference was observed prior challenge (DMI pre challenge; \(P = 0.43;\) Table 3-2). During the 10 d period of sequential increasing doses of LPS challenge, LPS injections had an hypophagic effect that tended to decrease DMI compared to the ones that only received saline (CTL+ compared to CTL-; 6.4 kg/d vs. 7.6 kg/d; \(P = 0.07;\) Table 3-1).

In addition, there was a treatment by time interaction (\(P = 0.05\)). Heifers that only received saline, CTL–, had no differences on DMI across the entire 12 d period (Fig 3-2a). On the contrary, treatments exposed to repeated LPS injection had a drop on intake every day of challenge that tended to become less abrupt with subsequential challenge days (Fig. 3-2b). Heifers exposed to LPS on the CTL + treatment had reduced intakes compared to CTL- on d 0, 1, 2, and 4 (\(P < 0.05;\) Fig 3-2b). Heifers supplemented with OBE-H, counteracted the suppressing effect of LPS on DMI on d 0, and d 1 (OBE-H compared to CTL +; 6.1, and 7.9 kg/d vs. 4.4, and 5.9 kg/d; \(P < 0.05;\) Fig 3-2b), and tended to have higher intakes on d 12 (OBE-H compared to CTL +; 7.9 vs. 6.5 kg/d; \(P = 0.07;\) Fig. 3-2b). On d 12, heifers supplemented with OBE-L had a tendency for greater intake compared to CTL + (8.0 vs 6.5 kg/d; \(P = 0.07;\) Fig 3-2b).

As expected, repeated LPS injections tended to decrease ADG on exposed heifers compared to the ones that only received saline (CTL- compared to CTL +; 0.4 kg/d vs. - 0.1 kg/d; \(P = 0.09;\) Table 3-2). This depressing effect of LPS on ADG was, at least numerically, partially offset on heifers supplemented with OBE (OBE compared to CTL +; - 0.1 kg/d vs 0.3 kg/d; \(P = 0.30;\) Table 3-2).
**Temperature**

Repeated exposure to LPS raised intravaginal body temperature confirming that the model was successful in inducing systemic inflammation (CTL+ compared to CTL-; 39.0 vs 38.6°C; \( P < 0.01 \); Table 3-3). Heifers exposed to LPS had higher maximum temperatures that were almost 1°C above non-exposed heifers (CTL+ compared to CTL-; 39.6°C vs. 38.8; \( P < 0.01 \); Table 3-3). CTL- heifers spent 0 h with fever, on the contrary, heifers exposed to LPS spent between on average 6 hours experiencing temperatures above 39.5°C, and tended to differ from CTL- \( (P < 0.07 \); Table 3-3). 

During every day of challenge, intravaginal temperature was evaluated during 36 h post challenged. Temperature was averaged every half an hour for 12 h after the challenge, then every hour up to 24 h post challenge, and the following day, data was average every 12 h. Exposure to LPS elevated intravaginal temperature for at least 6 h post challenge on every d of challenge \( (P < 0.05 \); Fig. 3-3a-f). On the contrary, heifers not exposed to LPS (CTL-), maintained relatively constant intravaginal temperature, and had lower temperature compared to the CTL+ for at least the first 6 h post challenge, every day of challenge \( (P < 0.05 \); Fig 3-3a-f). Supplementation of OBE-H had lowering effect on intravaginal temperature when compared to the non-supplemented ones, CTL+, between h 2 and 4, on the first day of challenge, d 0 \( (P < 0.05 \); Fig 3-3a), and on d 8 at least a tendency to have lower temperature was observed between hours 7.5 and 14 \( (P < 0.10 \); Fig 3-3e). Heifers supplemented with OBE-H had lower maximum temperature compared to CTL+ on d 9 (39.2 vs. 39.6°C; \( P = 0.03 \)), and a tendency on d 0 (39.9 vs 40.2°C; \( P = 0.08 \)).

**Haptoglobin**

Plasma concentrations of haptoglobin expressed as optical density x 100 (ODx100), is depicted in Table 3-4. CTL+ had higher concentrations of haptoglobin...
compared to CTL (4.7 vs. 2.8 OD×100; \(P < 0.01\)). In addition, heifer supplementation with OBE lowered the concentration of haptoglobin compared to CTL+ (3.3 vs. 4.7 OD×100; \(P < 0.01\); Table 3-4), and no difference was observed between the 2 doses of OBE (\(P = 0.72\); Table 3-4).

There was a Trt \(\times\) d interaction for plasma haptoglobin concentration (\(P < 0.01\); Fig. 3-4), where a biphasic increase in haptoglobin for the CTL+ was observed (Fig 3-4), reaching a maximum concentration by d 2, then decreased, and then by d 6 started to increase again to peak on d 10. OBE supplementation also followed this biphasic behavior, but ameliorated the increase on haptoglobin concentrations, and delayed the expression of the peaks compared to CTL+ (\(P < 0.03\); Fig. 3-4).

**Interleukin-6**

Interleukin-6 concentration expressed as pg/mL was measured the first and last day of challenge (d 0 and d 10), immediately before the challenge on h 0, and on h 1, 2, 4, and 8 post challenge. Heifers exposed to LPS had a 5 fold increase of IL-6 concentration on d 0, and a 7 fold increase on d 10 (CTL+ compared to CTL-; \(P < 0.01\); Table 3-5).

There was a Trt \(\times\) h interaction where heifers challenged with LPS rapidly increased IL-6 concentration, reaching a maximum at h 4 in both days (Fig 3-5a-b). Supplementation with OBE ameliorated the increase on IL-6 concentrations compared to the ones not supplemented (CTL+), between h 2 and 4, on d 0 and d 10 (\(P < 0.05\); Fig 3-5a-b). On the contrary, CTL- had no difference across the entire 8 h period both on d 0 and on d 10 (\(P > 0.80\); Fig 3-5a-b).

**Glucose**

Plasma glucose concentration expressed as mg/dL was measured every day during the entire 12-day period of challenge. Heifers exposed to repeated LPS
injections increased had increased plasma glucose concentration (CTL+ compared to CTL -; 69.4 mg/dL vs. 75.2 mg/dL; \( P < 0.02 \); Table 3-4). There was no difference in glucose concentration between heifers supplemented with OBE, and non-supplemented ones (\( P = 0.19 \); Table 3-4). OBE-H had higher plasma glucose concentration than OBE-L (\( P < 0.05 \), Table 3-4).

Additionally, plasma glucose concentration was measured on d 0, and d 10 immediately before the challenge on h 0, and on h 1, 2, 4, and 8 h post challenge. There was an interaction between treatment and hour on d 0 (\( P = 0.02 \); Fig. 3-6a), where heifers that received only saline, CTL-, maintained stable the concentration of glucose across the 8 h period (\( P > 0.28 \)). On the contrary, heifers exposed to LPS but not supplemented, CTL+, tended to increase glucose concentrations that peaked at h 2 (76.3 mg/dL; \( P = 0.10 \)), and then started to drop until h 8 (54.6 mg/dL; \( P < 0.01 \)). Heifers supplemented with OBE peaked before CTL+, at h 1 (\( P < 0.05 \)). From that point, OBE-L had a drop in plasma glucose that continued until h 8 (59.2 mg/dL; \( P < 0.04 \)). OBE-H, also had a drop, but recovered its initial plasma glucose concentrations by h 8 (66.9 mg/dL; \( P = 0.40 \)). On d 10, no treatment differed from each other (\( P = 0.28 \); Fig 3-6b).

**NEFA**

During the 12 d period of challenge, there was a tendency for a Trt effect for plasma NEFA concentrations, expressed as µEq/L. There was no difference on plasma NEFA concentration between treatments (\( P > 0.13 \); Table 3-4).

Plasma NEFA concentrations was also analyzed immediately before the challenge, and at h 1, 2, 4, and 8, on d 0 and d 10 of challenge. There was a Trt × h interaction both on d 0 and d 10. Heifers that only received saline, CTL-, had no significant changes on plasma NEFA concentration during the 8 h post challenge on d 0, and d 10 (\( P > 0.24 \); Fig. 3-6). On the contrary, on d 0, heifers treated with LPS, had
increased plasma NEFA concentration on h 4, and 8 \( (P < 0.05; \text{Fig. 3-6c}) \). Heifers supplemented with OBE-L had lower plasma NEFA concentration on h 8 compared to CTL+, and OBE-H \( (P < 0.03; \text{Fig. 3-6}) \) both on d 0 and d 10.

**Monocytes and Neutrophil Cell Counts**

Peripheral blood leukocyte and neutrophil counts were measured right before the LPS challenge at h 0, and at h 1, 2, 4, and 8 after the challenge on d 0, and on d 10. LPS exposure decreased lymphocytes, monocytes, and neutrophils counts both on d 0 and d 10 (CTL+ compared to CTL−; \( P < 0.01; \text{Table 3-6}) \). Supplementation with OBE had a 61% recovery of lymphocyte cell counts/µL compared with CTL+ on d 0 but not on d 10 \( (P = 0.05; \text{Table 3-6}) \).

There was a treatment × h interaction for lymphocyte, monocyte, and neutrophil cell counts on d 0 \( (P < 0.04; \text{Fig. 3-7}) \). At h 0 (prior to LPS challenge) no treatment differed from each other \( (P > 0.15) \). After the challenge, CTL− maintained greater lymphocyte, monocyte, and neutrophil cell counts from h 1 to h 8 compared to CTL+ \( (P < 0.03; \text{Fig. 3-7}) \). After the challenge on h 0, there was a drop on lymphocyte, monocyte, and neutrophil cell counts/µL on just 1 hour for CTL+, and remained below the its initial value the rest of hours measured on d 0 \( (P < 0.01; \text{Fig. 3-7}) \), and until h 4 on d 10 \( (P < 0.01; \text{Fig. 3-7}) \). On d 0, heifers supplemented with OBE-L tended to attenuate the drop on lymphocyte and neutrophils cell counts compared to CTL+ on h 2, 4, and 8 \( (P < 0.10; \text{Fig. 3-7}) \), and heifers supplemented OBE-H only on h 8 \( (P < 0.08; \text{Fig. 3-7}) \). On d 10, OBE-H heifers had higher neutrophil cell counts on h 8 compared with CTL+ \( (P = 0.04; \text{Fig. 3-7}) \).

**CD Markers Expression on Leukocytes cell surface**

The MFI of antibody staining for CD14, CD62L, and CD11b were analyzed on monocytes and neutrophil populations on d 0 and d 10 before the LPS challenge at h 0,
and at h 1, 2, 4, and 8 after the challenge. Heifers administered with LPS had almost a 2-fold increase in expression of the LPS receptor CD14 on monocytes on d 0, and a 1.5-fold increase on d 10 (CTL+ compared to CTL-; \( P < 0.01 \); Table 3-7). The adhesion molecule CD11b, had an increase in expression on monocyte cell surface for CTL+ heifers compared with CTL- on d 0, and on d 10 (\( P \leq 0.01 \); Table 3-7).

Heifers supplemented with OBE had 24% less expression of CD14 on the cell surface of monocytes compared with CTL+ on d 0 (\( P \leq 0.01 \); Table 3-7), and 26% less on d 10 (\( P \leq 0.01 \); Table 3-7). On d 0, OBE heifers tended to reduce the expression of the monocyte CD11b compared with CTL+ (MFI of 8176 vs. 10204; \( P < 0.07 \); Table 3-7). On d 10, OBE supplementation significantly reduced CD11b expression on monocytes when compared to CTL+ (MFI of 10349.6 vs. 13096; \( P < 0.01 \); Table 3-7). Heifers supplemented with OBE had less expression of L-Selectin (CD62L) on d 10, but not on d 0 when compared to CTL+ (5153.7 vs. 6330.3; \( P = 0.04 \); Table 3-7).

Expression of CD11b on neutrophil populations did not differ between CTL+ and CTL- on d 0 (\( P = 0.33 \); Table 3-7), however, on d 10, CTL+ had increased expression compared to CTL- (MFI of 22148 vs. 16699; \( P = 0.03 \)). On d 0, but not on d 10 OBE reduced the expression of CD11b when compared with CTL+ (MFI of 16587 vs. 20533; \( P = 0.03 \)). There was no treatment effect on CD62L MFI on neutrophil populations on d 0 (\( P = 0.28 \); Table 3-7). On d 10, OBE-H heifers had greater neutrophil CD62L MFI compared with OBE-L heifers (\( P = 0.05 \); Table 3-7).

**Discussion**

This study shows that supplementation with an olive oil bioactive extract was able to ameliorate some of the detrimental effects of repeated LPS injection on newly weaned heifers, by modulating the immune-inflammatory response and thereby inducing persistent effects on animal health and performance.
It has been previously shown that repeated injections of LPS at low doses represent a suitable approach to generate a phenotype exhibiting typical characteristics of low-grade sustained inflammation in weaned piglets, and in Holstein heifers (Rakhshandeh and de Lange, 2012; Liehr et al., 2017, Fernandes et al., 2018). These characteristics include partial suppression of feed intake, and reduction of BW gain. In contrast with models of acute inflammation, subacute inflammation contributes to chronic and progressive changes in tissue function (Bradford et al., 2015). The inflammatory response is less abrupt but sustained over time, and can be reflected on inflammatory markers concentration in peripheral circulation, including cytokines and APP (Rakhshandeh and de Lange, 2012; Kvidera et al., 2017; Liehr et al., 2017). In contrast to models of acute inflammation, in which LPS is usually administered in a high single dose of about 1–1.5 μg/kg BW (Carroll et al., 2009; Kvidera et al., 2017), in subacute models the reduction in DMI is less pronounced but more persistent over time. In line with these reports, we observed that repeated injections of LPS with increasing doses (from 0.10 to 1.25 μg/kg BW) over a 10 d period, decreased feed intake and BW gain, and that OBE supplementation attenuated the drop in feed intake in some, but not all days of challenge ($P < 0.05$; Fig. 3-2c). In addition, the drop on intake suffered by the heifers exposed to LPS was the highest on the first day of challenge, to become less abrupt in subsequent days, even though the doses were at least double on every new LPS injection.

The immune system first recognize LPS through specific pathogen sensing receptors, such us the LPS sensing receptor CD14, that transports LPS to the receptor MD-2, which forms a protein complex with the toll-like receptor 4 (TLR4). This event initiates the dimerization and activation of the TLR4 receptor, which will induce the
dimerization of its intracellular portion, TIR, that initiate a downstream signal cascade (Kim et al., 2007; Steimle et al., 2016). This result in the activation of the transcription factor NF-κB, by phosphorylation of the repressor subunit, liberating the transcription factor to translocate into the nucleus and bind to its promoter region. This, in turn, leads to the activation, and transcription of an array of genes involved in the inflammatory response, such as pro-inflammatory cytokines like TNF-α, IL-6, chemokines, and adhesion molecules such as L-selectin, and CD11b (Zebeli and Metzler-Zebeli, 2012; Guo et al., 2017). In our study, heifers exposed to LPS had increased CD14 MFI compared to CTL- on monocyte cell surface, and had more than a 10 fold increase on IL-6 concentration in plasma. The upregulation of the CD14 receptor is key to recognize LPS, and stimulates the production of pro-inflammatory cytokines. Pro-inflammatory cytokines such us IL-6, can activate leukocytes and promote the production of eicosanoids on endothelial cells, that in turn will act on neurons of the preoptic area of the brain to increase core body temperature (Nakamura, 2011; Bradford et al., 2015). In our study, heifers supplemented with OBE had reduced CD14 MFI, suggesting that OBE downregulated the expression of this pathogen-sensing receptor. In addition, supplementation of OBE reduced plasma concentration of IL-6 demonstrating the anti-inflammatory capacity of OBE. Intravaginal temperature was increased within 1 h after LPS injection, reaching a maximum at around five hours after the LPS administration, which coincides with the peak on plasma of IL-6 concentration. Heifers supplemented with OBE-H tended to have lower maximum temperature in some, but not all days of challenge, and at least numerically spent less time with fever (5 vs 5.8 h).

Interleukin-6, also promotes amino acids uptake by the hepatocytes, and synthesis of acute phase protein in expense of muscle catabolism, also dictated by the
action of IL-6 (Johnson, 1997). Haptoglobin is one of the most prominent APP on ruminants and one of the most widely used as a marker of inflammation. In our study, plasma concentration of haptoglobin from heifers exposed to LPS had a sustained elevated concentration throughout the entire period, suggesting the development of chronic inflammation, and further proving the success of the model on inducing a sustained inflammatory condition. Heifers supplemented with OBE had reduced concentration of haptoglobin in plasma compared with CTL+ heifers ($P < 0.01$; Table 3-4), most likely due to a reduction on circulating IL-6 concentration.

On the first and last day of challenge, leukocyte and neutrophil cell counts were measured. On both days, after 1 h of LPS injection a lymphopenia and neutropenia condition was observed. After immune cells recognize LPS, and become active, a rapid cell migration occur, in an attempt to localize and clear the infection (Griel et al., 1975; Bieniek et al., 1998). This is further corroborated by the increased expression of the adhesion molecule CD11b from heifers exposed to LPS, both on leukocyte and neutrophil populations. Adhesion molecules serves for immune cell transvasation from peripheral circulation into the site of infection, and their expression has been shown to be reduced by anti-inflammatory treatments (Pierce et al., 1996). Heifers supplemented with OBE suffered a similar drop of neutrophils and lymphocytes cell counts to those on the CTL+ treatment after the challenge, but were able to mediate a faster recovery (Fig. 3-7). This effect might be explain by the reduction of expression of adhesion molecules. A reduction of monocyte and neutrophil MFI for CD11b on d 0, and a reduction of MFI from L-Selectin (CD62L), and CD11b on monocytes on d 10 was observed; therefore reducing the ability of this cells to migrate out of circulation. In addition, a decrease on
CD14 MFI, and IL-6 concentration in plasma likely reduced the ability of leukocytes to recognize LPS, and activate immune cells.

The energy metabolism of heifers exposed to sequential increasing doses of LPS was largely affected ($P = 0.02$; Table 3-4). During an infection, the energy expenditure, and glucose utilization by the host is largely increased to support the demand of rapidly dividing immune cells, that become obligated glucose consumers (McGuinness, 2005; Burdick Sanchez et al., 2013; Kvidera et al., 2017). In our study, heifers exposed to LPS had a hyperglycemic phase after 1 h of immunostimulation, followed by hypoglycemia that lasted for the remaining 8 h of the sampling period. This is in agreement with Kvidera et al. (2017), where lactating cows exposed to LPS had increased glucose concentration in plasma during the first h post-challenge, to start to decrease thereafter. Endotoxemia stimulates glycogenolysis and gluconeogenesis in the liver and muscle, which increases plasma glucose concentrations to support the immune system (Burdick Sanchez et al., 2013; Kvidera et al., 2017). The hyperglycemic state is only possible because is coupled with insulin resistance in peripheral tissue to reprioritize, and spare fuels towards immune cells utilization. The hypoglycemic state is the outcome of the inability of the system to keep providing glucose at the same rate as needed by the immune system. In addition, in cattle, glycogen stores are very limited, hence, the capacity to mobilize glucose is very limited (Burdick Sanchez et al., 2013). Heifers supplemented with OBE-H recovered normal glycemia faster on the first day of challenge, when compared with CTL+. A reduction on inflammatory mediators and the development of a more moderate immune response probably demands less glucose for their metabolic purposes, therefore returning to baseline values faster than the CTL+.
Plasma concentrations of NEFA, followed an inverse relationship with glucose during the 8 h post challenge. Probably immediately after the challenge, glucose reserves began to be mobilize to support immune cell activation and rapid proliferation, high concentrations of glucose in plasma inhibits lipolysis, and NEFA concentration decreases. When plasma glucose concentration started to drop, fat reserves began to be mobilized, to provide a source of energy during the hypoglycemic state. This relationship has been previously shown by Burdick Sanchez et al., 2013 in an endotoxin challenge model with Brahman bulls. Furthermore, elevated concentrations of NEFA can impair glycogenolysis (McMahon et al., 1988), further compromising glucose availability.

In order to be able to regulate the magnitude of the inflammatory response, the host relies on several mechanisms to control overexpression of the immune response and endure a sustained presence of endotoxin in circulation, generating tolerance over time. The host system tries to maintain a balance between eliciting an inflammatory response to clear the potential pathogen, while trying to avoid overloading the system with inflammatory mediators when the triggering signal persists. Acyloxyacyl hydrolase (AOAH) is an enzyme that removes acyl chains from the lipid A disaccharide backbone of the LPS molecule. This enzyme is present in antigen-presenting cells and neutrophils (Munford and Hall, 1986), and can decrease the immunogenicity of the LPS molecule. In our model, we accounted for the development of endotoxin tolerance by increasing the dose at every new administration of LPS, however, the largest responses on intake, temperature, and inflammatory marker concentrations were developed by the heifers within the first 2 administrations of LPS. Heifers on the CTL+ group had a 26% reduction of maximum IL-6 concentration between d 0 and d 10 (360 vs. 265 pg/mL). Haptoglobin concentrations had the greatest concentration by d 2, to rebounce on d 9
with a smaller peak. In addition, heifers administered with LPS had the greatest drop in intake the first d of LPS administration, and the highest intravaginal temperatures post LPS administration were achieved during the first two days of challenge. Thus, even though between the first and last dose administered there was more than a 10-fold increase, the greatest responses to the administration of LPS were achieved on the first 2 challenges.

We conclude that supplementation of an olive oil bioactive extract can be a suitable approach to reduce the negative effects of subclinical chronic inflammation in newly weaned crossbreed heifers. The modulatory effect over the inflammatory response seems to be associated with a reduction in the expression of the LPS receptor CD14, partially blocking the downstream signaling cascade, and therefore decreasing the amount of pro-inflammatory cytokine synthesis. Finally, the reduction in body temperature, circulating APP, and the recovery of DMI, can be explained by the reduction of inflammatory mediators present in circulation.

Further research is needed to understand if this model accurately represents the natural challenges experienced by a weaned beef calf, and if supplementation with OBE can improve their performance in a commercial operation.
<table>
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<th>CTL+ % DM basis</th>
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NE, and NEg were calculated with NRC, 2016 (National Academies of Sciences and Medicine, 2016).
Table 3-2. Performance of newly weaned heifers before and during a challenge with increasing doses of LPS over a 10 d period (CTL+), or not challenge (CTL-), and challenged with LPS plus supplemented with olive bioactive extract at 0.04% (OBE-L) or 0.16% (OBE-H) of the diet dry matter.

<table>
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<th>OBE-L</th>
<th>OBE-H</th>
<th>SEM</th>
<th>CTLP + vs OBE</th>
<th>OBE-L vs OBE-H</th>
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<td>0.3</td>
<td>0.24</td>
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Table 3-3. Average intravaginal temperature and average maximum temperature on °C, and hours experienced fever of newly weaned heifers challenged with increasing doses of LPS over a 10 d period (CTL+), or not challenge (CTL-), and challenged with LPS plus supplemented with olive bioactive extract at 0.04% (OBE-L) or 0.16% (OBE-H) of the diet dry matter.

<table>
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<tr>
<th>Item</th>
<th>CTL -</th>
<th>CTL +</th>
<th>OBE-L</th>
<th>OBE-H</th>
<th>SEM</th>
<th>CTLP + vs OBE</th>
<th>OBE-L vs OBE-H</th>
<th>CTL - vs CTL +</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>38.6</td>
<td>39.0</td>
<td>39.2</td>
<td>39.0</td>
<td>0.16</td>
<td>0.49</td>
<td>0.02 &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>38.8</td>
<td>39.6</td>
<td>39.8</td>
<td>39.5</td>
<td>0.17</td>
<td>0.84</td>
<td>0.02 &lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>Time with fever²</td>
<td>0.0</td>
<td>5.8</td>
<td>9.9</td>
<td>5.0</td>
<td>3.41</td>
<td>0.56</td>
<td>0.12</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Time with fever= hours with intravaginal temperature over 39.5°C
Table 3-4. Responses of blood plasma metabolites of newly weaned heifers challenged with LPS (CTL+), or not (CTL-) on day 0 and 10, and challenged with LPS plus supplemented with olive bioactive extract at 0.04% (OBE-L) or 0.16% (OBE-H) of the diet

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatments</th>
<th>SEM</th>
<th>Contrasts and P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL -</td>
<td>CTL +</td>
<td>OBE-L</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>69.4</td>
<td>75.2</td>
<td>75.6</td>
</tr>
<tr>
<td>NEFA µEq/L</td>
<td>1.2</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Haptoglobin ODx100</td>
<td>2.8</td>
<td>4.7</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Table 3-5. Responses of blood plasma metabolites of newly weaned heifers challenged with LPS (CTL+), or not (CTL-) on day 0 and 10, and exposed to LPS plus supplemented with olive bioactive extract at 0.04% (OBE-L) or 0.16% (OBE-H) of the diet

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatments</th>
<th>SEM</th>
<th>Contrasts and P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTL -</td>
<td>CTL +</td>
<td>OBE-L</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>71.8</td>
<td>67.3</td>
<td>67.0</td>
</tr>
<tr>
<td>NEFA µEq/L</td>
<td>1.2</td>
<td>1.3</td>
<td>1.2</td>
</tr>
<tr>
<td>IL-6 pg/mL</td>
<td>26.8</td>
<td>136.6</td>
<td>77.3</td>
</tr>
<tr>
<td></td>
<td>day 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEFA µEq/L</td>
<td>1.3</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>IL-6 pg/mL</td>
<td>17.2</td>
<td>115.2</td>
<td>86.7</td>
</tr>
</tbody>
</table>

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Table 3-6. Responses of blood cell types of newly weaned heifers challenged with LPS (CTL+), or not (CTL-) on day 0 and 10, and challenged with LPS plus supplemented with olive bioactive extract at 0.04% (OBE-L) or 0.16% (OBE-H) of the diet.

<table>
<thead>
<tr>
<th>day 0</th>
<th>Treatments†</th>
<th></th>
<th></th>
<th></th>
<th>SEM</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CTL -</td>
<td>CTL +</td>
<td>OBE-L</td>
<td>OBE-H</td>
<td></td>
<td>CTL + OBE-L vs</td>
<td>CTL - vs OBE</td>
<td>OBE-H</td>
<td>CTL +</td>
</tr>
<tr>
<td>Lymphocytes × 1,000/μL</td>
<td>2.5</td>
<td>0.8</td>
<td>1.5</td>
<td>1.3</td>
<td>0.21</td>
<td>0.05</td>
<td>0.52</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>Monocytes × 1,000/μL</td>
<td>0.6</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.10</td>
<td>0.54</td>
<td>0.62</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>Neutrophils × 1,000/μL</td>
<td>2.4</td>
<td>0.8</td>
<td>1.6</td>
<td>1.3</td>
<td>0.35</td>
<td>0.12</td>
<td>0.51</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>day 10</td>
<td>Lymphocytes × 1,000/μL</td>
<td>2.7</td>
<td>0.9</td>
<td>1.2</td>
<td>1.2</td>
<td>0.48</td>
<td>0.37</td>
<td>0.99</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Monocytes × 1,000/μL</td>
<td>0.4</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.07</td>
<td>0.88</td>
<td>0.98</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>Neutrophils × 1,000/μL</td>
<td>1.8</td>
<td>0.3</td>
<td>0.5</td>
<td>0.6</td>
<td>0.20</td>
<td>0.41</td>
<td>0.62</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
</tbody>
</table>
Table 3-7. Mean fluorescence intensity of expression of cluster of differentiation (CD) 14, 62L, and 11b by peripheral blood monocytes and neutrophils of newly weaned heifers challenged with LPS (CTL+), or not (CTL-) on day 0 and 10, and challenged with LPS plus supplemented with olive bioactive extract at 0.04% of diet (OBE-L) or 0.16% of the diet (OBE-H).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Contrasts</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>d 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocyte</td>
<td>MFI CD14 ×</td>
<td>1,000</td>
</tr>
<tr>
<td></td>
<td>MFI CD62L ×</td>
<td>1,000</td>
</tr>
<tr>
<td></td>
<td>MFI CD11b ×</td>
<td>1,000</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>MFI CD11b ×</td>
<td>1,000</td>
</tr>
<tr>
<td></td>
<td>MFI CD62L ×</td>
<td>1,000</td>
</tr>
<tr>
<td>d 10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MFI CD14 ×</td>
<td>1,000</td>
</tr>
<tr>
<td></td>
<td>MFI CD62L ×</td>
<td>1,000</td>
</tr>
<tr>
<td></td>
<td>MFI CD11b ×</td>
<td>1,000</td>
</tr>
<tr>
<td>Neutrophil</td>
<td>MFI CD11b ×</td>
<td>1,000</td>
</tr>
<tr>
<td></td>
<td>MFI CD62L ×</td>
<td>1,000</td>
</tr>
</tbody>
</table>
Figure 3-1. Timeline of the study.
Figure 3-2. Dry matter intakes of newly weaned heifers exposed to saline, control negative (CTL -), exposed to sequential increasing doses of LPS (CTL +), or CTL + plus supplementation with low or high dose of a olive oil bioactive extract (OBE-L or OBE-H respectively). Panels: (a) Dry matter intake pre challenge, from d -14 until d -1. Effect of treatment ($P = 0.43$). (b) Dry matter intake during the 12 d challenge of sequential increasing doses of LPS, from d -1 until d 12. Effect of treatment ($P = 0.07$). Error bars denote SEM.
Figure 3-3. Intravaginal temperature of newly weaned heifers exposed to saline, control negative (CTL -), exposed to sequential increasing doses of LPS (CTL +), or CTL + plus supplementation with low or high dose of an olive oil bioactive extract (OBE-L or OBE-H respectively). Panels: (a-f) Temperature during 36 h post-challenge on d 0, 2, 4, 8, and 10 respectively. Effects of treatment × time (P < 0.01). Error bars denote SEM.
Figure 3-4. Mean plasma haptoglobin concentrations of newly weaned heifers exposed to saline (control negative; CTL -), exposed to sequential increasing doses of LPS (control positive; CTL +), or CTL + plus supplementation with low or high dose of an olive oil bioactive extract (OBE-L or OBE-H respectively). Effect of treatment × time \( (P < 0.01) \). Error bars denote SEM.
Figure 3-5. Mean plasma IL-6 concentrations of newly weaned heifers exposed to saline (control negative; CTL -), exposed to sequential increasing doses of LPS (control positive; CTL +), or CTL + plus supplementation with low or high dose of an olive oil bioactive extract (OBE-L or OBE-H respectively). Panels: (a) Mean plasma IL-6 concentrations immediately before LPS challenge on h 0, and at h 1, 2, 4, and 8 post-challenge on d 0. Effect of treatment × time ($P < 0.01$). (b) Mean plasma IL-6 concentrations immediately before LPS challenge on h 0, and at h 1, 2, 4, and 8 post-challenge on d 10. Effect of treatment × time ($P < 0.01$). Error bars denote SEM.
Figure 3-6. Mean plasma glucose and NEFA concentrations of newly weaned heifers exposed to saline (control negative; CTL -), exposed to sequential increasing doses of LPS (control positive; CTL +), or CTL + plus supplementation with low or high dose of an olive oil bioactive extract (OBE-L or OBE-H respectively). Panels: (a) Mean plasma glucose concentrations immediately before LPS challenge on h 0, and at h 1, 2, 4, and 8 post-challenge on d 0. Effect of treatment × time ($P = 0.02$). (b) Mean plasma glucose concentrations immediately before LPS challenge on h 0, and at h 1, 2, 4, and 8 post-challenge on d 10. Effect of treatment × time ($P = 0.29$). (c) Mean plasma NEFA concentrations immediately before LPS challenge on h 0, and at h 1, 2, 4, and 8 post-challenge on d 0. Effect of treatment × time ($P < 0.01$). (d) Mean plasma NEFA concentrations immediately before LPS challenge on h 0, and at h 1, 2, 4, and 8 post-challenge on d 10. Effect of treatment × time ($P = 0.01$). Error bars denote SEM.
Figure 3-7. Responses of white blood cell types of newly weaned heifers exposed to saline (control negative; CTL -), exposed to sequential increasing doses of LPS (control positive; CTL +), or CTL + plus supplementation with low or high dose of an olive oil bioactive extract (OBE-L or OBE-H respectively). Panels: (a,b) Cell counts of lymphocytes immediately before LPS challenge on h 0, and at h 1, 2, 4, and 8 post-challenge. Effect of treatment × time on d 0 (a; P < 0.01). Effect of treatment × time on d 10 (b; P < 0.01). Panels: (c,d) Cell counts of monocytes immediately before LPS challenge on h 0, and at h 1, 2, 4, and 8 post-challenge. Effect of treatment × time on d 0 (c; P < 0.01). Effect of treatment × time on d 10 (d; P = 0.27). Panels: (e,f) Cell counts of neutrophils immediately before LPS challenge on h 0, and at h 1, 2, 4, and 8 post-challenge. Effect of treatment × time on d 0 (e; P = 0.04). Effect of treatment × time on d 10 (f; P = 0.07). Error bars denote SEM.
CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

We showed in this study that dietary supplementation of OBE to newly weaned heifers exposed to sequential increasing doses of lipopolysaccharide contributed to reducing some of the negative effects of sustained immune activation. The modulatory effect over the inflammatory response seems to be associated with a reduction in the expression of the LPS receptor CD14, partially blocking the downstream signaling cascade, and therefore decreasing the amount of pro-inflammatory cytokine synthesis. Finally, the reduction in body temperature, circulating APP, and the recovery of DMI, can be explained by the reduction of inflammatory mediators present in circulation.

Additionally, the in vitro batch culture study showed that OA was able to modulate some ruminal fermentation parameters, particularly with forage-based substrates.

These conclusions raise the question of whether OBE modulatory effects on systemic inflammatory response, and the capacity of OA to modify rumen fermentation in vitro can translate into an increase in animal performance, efficiency of feed conversion into kilograms of BW gain, and the shift towards a more contained immune response. Further research is needed to understand if the model used in this study accurately represents the natural challenges experienced by a weaned beef calf, and if supplementation with olive bioactive extracts can improve their performance in a commercial operation.


Dewick, P. M. No Title.


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BIOGRAPHICAL SKETCH

Lautaro Rostoll-Cangiano is originally from San Luis, Argentina. In 2009, he began studying at the National University of Córdoba (UNC) and graduated in 2014 with a degree in agricultural engineering. During his time at UNC he was a TA in ruminant nutrition for undergrads for two years (2013-2015). He worked for the National Institute of Agriculture Technology (INTA) in the area of Animal Production and providing private consulting in cattle nutrition from 2014 until 2016. In 2015, he did an internship at the North Florida Research and Education Center for 6 months, and he came back to Florida in 2016 to pursue his master’s in animal science.