Feeding strategies to improve health and sustainability of dairy cattle

by

Caio Seiti Takiya

B.S., University of São Paulo, 2013
M.S., University of São Paulo, 2016

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences & Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2020

Abstract

Dairy farming is a multi-billion industry that produces high-quality sources of protein, vitamins, and minerals in the form of dairy products to an ever-growing world population. The dairy industry, however, has been challenged to produce more milk using feeds that are not desirable for other livestock (i.e. swine and poultry) or human nutrition. High milk producing cows have greater risk of developing metabolic/infectious diseases and reproductive problems, often associated with immune system dysfunction. Feeding strategies during the transition period primarily targeting the modulation of the immune system have shown positive effects on controlling inflammation, measures of cellular immune response, and overall performance of cows. An immunomodulatory feed additive was fed to cows during the dry period and early lactation to evaluate immune cell responses and overall performance. Immune cells were collected from peripheral blood and uterus of cows. The feed additive modulated uterine immune cell response after parturition, where this effect was dependent on parity and day of lactation. Milk responses to this feed additive were influenced by environmental temperature and humidity that cows experienced during 2 wk before and 2 wk after parturition. The administration of sodium salicylate via drinking water during the first week after parturition altered the abundance of inflammatory mediators in subcutaneous adipose tissue of cows. Changes included an increase in abundance of complement system proteins and interleukin-10 signaling, and evidence for greater monocyte infiltration. The metabolic effects of sodium salicylate observed in earlier studies might be associated with changes promoted in adipose tissue. Finally, two experiments were carried out to demonstrate that conventional diets for lactating cows are relatively efficient at converting human-edible foods in milk, returning greater amounts of digestible essential amino acids than the human-edible feeds provided to cows. Findings such as these can provide the dairy industry more tools for improving herd health and promoting sustainability of the dairy industry as a whole.

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Dr. Barry Bradford

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Acknowledgements

The acknowledgements for my family need to be eternalized in this dissertation. I much appreciate their support and encouragement during my entire life.

To my fellow graduate students/lab mates - Katie, Holly, Billy, Caroline, Steven, Sarah, Turner, Miriam, Eren, Alfredo, all people from the Dairy Research and Teaching Unit, and undergraduate students that worked so hard in all experiments (Amanda, Dani, and Brigid – you rock!) - I would like to say thank you for being part of this journey and helping me so much! My dear friends from Brazil (Dado, Jefferson, Lucas, Renno, Del Valle, and Jose Esler) and the new friends that I met in the US (Aurelio, Joaquin, Mike, Steven, Raphael, Douglas, Oscar, Santiago, Bade, Imalka, Marianna, Julia, and Laura), you made my time in the US much more pleasant that I would expect.

I am thankful to my Committee members that taught me so much and were always present when some unexpected situations came up. Dr. Mendonca, Dr. McGill, and Dr. Drouillard, I have learned more than I would have thought possible with you – Thank you!

I am much appreciated for Phibro research support and funding, in special to Dennis Nuzback. I acknowledge the Coordination for the Improvement of Higher Education Personnel (CAPES, Brasilia, Brazil) for the fellowship (88881.170046/2018-01).

Laman, you are the best! Thanks for all laughs and for making the lab tasks easier and funny. Last but not least, a special thanks to Dr. Bradford for the opportunity to return to KSU as his student and for everything that I have learned with you.

Dedication

This Dissertation is dedicated to my grandmother Emilia – *in memoriam*

Chapter 1 - Review of the literature: Balancing immunity in dairy cattle reproduction

INTRODUCTION

The state of pregnancy represents an extreme challenge for the immune system. From the perspective of the pregnant female's immune system, the fetus is an allograft that contains foreign antigens from the sire. Uterine functions such as implantation and placental growth are complex processes tightly regulated by the interaction between the endocrine and immune systems. There is increasing evidence that this interaction is essential for effective ovarian and uterine functions (Henderson et al., 2003; Padua et al., 2005; McDonald et al., 2006). To support a successful pregnancy, it is advantageous for a dam's immune responses to shift away from inflammatory responses that contribute to fetal rejection and toward anti-inflammatory tolerance (Raghupathy, 1997). Importantly, pregnant females are not immunosuppressed, but rather their immune responses are biased toward an anti-inflammatory phenotype.

For example, transcriptome studies have shown an enrichment of immune-related genes in the endometrium of pregnant cows compared to non-pregnant counterparts (Mansouri-Attia et al., 2009). Mechanisms for uterine tolerance in the cow include reduced expression of major histocompatibility proteins by the trophoblast (outer cell layer of the embryo), recruitment of macrophages (primarily M2 phenotype) to the pregnant endometrium, and modulation of immune-related genes in response to the presence of the conceptus (Oliveira et al., 2012). On the other hand, as gestation comes to an end, cows need to mount an inflammatory response to expel the placenta and debris, to eliminate bacterial contamination, and to promote endometrium regeneration.

Reprogramming immune cell function is likely essential for pregnancy, and its failure or inadequate modulation contributes to embryo loss (Kamat et al., 2016). It is estimated that fetal viability is only achieved in about 55% of fertilizations in healthy cattle, whereas around 70-80% of the total embryonic loss occurs between days 8 and 16 after insemination [day 16 corresponding to the day of maternal recognition of pregnancy; (Diskin and Morris, 2008)]. Optimization of reproductive efficiency is essential for dairy farms to remain economically viable and sustainable (Ribeiro et al., 2012) and understanding the factors that can influence oocyte fertilization and gestation maintenance is of major interest. Therefore, this review will describe the major interactions between the immune and reproductive systems.

IMMUNOREGULATION OF CONCEPTION

The female genital tract is naturally equipped to recognize pathogens and tissue damage (Sheldon et al., 2018). Some uterine, tubal and ovarian cells of the cow express receptors (pattern recognition receptors, PRR; sensors of ‘danger’) recognizing highly conserved microbial molecular signatures (MAMP: microbe-associated molecular patterns) or host-derived molecules indicative of cellular injury (DAMP: damage-associated molecular patterns; DNA fragments, mitochondrial content, but also free fatty acids and carbohydrates). Transmembrane toll-like receptors (TLR) are probably the most classical PRR and are expressed by bovine granulosa cells (Price and Sheldon, 2013), bovine oviductal epithelial cells, and epithelial and stromal cells of the endometrium (Herath et al., 2009; Turner et al., 2014; Danesh Mesgaran et al., 2018). These different compartments are able to mount an early immune response: recognition of MAMP or

DAMP by the genital cells initiate several signaling cascades (through NFκB or MAP kinase pathways, for example), resulting in the expression of pro-inflammatory mediators [e.g. tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-8], antimicrobial peptides and anti-apoptotic factors.

Several studies have demonstrated that insemination leads to a maternal immune response in the female reproductive tract (Schjenken and Robertson, 2014; Bromfield, 2018). One of the first players in controlling the immune response that allows pregnancy is the semen (consisting of spermatozoa and seminal plasma). The presence of spermatozoa in the uterus induces rapid chemotaxis of polymorphonuclear cells (PMN, especially neutrophils), which are detected in the uterus already 0.5 h after AI in horses and pigs (Kaeoket et al., 2003). In cattle, the peak uterine PMN influx occurs in < 2 h (Alghamdi et al., 2009). In all these species, the onset of PMN chemotaxis is rapid and the duration of PMN infiltration relatively short. This ensures effective removal of sperm and bacteria and subsequent return of the endometrium to a normal state, prepared to receive the embryo.

Spermatozoa are not inherently chemotactic, but they activate a complement cascade in uterine secretions, at least in horses and pigs (Troedsson et al., 2005). The complement cascade mediates a series of biological reactions, including vascular permeability, chemotaxis and opsonization for phagocytosis. Activation of the complement (C) cascade cleaves factor C5 into C5a and C5b, of which C5a mediates chemotaxis of PMN (Tizard, 2017). Activated PMN bind to and phagocytize spermatozoa. Rapid removal of sperm is thought to prevent acquired immune responses against sperm (Hansen, 2011). Thus, the fate of most sperm is elimination. However, there is evidence that seminal plasma can also down-regulate immune responses. Gilbert and Fales (1996) reported that seminal plasma inhibited neutrophil aggregation and down-regulated the C3bi

receptor, which is known as complement receptor-3 and mediates phagocytosis of organisms opsonized by complement proteins. This may indicate impaired phagocytic ability of PMN exposed to seminal plasma. Indeed, implantation and live birth rates following in vitro fertilization treatment are significantly improved when woman are exposed to semen at the beginning of pregnancy (Crawford et al., 2015). Aloé et al. (2012) added PMN to bovine uterine epithelial cells in vitro and found the epithelial cells to be capable of producing IL-8 (a chemokine) in response to PMN. Seminal plasma blocked IL-8 stimulated PMN transmigration and reduced the production of reactive oxygen species. However, bull semen has a high sperm concentration and small volume and thus very little seminal plasma. In addition, the fact that embryo transfer results in high pregnancy rates suggests that a maternal immune response is not required to establish pregnancy, at least not before the onset of conceptus elongation.

After ovulation, the oocyte enters the uterine tube (oviduct); the mammalian oviduct is considered a refuge for sperm, and it does not respond to insemination with a high influx of immune cells like the vagina, cervix, and uterus do (Rodriguez-Martinez et al., 1990). In cows, it takes about 6-8 h until enough sperm reach the oviduct for successful fertilization (Hunter and Wilmut, 1983). Although neutrophils are present in the oviduct, prostaglandin E2 released by uterine tube epithelial cells [after luteinizing hormone (LH) stimulation] suppresses the phagocytosis of sperm by neutrophils, thereby supporting sperm survival in the uterine tube (Marey et al., 2014). The interaction between sperm and bovine oviduct epithelial cells in vitro also results in an anti-inflammatory phenotype with an up-regulation of TGFB1 and IL10 (anti-inflammatory cytokines) and down-regulation of TNF and IL1B (pro-inflammatory cytokines; Yousef et al., 2016), which likely increase the tolerance to sperm until fertilization. The LH surge also stimulates localized production of IL-8 and angiogenic factors, basic fibroblast growth factor

and vascular endothelial growth factor A, which are essential for the development of the corpus luteum (CL) and further maintenance of pregnancy (Schams et al., 2001; Berisha et al., 2006; Jiemtaweeboon et al., 2011).

IMMUNOREGULATION OF PREGNANCY MAINTENANCE

In cattle, the first three to four cell divisions post-fertilization occur in the oviduct, such that the embryo enters the uterus approximately 4 days post-fertilization (at the 16-cell to early morula stage). There it undergoes a number of cell divisions to form the morula which, after differentiation, forms a blastocyst consisting of the inner cells mass (which will eventually give rise to the embryo/fetus) and an outer cell mass consisting of trophectoderm cells which ultimately gives rise to the placenta (Fair, 2016). Up to this stage, the embryo is encased in the glycoprotein shell (the zona pellucida) and thus, the endometrial lining is not exposed to paternal antigens until hatching. Following hatching, the conceptus has several hurdles to overcome during the peri-attachment period. It must guarantee consistent progesterone production by the CL while avoiding attack by the maternal immune system (Fair, 2016).

A blastocyst hatches in the uterus at day 9 of pregnancy and initiates the rapid elongation of the trophectoderm at around day 12 (Sakurai et al., 2012). The extraembryonic membrane extends throughout the entire uterine horns by day 24 and subsequently attaches to endometrial cells (Degrelle et al., 2005). During this peri-implantation period, trophoblastic cells produce several molecules such as interferon-tau (IFN-τ), placental lactogen, prolactin related proteins, and pregnancy-associated glycoprotein (PAG). This glycoprotein is a hormone that directs the CL to survive, enlarge, continue producing progesterone and estrogen to suppress menses, and to create an environment suitable for embryo development. Studies have shown that PAG increases from

the beginning to the end of pregnancy, reaching its maximum at parturition (Valadão et al., 2019). Once hatched, the blastocyst forms an ovoid-shaped conceptus between days 12-14 and the elongation process begins. Elongation entails rapid proliferation of the conceptus trophectoderm cells, reaching 3-4 mm or more in length by day 14, and 25 cm or more in length by day 17 (Randi et al., 2016). As the embryo elongates, the trophectoderm and endometrial luminal epithelium become closely apposed. During this period, the conceptus relies on maternal secretions collectively termed histotroph for survival (Bazer, 1975). In contrast to mouse and human species, implantation in cattle is non-invasive. It is characterized by a superficial attachment and adhesion of the trophectoderm to caruncular and intercaruncular areas, commencing about day 19 (Brooks et al., 2014).

Expression of major histocompatibility complex (MHC) antigens on the trophoblast is largely down-regulated although, at least in the cow, there is limited expression of class I MHC molecules by the trophoblast in later pregnancy (Rutigliano et al., 2016). Major histocompatibility class I molecules are expressed on the cell surface of all nucleated cells (i.e. somatic or nonimmune cells) and present peptide fragments derived from intracellular proteins. These peptides are normally derived from the cell's own 'house-keeping proteins' and therefore fetoplacental production of MHC may elicit an immune response by the dam's cytotoxic CD8+ T cells, resulting in cell killing. In other words, class I MHC is a type of cellular identification to distinguish "self" from non-self and avoid natural killer cell attack. However, the lack or low expression of MHC class I molecules also makes the embryo susceptible to lysis by natural killer cells (CD8+ T cells); MHC class I molecules originally inhibit lytic activity through binding to inhibitory receptors (Orr and Lanier, 2010). In the bovine embryo, a non-classical MHC class I antigen (NC1) is expressed (Birch et al., 2008) beginning at the morula stage (Doyle et al., 2009). This NC1 antigen inhibits

the lytic capacity of natural killer (NK) cells, whereas the expression of NC1 can be enhanced with progesterone, IFN-γ, IL-3 or IL-4 (O'Gorman et al., 2010). Down-regulation of MHC antigen expression may be an important requirement for successful pregnancies: cloned bovine conceptuses, which experience high rates of fetal loss, can express aberrantly high levels of MHC class I protein associated with increased accumulation of maternal lymphocytes in endometrial stroma (Hill et al., 2002).

Interferon-τ is secreted by the elongating conceptus, specifically the trophectoderm (Robinson et al., 2006). Recently, researchers demonstrated that embryos at the early morula stage also express IFN-τ when cultured with oviduct epithelial cells (Talukder et al., 2018). It is believed that the luminal epithelium of the uterine endometrium is the primary target for IFN-τ (Roberts et al., 1992; Imakawa et al., 2005). However, IFN-τ can reach the stroma, the uterine myometrium (Johnson et al., 1999; Hicks et al., 2003), circulating immune cells and the ovaries (Shirasuna et al., 2012). IFN-t released by the trophoblast prevents the development of the luteolytic mechanism by inhibiting the expression of the oxytocin receptor gene in the endometrial epithelium, which prevents the release of luteolytic pulses of prostaglandin F2α (Dorniak et al., 2013). Interferon-τ triggers the expression of genes in the endometrium to promote conceptus growth and development and induce uterine receptivity (Song et al., 2007; Mansouri-Attia et al., 2012). The conceptus INF-τ also affects immune function genes in both the uterus and peripheral blood (Yankey et al., 2001; Han et al., 2006; Gifford et al., 2008).

Type I interferons, including IFN-τ, promote immunosuppressive functions including the induction of regulatory T cell differentiation. Regulatory T cells can be defined as a T-cell population that functionally suppresses an immune response by influencing the activity of another cell type (Shevach, 2004). Regulatory T cells have a major role in inducing and maintaining

peripheral tolerance. For instance, CD4+ cells treated with IFN-τ exhibit regulatory activity by expressing transforming growth factor beta (TGFB) and IL-10 (Mujtaba et al., 1997). Indeed, there is evidence that IFN-τ generates anti-inflammatory immune responses in the bovine uterus (Rashid et al., 2018). These authors cultured blood mononuclear cells (PBMC) with an uterine flush with multiple embryos collected on d 7 from donor cows where they found a down-regulation of pro-inflammatory cytokines (TNF-α and IL1-β) and reported an up-regulation of IL-10 (an anti-inflammatory cytokine). Furthermore, an addition of specific anti-IFN-τ antibody to the uterine flush inhibited the effects on PBMC, indicating that IFN-τ is a major factor for such immune modulation (Rashid et al., 2018).

Although studies on the involvement of the maternal immune system in the establishment of pregnancy in cattle are few in number, particularly for early pregnancy, monocytes, macrophages and dendritic cells (DC) appear to be the key actors during the implantation period (Fair, 2015). Macrophage recruitment at the early pregnancy stage is associated with clearing cellular debris and regulating apoptosis (Straszewski-Chavez et al., 2005), as well as regulation of placental lactogen concentrations at the fetal-maternal interface. However, these roles may be more important for mice and humans, where implantation is quite invasive. The Bovidae diverged as a separate family of pecoran ruminants about 24-29 million years ago during the Late Oligocene epoch (Hassanin and Douzery, 2003). While the basic pattern of reproduction in ruminants is like other mammals, there are distinct features including placental anatomy. Ruminants possess an epitheliochorial placenta characterized by apposition of fetal and maternal tissues. Invasion of the maternal system either does not occur or is limited to migration of trophoblast cells into the maternal endometrial epithelium to form a syncytium (Vogel, 2005; Wildman et al., 2006). As compared to species with invasive placenta, access of maternal leukocytes to fetal placental tissue

is restricted physically by several cells layers and there may be reduced opportunity for pieces of trophoblast tissue to enter the lymph and peripheral circulation of the dam.

In cattle, using immunofluorescent labeling of immune cell markers, authors observed an initial expansion of monocytes, macrophages, and DC populations in the endometrial stroma on day 13 of pregnancy (Mansouri-Attia et al., 2012). At the same time there was a decline in the number of CD11b positive cells; the loss of CD11b expression is characteristic of monocytes acquiring a stationary phenotype (Mansouri-Attia et al., 2012). This observation suggests that monocyte-derived cells accumulate in the endometrial stroma in response to the embryo. Similarly, a human and mouse specific role of dendritic cells is the involvement in decidua (modified mucosal lining in the uterus for pregnancy preparation) formation (Blois et al., 2007). Leung et al. (2000) also demonstrated the presence of CD4+ T cells, CD21+ B-cells, and CD14+ macrophages in the endometrium of cows on day 16 of pregnancy. In cattle, macrophages are rare in the endometrium of non-pregnant cows but accumulate in large numbers during pregnancy (Oliveira and Hansen, 2008). In the absence of infection, endometrial macrophages would be preferentially directed towards an M2 phenotype, promoting inhibition of inflammation, angiogenesis, and tissue remodeling (Allavena et al., 2008; Zhang et al., 2011). Oliveira et al. (2010) described several genes associated with an M2 macrophage phenotype that were upregulated in endometrial CD14+ cells.

Uterine DC have been proposed to serve as a switchboard between fetal rejection and tolerance (Kammerer et al., 2000; Gardner, 2003; Blois et al., 2005). Dendritic cells are the most potent antigen presenting cells (APC) involved in the innate immune response and in the maintenance of tolerance (Moretta et al., 2005). Immature DC exhibit a tolerogenic phenotype, characterized by low expression of costimulatory molecules (CD40, CD80, and CD86), low

production of proinflammatory cytokines, increased production of IL-10, and capacity to induce regulatory T cells, all of which promote pregnancy maintenance (Moretta et al., 2005). The expansion of these populations in the maternal endometrium is likely to be induced by IFN-τ. In murine pregnancies with high abortion rates, an increase of mature APC can be observed (Blois et al., 2005). By blocking crucial ligands required on APC to induce T-cell activation, mechanisms of fetal tolerance are restored in abortion-prone pregnancies (Zhu et al., 2005). Progesterone has been shown to inhibit mature DC as well as DC-stimulated proliferation of T cells in a receptor-mediated fashion (Butts et al., 2006).

Progesterone is critical for not only the establishment but also for the maintenance of pregnancy, as its functions support ovulation and uterine as well as mammary gland development. Compared to the low levels (1-2 nmol/L) during the follicular phase of the menstrual cycle, progesterone concentrations increase to 15-20, 35-50, and 20-40 nmol/L in the early- mid-, and late-luteal phases, respectively. The major source of progesterone during pregnancy is the corpus luteum of the ovary and, if pregnancy occurs, progesterone production is eventually sustained by the placenta in many species, including humans and rodents (Tibbetts et al., 1999). In cattle, the placental contribution to progesterone synthesis is low throughout most of pregnancy. The need for progesterone in maintaining pregnancy is shown by the fact that blocking progesterone binding sites causes abortion in humans and in various animal species. Besides its endocrine effects, progesterone acts as an ‘immunosteroid’.

Progesterone blocks very early T-cell lymphopoiesis during pregnancy (Mesiano et al., 2002) and controls the bias towards a pregnancy-protective immune milieu (Tan et al., 2012), which involves an immunomodulatory protein known as progesterone-induced blocking factor (Hudić et al., 2009). Progesterone-induced blocking factor (PIBF1) changes cytokine ratios to

decrease cell-mediated responses and affects the helper T cell 1 (Th1)/ Th2 balance during pregnancy in mice (Szekeres-Bartho and Wegmann, 1996). T helper cells are classified into Th1 and Th2 phenotypes based on the profile of cytokines that they produce. Th1 cells generate cytokines such as IFN-γ, IL-1β, and TNF-α and support cell-mediated immunity (Mosmann et al., 1986). On the other hand, Th2 cells produce IL-4, IL-5, and IL-10, and down-regulate Th1-cell responses (Adkins et al., 2004). Th1 cytokines inhibit the outgrowth of trophoblast cells (Berkowitz et al., 1988) and play a central role in acute allograft rejection (Burns et al., 2005), whereas Th2 cytokines induce immunological allograft tolerance (Li et al., 1998). Demonstrating the capacity of progesterone in influencing immune tolerance, the administration of progesterone blocked tissue graft rejection in the uterus of sheep (Majewski and Hansen, 2002). Treating ovariectomized animals with progesterone decreases the ability of cows and sheep to overcome uterine infections (Hansen, 2011).

The embryo employs several mechanisms to avoid dam's rejection, including the production and release of proteins (PAG) to regulate ovarian function and maintain CL progesterone production, altered expression of MHC antigens to avoid the attack of immune cells, and an overall anti-inflammatory response triggered by IFN-τ. Endometrial macrophages with M2 phenotype and immature DC also attenuate pro-inflammatory responses of the dam during early pregnancy.

INFLAMMATION AND INFERTILITY

Inflammatory responses in the reproductive tract can also reduce fertility, as described for the induction of inflammation by administration of lipopolysaccharide (LPS) or killed sperm (Rozeboom et al., 2000; Alghamdi et al., 2004). After calving, the uterine lumen of cows presents

cellular debris of placental and maternal origin and it is commonly colonized by microorganisms. Because of the bacterial infiltration, postpartum cows mount an early inflammatory response to rapidly clear the uterine environment; endometrial cytology assessments often reveal that 35-40% of cells are PMN around 7 d postpartum (Gilbert and Santos, 2016; Cheong et al., 2017). Cows with a more robust recruitment of PMN into the uterus on the day of calving were more likely to have ovulatory first dominant follicles (Cheong et al., 2017).

Although an early inflammatory response is beneficial for cows, it is important to distinguish local intrauterine cell mobilization and systemic inflammation. While virtually all cows have intrauterine inflammation early post-partum, a smaller proportion of cows will have a systemic immune response characterized by elevation of circulating acute phase proteins such as haptoglobin (Bionaz et al., 2007; Krause et al., 2014). A persistent high blood haptoglobin concentration during early lactation is negatively associated with ovulation rates, whereas a rapid intrauterine inflammatory response is associated with high odds of ovulation of the first dominant follicle in cows (Cheong et al., 2017). Because ovulation, oocyte fertilization, and maintenance of gestation require a fine-tuned immune response, it is expected that excessive or persistent inflammation has deleterious impacts on fertility. Inflammatory disorders may influence several components of the reproductive processes including endocrine function, ovarian function and oocyte quality, uterine function, and implantation.

Hypothalamic and pituitary function

Reproduction in mammals is dependent on the regulation of the neurohormonal system called the hypothalamic-pituitary-gonadal axis. Gonadotropin releasing hormone (GnRH) is a decapeptide synthesized in the hypothalamus and released into the hypophyseal portal system to

act on gonadotropic cells in the anterior pituitary gland (Jiang et al., 2001). In the pituitary, GnRH regulates the secretion of LH and follicle-stimulating hormone (FSH; Jiang et al., 2001), both essential for steroidogenesis, gametogenesis, and ovulation (Durán-Pastén and Fiordelisio, 2013). Inflammatory challenges through administration of LPS have suppressed the circulating concentrations of LH in several species, including sheep and cattle (Harris et al., 2000; Suzuki et al., 2001).

Administration of LPS blocked the preovulatory rise in estradiol and blocked or delayed the LH surge in ewes (Battaglia et al., 2000). Endotoxin inhibition of the estradiol spike was partially due to inhibition of LH pulsatility (Battaglia et al., 2000). Evidence that endotoxin alters circulating concentrations of LH, acting at the hypothalamus through altered GnRH production and/or release has been reported in some studies. Culture of dispersed sheep pituitary cells with endotoxin increased accumulation of LH in the cell culture media after 24 h (Coleman et al., 1993). Endotoxin lowered the collection rate of GnRH from the hypophyseal portal blood as well as GnRH pulse amplitude in sheep (Battaglia et al., 1997). This study also revealed suppressed LH pulse frequency and stimulated increased cortisol, progesterone, and body temperature (Battaglia et al., 1997). The administration of endotoxin increases the plasma concentration of TNF-α (Coleman et al., 1993). In ovariectomized rats, central administration of TNF-α antibody attenuated endotoxin suppression of mediobasal hypothalamus electrical activity and improved LH pulsatility (Yoo et al., 1997). Similar effects have been reported in cows; injecting cows with endotoxin caused a systemic inflammatory response characterized by fever and decreased paraoxonase in serum and indirectly affected follicle function by depressing LH pulse secretion from the anterior pituitary (Peter et al., 1990; de Campos et al., 2017). Decreased LH pulse

frequency is an important feature of non-ovulatory cows (Canfield and Butler, 1990; Cheong et al., 2016).

Ovarian function and oocyte quality

Ovarian follicular development involves a complex series of coordinated events, including primordial follicle activation, growth of primary and secondary follicles, formation of an antrum cavity, oocyte maturation, steroidogenesis, and ovulation (Silva et al., 2020). During these growth and differentiation processes, paracrine factors mediate the communication among oocyte, granulosa, and theca cells (Hsueh et al., 2015). Cytokines such as TNF-α and IL-1β are involved in controlling follicular development and ovulation. Their functions include regulation of cellular proliferation/differentiation, follicular survival/atresia, and oocyte maturation (Field et al., 2014). It has been reported that, depending on the stage of development, TNF-α may regulate differentiation of granulosa cells and apoptosis (Manabe et al., 2008; Glister et al., 2014). An in vitro study demonstrated that TNF-α promotes growth and antrum formation in bovine secondary follicles (Casali et al., 2008). The ability of TNF-α to promote the increase in follicular diameter it is likely because these follicles have receptors for TNF-α (TNFR1 and TNRF2) in their oocyte and granulosa cells (Silva et al., 2017). Authors demonstrated that TNFR2 can induce gene transcription associated with cell survival, growth, and differentiation (Wajant et al., 2003). Interleukin-1β acts in the control of follicle development by facilitating granulosa cell proliferation and preventing premature differentiation (Silva et al., 2020). Although inflammation mediators are required for folliculogenesis, the excessive concentration of these metabolites may be detrimental to ovarian function and oocyte development.

Because of the intimate anatomic relationship between the utero-ovarian vein and the ovarian artery in cattle that allows the counter-current transfer of metabolites, the ovary is exposed to higher concentrations of bacterial and other inflammatory products from the uterus during the early post-partum period. Exposure to high concentrations of pro-inflammatory mediators likely influence hormonal secretions in ovaries. For instance, in vitro exposure of theca interna and granulosa cells to TNF-α decreased androstenedione and estradiol secretion (Williams et al., 2008). In vitro exposure of granulosa cells to LPS resulted in reduced expression of aromatase and lesser estradiol secretion (Herath et al., 2007). Elevated follicular fluid TNF-α concentrations are associated with recovery of poor-quality oocytes in women (Lee et al., 2000). The exposure of porcine oocytes to TNF-α delays oocyte maturation and promote abnormalities in chromosomal alignment (Ma et al., 2010). Tumor necrosis factor-α has been shown to inhibit secretion of estradiol and progesterone in bovine granulosa cells in vitro (Spicer, 1998). In cows, the increased expression of pro-inflammatory cytokines in the granulosa is associated with ovulation failure and follicular cyst formation (Baravalle et al., 2015). Taking all these results together, aberrant inflammation signals can alter normal ovarian hormone production and follicular dynamics, resulting in decreased oocyte quality, anovulation, and infertility.

Distant organ diseases are detrimental for fertility

Further evidence of indirect detrimental effects of inflammation on reproductive processes comes from the observation that inflammation in distant sites - especially mastitis - impairs reproductive performance. Several studies in which cows have been classified based on the presence of clinical or subclinical mastitis indicate that occurrence of mastitis is associated with reduced probability of establishment of pregnancy and increased odds of pregnancy loss (Hansen

et al., 2004). Ribeiro et al. (2013) evaluated the effects of post-partum diseases on pregnancy rates after the first insemination of grazing cows. As expected, authors reported lower risk of pregnancy when cows had calving problems [0.38 odds ratio (OR), 95% confidence interval (CI): 0.22 to 0.66), metritis (0.27 OR, 95% CI: 0.13 to 0.54), or clinical endometritis (0.55 OR, 95% CI: 0.37 to 0.81). However, cows that experienced mastitis (0.71 OR, 95% CI: 0.49 to 1.05), respiratory problems (0.44 OR, 95% CI: 0.17 to 1.11), or digestive problems (0.24 OR, 95% CI: 0.09 to 1.57) after calving also had lesser chances of being pregnant after artificial insemination. More recently, a meta-analysis showed decreased risk of conceiving at first service (0.84 OR; 95% CI: 0.77 to 0.91) and greater risk of pregnancy loss (1.81 OR; 95% CI: 1.50 to 2.19) in cows that experienced mastitis compared with cows that not experienced mastitis (Dolecheck et al., 2019).

Several epidemiological studies have shown that mastitis decreases conception risk (Lavon et al., 2011) and increases the number of inseminations required per conception as well as the interval from calving to conception (Barker et al., 1998; Schrick et al., 2001). In a study controlling for endometritis and other variables, such as post-partum uterine disease, parity, body condition score and days in milk at first service, somatic cell count remained a significant contributor to reduced risk of pregnancy (Cheong et al., 2011). The mechanism by which an immune response in one region of the body causes changes in the reproductive tract is likely to involve release of bacterial products, cytokines and chemokines from the site of infection that affect the hypothalamus, pituitary and/or reproductive tract (Hansen, 2011).

Studies have shown that intramammary LPS may translocate into systemic circulation, reaching 5- to 18-fold greater plasma concentrations compared with non-infected cows (Hakogi et al., 1989; Dosogne et al., 2002). Interestingly, clinical mastitis caused by Gram-negative bacteria had a more detrimental effect on probability of conception than clinical mastitis caused by Gram-

positive bacteria or other organisms (Hertl et al., 2010). Endotoxin has been reported to affect various tissues involved in reproduction, including the hypothalamus, ovary, and endometrium, as described earlier. The key cell wall component of Gram-positive bacteria that is involved in induction of inflammation is lipoteichoic acid (LTA). Although LTA has been shown to affect fertility in the same manner as LPS in mice, a much greater dose of LTA was needed to show a similar effect as that of LPS (Kajikawa et al., 1998). This may explain differences in the effects of Gram-negative and Gram-positive mastitis on the probability of conception. Furthermore, there is evidence in the literature that mastitis has a carryover effect on fertility. For instance, mastitis had a negative impact on reproductive outcomes regardless of whether it occurred before the first insemination, between first insemination and conception, or after conception (Perrin et al., 2007; Lavon et al., 2011; Albaaj et al., 2017). The negative impacts of mastitis might be associated with impaired ovarian function and embryo development after clinical or subclinical mastitis events. Authors reported a 40% drop in follicular steroids concentration and depressed follicular growth of synchronized cows experimentally induced subclinical mastitis (Furman et al., 2014; Rahman et al., 2012). There is evidence that non-uterine diseases also impairs the early conceptus elongation and secretion of IFN-τ (Ribeiro et al., 2016).

Anti-inflammatory treatments

The bovine endometrium contains relatively large amounts of arachidonic acid that can be rapidly converted to different products such as prostaglandin $F_{2\alpha}$ and prostaglandin E_2 (Salamonsen and Findlay, 1990). Free arachidonic acid (derived from membrane phospholipids) is converted to prostaglandins by the action of cyclooxygenase (COX) enzymes COX-1 and COX-

2. Non-steroidal anti-inflammatory drugs (NSAID) may inhibit both COX-1 and COX-2 enzymes and thus, decrease prostaglandin release (Cheng et al., 1998).

Despite the increased data associating inflammation with infertility, treatments with anti-inflammatory drugs to improve reproductive outcomes have generated inconsistent outcomes. The administration of NSAID before ovulation increased the incidence of follicular cysts (Pugliesi et al., 2012), and their administration at the time of artificial insemination did not improve pregnancy rate (Heuwieser et al., 2011). On the other hand, NSAID administration at the time of embryo transfer improved pregnancy rates (10 to 25%), especially when transferring low-quality embryos (Scenna et al., 2005; Aguiar et al., 2013). The administration of ibuprofen lysinate 1 h before embryo transfer improved pregnancy rates in comparison with control in heifers (82 vs. 56%; Elli et al., 2001).

Studies have suggested that controlling inflammation during clinical presentation of an inflammatory disease may improve subsequent reproductive performance of dairy cows. For instance, fewer cows that were treated with meloxicam (an NSAID) during clinical mastitis were removed from herds due to failing to conceive [3.4% vs 8.4% for meloxicam and control groups, respectively; OR = 0.37, 95% CI: 0.16 to 0.86 (McDougall et al., 2009)]. McDougall et al. (2016) demonstrated in a randomized clinical trial that the administration of meloxicam as an adjunct during antimicrobial treatment of clinal mastitis increased the conception risk at the first insemination postpartum as well as the proportion of cows pregnant by day 120 of lactation.

CONCLUSIONS

The immune system plays an integral role in affecting reproductive function. Although an immune response is elicited following insemination, spermatozoa that reach an oviduct seems to

be protected from immune cells due to suppressive effects of prostaglandin E_2 on phagocytic capacity of neutrophils. After fertilization, the embryo develops an outer cell mass called the trophoblast that express a non-classical MHC class I that avoids killing by natural killer cells. Trophoblastic cells also produce IFN-τ, responsible for maintaining CL function and at the same time generating anti-inflammatory immune responses in the bovine uterus.

Because of the anatomic relationship between uterine and ovarian vessels, compounds from the uterus after parturition (such as endotoxins and pro-inflammatory mediators) may appear in high concentrations at the ovary and negatively influence ovarian function and folliculogenesis. Diseases in distant organs can cause negative impacts in reproductive performance such those observed in cows with metritis or endometritis. Several studies have associated clinical mastitis with subsequent poor reproductive outcomes; however, when adding anti-inflammatory drugs to antibiotic treatments for mastitis, cows had greater chances for pregnancy in comparison with cows that did not receive anti-inflammatory drugs.

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Chapter 2 - Effects of an immunomodulatory feed additive on performance, inflammation biomarkers, and uterine immunity of transition cows

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ABSTRACT

OmniGen-AF® (OMN, Phibro Animal Health, Teaneck, NJ) is an immunomodulatory feed additive that has shown positive effects on performance, immunity, and health of mammals. The main objective of this study was to determine the influence of feeding OMN during the dry period and early lactation on uterine health by measuring the oxidative burst response of granulocytes and the presence of pathogenic bacteria in the endometrium of postpartum cows. We also evaluated feed intake and feeding behavior, milk yield and composition, blood metabolites including markers of body fat mobilization, acute-phase proteins, fatty acids, and oxylipids. Forty-two cows entering in their second lactation were blocked by the expected calving date and assigned to control (CON) or top-dressed OMN (56 g/d) from dry-off until 49 days in milk. Due to diseases and twins, only the data from 34 cows were used for pre-partum analysis and data from 28 cows were used for post-partum analyses. Statistical analyses were performed using mixed models with covariate adjustments [e.g. previous milk production, parity group, temperature-humidity index (THI) around parturition, etc.]. Stepwise backward elimination was performed in statistical models when fixed effects had *p*-values > 0.15. Treatments did not alter feed intake either during pre-partum or

post-partum periods, however, OMN cows had larger and fewer meals throughout the day in post-partum comparison with CON (1.72 vs 1.82 kg and 14.9 vs 13.3 meals/d, for CON and OMN, respectively). Milk yield was reduced in OMN group compared with CON group (51.8 ± 1.19 vs. 53.3 ± 1.22 kg/d), and blood markers (free fatty acids and beta-hydroxybutyrate) of adipose tissue mobilization were also lower in OMN cows. There was an interaction between THI around parturition and treatments for milk yield, where CON cows produced more milk in thermoneutral conditions, but OMN cows better maintained milk yield when transitioning at a mean THI > 66. There was no evidence for treatment effects on blood acute-phase protein haptoglobin and α-1-glycoprotein concentrations. Cows fed OMN had greater plasma concentrations of palmitic acid, linoleic acid, and stearic acid. OmniGen-AF increased plasma concentrations of oxylipids downstream of arachidonic acid metabolism, including 6-keto-prostaglandin F1α and thromboxane B2. Uterine cytobrush did not show differences in granulocyte infiltration, but flow cytometry analysis revealed an interaction between treatment and time for oxidative burst potential in endometrial granulocytes, where OMN cows reduced oxidative burst potential of granulocytes collected on d 10 of lactation, but increased the same variable on d 38 of lactation. The presence of pathogenic bacteria related with metritis/endometritis was not altered by treatments. This study did not show evidence that OMN can alter granulocyte response in peripheral blood but modulated uterine granulocyte response. However, OMN adversely affected milk yield, which might be associated with altered feeding behavior during lactation and lower blood concentrations of free fatty acids and beta-hydroxybutyrate.

INTRODUCTION

The transition from a pregnant and non-lactating stage to a lactating and non-pregnant state is the most tumultuous period experienced by a dairy cow, where several metabolic and hormonal changes occur to direct stored and consumed nutrients to colostrum and milk synthesis. The sudden increase in nutrient requirements for lactation is not followed by an increase in nutrient consumption to the same extent, resulting in a negative energy balance for some weeks (Bell, 1995). To compensate for the deficit between nutrient intake and requirements, adipose tissue is broken down and free fatty acids (FFA) are released into the bloodstream. Free fatty acids can undergo β-oxidation in the mitochondria of cells and supply acetyl-CoA to the TCA cycle. Free fatty acids can also be metabolized in the liver to triglycerides and ketone bodies [if the mitochondrial β-oxidation capacity is exceeded; (Drackley, 1999)].

Although the mobilization of adipose tissue is a physiological adaptation, accumulation of FFA and ketone bodies in the bloodstream have been associated with increased risk of diseases (Contreras and Sordillo, 2011a), potentially because these metabolites can lead to immune dysfunction. For instance, neutrophils cultured in media with increasing FFA concentrations (ranging from 0 to 2 m*M*) had decreased viability (%) at the highest FFA concentration (Scalia et al., 2006). Culturing bovine endothelial cells with high concentrations of FFA increased the expression of pro-inflammatory cytokines and chemokines, similar to what is observed when culturing cells with lipopolysaccharides (LPS) (Contreras et al., 2012). Moreover, induced hyperketonemia in dairy cows impaired neutrophil migration to the mammary gland of dairy cows after intramammary lipopolysaccharide challenge (Zarrin et al., 2014).

In addition to metabolic and hormonal changes, parturition is a stressful event that often causes endometrial damage and facilitates uterine infections. If the innate immune response of

cows is not able to rapidly neutralize pathogens, uterine disease can become established. The necessity of cow survival following pathogen invasion alters glucose partitioning to the immune system instead of nonessential physiological processes such as reproduction and lactation. For example, an artificially triggered immune response by lipopolysaccharide administration in mid-lactation cows consumed more than 1 kg of circulating glucose in blood within 6 h (Kvidera et al., 2017). An activated immune system may therefore decrease productivity of dairy cattle with health disorders. Besides the costs associated with milk loss, clinical treatments, culling and maintenance of replacement animals may have a financial impact estimated at approximately $650 million annually in the United States (Sheldon et al., 2009).

OmniGen-AF® (OMN; Phibro Animal Health, Teaneck, NJ) is an immune-modulating feed additive composed of a blend of ingredients that includes silicon dioxide, aluminosilicate, brewers dehydrated yeast, dried *Trichoderma longibrachiatum* formation products, and B-complex vitamins (the complete formulation is proprietary). Promising results with OMN supplementation, such as increased transcript abundance of L-selectin (adhesion molecule critical for migration) and oxidative burst (killing mechanism) intensity of blood neutrophils from ruminants, have been reported in the literature (Wang et al., 2004; Ryman et al., 2013). Histological analysis of uteri of murine females fed OMN revealed a trend to decrease the number of endometrial fibrotic lesions and decreased incidence and severity of myometrial vascular fibrosis compared to the control group (Jorgensen-Muga et al., 2018). Although there are reports associating OMN supplementation with peripheral neutrophil function and with improvements in uterine health, literature lacks studies evaluating neutrophil function in the endometrium of early lactation cows. We hypothesized that OMN dietary supplementation during the dry period and early lactation would promote neutrophil activity both systemically (blood) and locally

(endometrium), reducing the presence of potentially pathogenic bacteria in the uterus and improving overall performance of cows. This study evaluated effects of OMN on feeding behavior, milk yield and composition, blood analytes (including markers of inflammation and adipose tissue mobilization), complete blood count (CBC), peripheral and uterine granulocyte oxidative burst potential, and presence of pathogenic bacteria in the uterus of transition dairy cows.

MATERIALS AND METHODS

This study was carried out under the approval of the Institutional Animal Care and Use Committee of Kansas State University (protocol #3933.1). This study was carried out between June 2018 and June 2019 at the Dairy Teaching and Research Center of Kansas State University, Manhattan.

Animals, design, treatments, and management

Forty-two Holstein cows, entering at least their second lactation, were enrolled at dry-off in a randomized block design. Cows were blocked by expected calving date and parity and assigned randomly to treatments within block. Treatments were either control or OMN (56 g/d OmniGen-AF, item #17-3527; Phibro Animal Health) from the day of dry-off (67 ± 3.0 d before the actual calving date) until 49 DIM. OMN was provided as odorless gray granules. Cows were housed in a free stall pen equipped with headlocks from dry-off until d -30 relative to the expected calving date (far-off period). To individually provide OMN without cross-feeding, cows assigned to OMN treatment were allowed to consume the top-dressed OMN before the other cows had access to feed. Cows were fed once daily with a TMR formulated according to NRC (2001; Table 2-1), and OMN was top-dressed on the feed in front of designated cows immediately after feeding

(0700 h). After the far-off period, cows were moved into a maternity barn with access to feed and treatments controlled by an electronic gating system (Roughage Intake System; Insentec B. V., Marknesse, the Netherlands) until calving (close-up period). Cows were fed twice daily (0800 and 1700 h) in equal proportions with a diet similar to that in the far-off period except for the addition of anionic salts (660 g/d Animate, Phibro Animal Health) hand-mixed into the TMR to maintain urine pH between 5.5-6.0. Urine pH was measured twice per wk over the close-up period using pH tapes with 0.5-unit increments.

Upon calving, all cows were offered 20 L Bovi Fresh solution (22.5 g/L, Aspen Veterinary Resources, Ltd., Liberty, MO), milked, and moved into a tie-stall facility within 12 h. Cows were fed *ad libitum* a lactation TMR twice daily (0500 and 1800 h, 70% of total daily ration provided at a.m. feeding) and milked thrice daily (0400, 1100, and 1800 h). OmniGen was top-dressed on the feed twice daily (28 g/feeding) and mixed with the upper 2/3 of the TMR. The diet was formulated to meet or exceed nutrient requirements of lactating cows according to NRC (2001). Cows with high urinary ketone concentrations (≥80 mg/dL) for 1 d or moderate ketones (40–80 mg/dL) for 2 consecutive d were treated with Keto-gel (310 g/treatment; 94% propylene glycol, 1.9% niacin, 1.9% choline, 1.6% methionine, 129 IU/g of vitamin A, 32 IU/g of vitamin D, 0.32 IU/g of vitamin E; Jorgensen Labs, Loveland, CO) for 3 d at 0700 h (CON: n = 3 and OMN: n = 1). Cows exhibiting a temperature >39.4°C were treated with either i.m. ceftiofur hydrochloride (Excenel RTU, Zoetis Animal Health, Florham Park, NJ) or ampicillin (Polyflex, Boehringer Ingelheim, Ingelheim am Rhein, Germany) per label instructions (CON: n = 5 and OMN: n = 2). Cows calving with twins and cows that become chronically ill with periparturient health problems were removed from the study, although prepartum data and clinical outcomes of those removed for health reasons were included in analyses. Data from 34 cows (CON = 15 and OMN = 19) pre-

partum and 28 cows post-partum (CON = 15 and OMN = 13) were used for statistical analysis and reasons for cow removal included: left displaced abomasum (n = 1 OMN), failure to adapt to the electronic bunk system (n = 1 OMN), milk fever (n = 1 OMN), joint issues and hock abscess (n = 2 OMN; far-off period), twins (n = 3; 1 CON and 2 OMN), not pregnant (n = 2 OMN), large ketones (n = 1 OMN), mastitis (n = 1 OMN), suspected milk fever (n = 1 OMN), and retained placenta (n= 1 OMN). Previous mature equivalent milk (Prev305ME), fat (PrevF305M) and protein previous (PrevP305M) were retrieved from PCDART software (Dairy Records Management Systems, Raleigh, NC) and used in milk yield and component statistical models. Control group cows had (mean±SD) 711 ± 59.9 kg BW, 2.93 ± 0.29 BCS, 14,925 ± 1,416.7 kg Prev305ME, and 1.73 ± 0.96 parities at enrollment. Cows in OMN group had 677 ± 83.7 kg BW, 2.92 ± 0.21 BCS, 14,325 ± 1,800.5 kg Prev305ME, and 1.46 ± 0.66 parities at enrollment.

Dietary ingredients were collected every week throughout the experimental period, pooled by trimester, and analyzed for chemical composition using wet chemistry methods by the Dairy One Forage Laboratory (Ithaca, NY). Chemical composition of diets used throughout the experiment are shown in Table 2-1. During the summer months, the maternity barn and tie-stall facility were both cooled using evaporative pads, whereas the pen where far-off cows were housed was equipped with water sprinklers. Temperature and relative humidity (RH) were recorded every hour in maternity and tie-stall barns using data loggers (UX100-003 Onset HOBO Data Loggers, Bourne, MA). Temperature-humidity index (THI) was calculated and the average THI from 2 wk before to 2 wk after parturition were included in statistical model. THI was calculated according to Ravagnolo et al. (2000) as follows: $THI=[(1.8\times Tdb)+32] - (0.55 - 0.0055\times RH)\times(1.8\times Tdb-26)$, where Tdb is the dry bulb temperature (°C). Rectal temperature was measured daily (0700 h)

during lactation using a veterinary thermometer (0.1°C accuracy; GLA M700, GLA Agricultural Electronics, San Luis Obispo, CA).

Feeding behavior, milk composition, and blood variables

Individual feed intake and feeding behavior were recorded from close up period until 49 DIM by electronic systems. The tie-stall facility was equipped with individual feed bunks suspended from load cells with bunk weight monitored continuously by computer. Feeding behavior variables were calculated according to Mullins et al. (2012). All feeding activity, including meal length and size, was recorded electronically. Meals were combined if the inter-meal interval was less than 12 min. As-fed ration intake was adjusted for DM content to determine meal and daily DMI. Cows were milked thrice daily in a double-6 herringbone milking parlor, and milk yield was recorded at each milking. Milk samples were collected at each milking on 3 consecutive days each week and were analyzed for concentrations of fat, true protein, lactose (B-2000 Infrared Analyzer; Bentley Instruments, Chaska, MN), somatic cells (SCC 500, Bentley Instrument), and urea nitrogen (MUN spectrophotometer, Bentley Instruments) by MQT Labs (Kansas City, KS). Somatic cell linear score was calculated as described by Shook (1993): $\log_2$(somatic cell count ÷ 100). Energy-corrected milk (ECM) production was calculated as: ECM = 0.327 × milk yield + 12.86 × fat yield + 7.65 × protein yield. Body weight and body condition score were recorded at enrollment and on days -30, -14, 1, 14, 28, and 42 relative to parturition using a 0 to 5 scale in 0.25-unit increments (Wildman et al., 1982).

Blood samples were collected from all cows into evacuated tubes (BD Vacutainer, BD Biosciences, San Jose, CA) by puncture of coccygeal vessels, always before the morning feeding. Serum (BD# 368660) and plasma (K_2 EDTA; BD# 366643) tubes were centrifuged at 2,000 × *g* for 15 min at 22°C. Blood in serum tubes was allowed to clot before centrifugation. Serum and

plasma samples were collected and stored at -20°C until analyzed. Blood plasma samples collected at enrollment and on days -30, -14, 1, 7, 14, 28, and 42 relative to parturition were analyzed for beta-hydroxybutyrate (BHB), urea nitrogen, haptoglobin (Hp), and alpha 1-acid glycoprotein (AGP). Plasma was analyzed using enzymatic colorimetric procedures to determine concentrations of glucose (kit #439– 90901, Wako Chemicals USA Inc., Richmond, VA), FFA (NEFA-HR, Wako Chemicals USA Inc.), and BHB (#H7587–58, Pointe Scientific Inc., Canton, MI), absorbance was read on a spectrophotometer (Powerwave XS, Biotek Instruments, Winooski, VT), and calculations were conducted using Gen5 software (Biotek Instruments). Plasma Hp concentration was determined by the colorimetric method described by Cooke and Arthington (2013). Blood urea nitrogen was analyzed in serum samples using a colorimetric kit (EIABUN, Invitrogen, Carlsbad, CA). Serum samples were analyzed for AGP using a bovine-specific ELISA kit (ICL Inc., Portland, OR) at a dilution rate of 1:20,000. An electronic multichannel pipetting system was utilized to achieve the final concentration (Integra Viaflo Assist, INTEGRA Biosciences Corp., Hudson, NH). The kit was validated using spike and recovery, and linearity of dilution techniques (Brown et al., *in preparation*).

Complete blood counts were performed on samples collected in 5-mL potassium EDTA tubes at enrollment and on days -14, 1, 7, 14, and 42 relative to parturition. Samples were analyzed at the Kansas State Veterinary Diagnostic Laboratory using an electronic hematology system (ADVIA2120i Hematology System, Siemens Healthineers, Tarryton, NY).

Blood samples collected on d -14, 1, 7, and 14 relative to parturition were analyzed for fatty acid and oxylipid profiles using LC/MS/MS as described previously in Kuhn et al. (2017). During processing, samples were thawed on ice and combined with a mixture of standards containing 5(S)-HETE-d8 (0.25 μ*M*), 15(S)-HETE-d8 (0.25 μ*M*), 8(9)-EET-d11 (0.5 μ*M*), PGE2-

d9 (0.5 µ*M*), 8,9-DHET-d11 (0.25 µ*M*), AA-d8 (50 µ*M*), 2-AG-d8 (2 µ*M*), and AEA-d8 (0.25 µ*M*). Solid phase extraction was carried out with Oasis HLB 12CC LP extraction columns (Waters, Milford, MA, USA) preconditioned with methanol and HPLC water. Supernatants were loaded into the columns, washed with methanol, and eluted with a 50:50 mixture of methanol and acetonitrile. Volatile solvents were removed using a Savant SpeedVac, and residues were reconstituted in methanol, mixed at a 1.5:1 ratio with HPLC water, and stored in glass chromatography vials with glass inserts at −20 °C until analysis. Data analysis was performed by generating 7-point linear curves with standards purchased from Cayman Chemical (Ann Arbor, MI). The curves for oxylipids were 5-fold dilutions ranging from 100 n*M* to 0.01 n*M*. The curve for PUFA, monosaturated fatty acids, and saturated fatty acids was a 5-fold dilution ranging from 500 µ*M* to 0.001 µ*M*. The linear curves generated produced R^2 values of 0.99. Detailed LC/MS/MS analysis was as described (Mavangira et al., 2015).

Uterine polymorphonuclear cells percentage and granulocyte oxidative burst

Endometrial samples were collected (0630 h) on d 10 to 13, 21±3 and 38±3 postpartum using the cytobrush technique as described in Melcher et al. (2014). Briefly, a sterile cell collector (Cytobrush Plus; Medscand Medical, Trumbull, CT) was attached to a sterile stainless-steel rod and catheter (similar to an insemination gun) covered with a plastic sanitary sheath (80 A.I./E.T. Sanitary sheaths; IMV technologies, Normandy, France) to protect the brush from contamination. Epidural lidocaine (3 mL 2% lidocaine; Vetone, Boise, ID) anesthesia was performed 5 min before transrectal palpation and instrument insertion into the body of the uterus. In the uterine cavity, the sanitary sheath was retracted, and the brush was rotated on the endometrium. The brush was then retracted into the catheter and removed from the cow. Afterwards, the brush was swabbed (Isohelix

DNA Buccal Swabs, Kent, UK) and stored sterile at -80°C for DNA isolation and quantification of bacterial populations. The brush was rolled onto a clean microscope slide, which was fixed and stained (J0322 Dip quick stain kit; Jorgensen Laboratories, Inc., Loveland, CO). Slides were examined for the percentage of polymorphonuclear cells (PMN; granulocytes) under the microscope using 400× magnification, counting 300 cells according to Melcher et al. (2014).

Finally, the brush was immersed in complete media (cRPMI 1640 Medium; Gibco Thermo Fisher Scientific, Waltham, MA) until cell preparation for flow cytometry. Blood samples for flow cytometry were collected into ACD vacutainer tubes (BD Biosciences) by puncture of coccygeal vessels after the cytobrush was performed. Brush and blood samples were stored on ice while in transit to the laboratory. Brushes with complete media were dumped in 50-mL conical tubes with cell strainers (40 µm nylon mesh – Cat. No 22363547, Fisherbrand, Pittsburg, PA). Endometrial cells were washed from the brush with the aid of a 25-mL pipette filled with 5 mL of RPMI media (moved in and out to clean off brush). Conical tubes were centrifuged (5 min, 1200 × g at room temperature) and cells re-suspended in Hanks balanced salt solution (400 µL HBSS 1X with calcium chloride and magnesium chloride; Gibco, Carlsbad, CA). Cell suspension (200 µL) from endometrial samples was transferred to 2 microcentrifuge tubes. Whole blood sample aliquots (100 µL) were transferred into 3 microcentrifuge tubes containing 100 µL of HBSS. In order to measure oxidative burst capacity, 2 endometrial and 2 whole blood samples were incubated with DHR (2.8 µL at 30 m*M*; dihydrorhodamine 123 – Cat. No D623, Invitrogen, Carlsbad, CA) at 37°C for 20 min. After the incubation period, one endometrial sample and one whole blood sample were treated with PMA (25 µL at 10 m*M*; phorbol 12-myristate 13-acetate – Cat. No P8139-1MG, Sigma-Aldrich, St. Louis, MO) and then all tubes were incubated at 37°C for 20 min. Tubes were stored at 4°C for 5 min to stop reactions. Afterwards, 1 mL of red blood cell lysis buffer (ACK Lysing

Buffer, Gibco) was added to all tubes and held at room temperature for 5 min. Tubes were then centrifuged (5 min, 800 × *g* at room temperature), the supernatant discarded, and cells washed twice with HBSS (400 µL).

Cell pellets were resuspended with a master mix solution containing 400 µL FACS buffer (90% PBS, 10% fetal bovine serum, 0.1% NaN_3) and 1 µL of each monoclonal antibody per tube. Primary monoclonal antibodies used were bovine granulocyte monoclonal antibody (CH138A-IgG1, Cat. No WS0608B-100, Kingfisher Biotech, Inc.) and bovine CD45 (leukocytes) monoclonal antibody (clone CACTB51A-IgG2a, Cat WS0544B-100, Kingfisher Biotech, Inc.). All samples were incubated in the dark at 4°C for 20 min, centrifuged (5 min, 800 × *g* at room temperature), and washed once with FACS buffer (400 µL). Cell pellets were resuspended with another master mix solution containing 400 µL FACS buffer and 1 µL of each secondary-conjugated antibody. Secondary antibodies added were goat anti-mouse IgG1 Human ads-AF647 (Cat. No 1070-31, Southern Biotech) and goat anti-mouse IgG2 Human ads-PE (Cat. No 1080-09, Southern Biotech). Samples were incubated in the dark at 4°C for 20 min, centrifuged (5 min, 800 × *g* at room temperature), and washed once with FACS buffer (400 µL). Cell pellets were resuspended with 400 µL PBS and transferred to polypropylene tubes (Cat. No 352054; BD Biosciences, Bedford, MA) suitable for flow cytometry. One whole blood sample without DHR, PMA, and antibodies passed through all incubations and washing procedures and was defined as unstained control. Pooled whole blood samples were used as single stained controls for DHR (stimulated with PMA), PE, and AF647.

Samples were analyzed using a flow cytometer (BD LSRFortressa® X-20, BD Biosciences) installed at the Department of Diagnostic Medicine/Pathobiology at Kansas State University, Manhattan KS. Flow cytometer function was consistently evaluated by the research

associate of the flow cytometer laboratory. The flow cytometer was connected to a computer with BD FACSDiva^{TM} software (BD Biosciences). Compensation was calculated (5,000 events) for every experiment using unstained and single stained controls. The population of interest (granulocytes) was gated using forward scatter and side scatter. Dot plots (PE × AF647 - % granulocytes of total leukocytes) and histograms (AF488 for DHR intensity) were created to visualize data. Samples were analyzed using the following laser channels: 488-nm laser (DHR/AF488 - Ex-Max 495 nm/Em-Max 519 nm), 650-nm laser (AF647 - Ex-Max 650 nm/Em-Max 668 nm), and 560-nm laser (PE - Ex-Max 496 nm/Em-Max 578 nm). A total of 10,000 gated events were recorded and analyzed using FlowJo Software (BD Biosciences). Data were reported as percentage of cells and median fluorescent intensity (MFI).

DNA extraction and bacteria quantification

Microbial DNA from swabs was extracted using a commercial kit (Quick-DNA Fungal/Bacterial Microprep kit cat# D6007; Zymo Research, Irvine, CA). Isolated DNA concentration was measured using a Take3 microvolume plate and SynergyHTX plate reader (BioTek Instruments). Quantitative real-time PCR was used to quantify abundance of total bacteria (16S ribosomal RNA gene), and virulence factors associated with cattle uterine diseases in *Trueperella pyogenes* (fimA), *Fusobacterium necrophorum* (IktA), and *Escherichia coli* (fimH). Forward and reverse primer sequences are listed in Table 2-2. Quantitative real-time PCR was performed on a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Inc.) using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc.). Normalized transcript abundance was calculated using total bacterial 16S abundance to normalize results, and a composite of all samples was used as a reference sample. The slope derived from regressing C_T values against their

log-transformed dilution coefficients was used to calculate efficiency according to the equation E = −1 + 10(−1/slope). Because estimated efficiencies were relatively uniform across taxa (Table 2-2), the relative abundance of each population was calculated ($2^{-\Delta C_T}$) without attempting to adjust for differences in efficiency. Specificity of amplification was assessed by melt curve analysis, and no evidence of off-target amplification was observed. Each sample was run in duplicate.

Statistical analyses

Data were submitted to the Mixed procedure of SAS (version 9.4, SAS Institute, Cary, NC) to model the fixed effects of treatment, time, interaction between treatment and time, and the random effects of cow and block. Data at the enrollment date were used for covariate adjustments. We also tested in the model the fixed effects of parity group at enrollment (1 or ≥2 lactation number), average THI during the 2 weeks before and 2 weeks after parturition (linear and quadratic terms), daily THI (linear and quadratic terms), and their interactions with fixed effects described above. For milk parameters, Prev305ME, PrevF305M, and PrevP305M, and interactions were included in the model for covariate adjustments. Energy-corrected milk data were averaged by week prior to analysis. Stepwise backward elimination was performed when *p*-value for fixed effects were >0.15, except for treatment, time, and treatment by time interaction effects, which remained in all models. The between-within degrees of freedom method was used to determine denominator degrees of freedom. Studentized residuals were generated using the residual command in the MIXED procedure, and observations with conditional Studentized residuals <-4 or >4 were considered outliers and removed from statistical analyses. Equally spaced repeated measures over time were tested with autoregressive [AR(1)] and heterogenous autoregressive [ARH(1)] covariance structures, selected based on the least BIC value. Unequally spaced repeated

measures were evaluated with spatial power covariance structures. When Studentized residuals were not normally distributed, data were transformed for analysis [including oxidative burst capacity (log) and percentage of PMN in smears (arcsine)]. Parameter solutions for fixed effects were plotted to show interactions between treatment and continuous variables. Significance was set at $P < 0.05$.

Uterine bacterial data were analyzed using JMP (version 14, SAS Institute). The presence of pathogenic bacteria in the uterus (defined as a C_T value < 36 cycles) was analyzed through logistic regression using the fixed effects of treatment, sampling day, parity group, and their interactions in the model. Odds ratio and confidence interval were calculated for each virulence factor. When presence/absence data were too one-sided for robust logistic modeling, Fisher's exact test was performed after contingency analysis.

RESULTS

Intake, feeding behavior, milk production, and blood variables

Treatments did not affect ($P = 0.880$) urine pH during the dry period (5.87 ± 0.11 vs 5.85 ± 0.12 for OMN and CON, respectively). Feed intake was not affected by treatment during the dry period and early lactation (Figure 2-1 A and B). An interaction effect between treatment and THI (quadratic term) was observed for feed intake during early lactation ($P = 0.013$), where similar DMI were observed at THI ~53, but CON cows consumed more DM when transition THI was greater (Figure 2-1 C). Regarding the feeding behavior during the dry period, cows fed OMN exhibited lower average meal weight in comparison with CON (1.63 vs 2.16 ± 0.33 kg for OMN and CON, respectively), especially on d -27, -25 to -23, -19, -17, and -7 relative to calving date (Figure 2-2A). No differences were observed on meal length and intermeal interval (Figure 2-2 B

and C). Cows fed OMN had more meals/day (P = 0.023) in comparison with CON (7.88 vs 7.07 ± 0.55 /d for OMN and CON, respectively; Figure 2-2 D).

Cows fed OMN had greater average meal weight (1.72 vs 1.82 ± 0.93 kg for CON and OMN, respectively) in comparison with CON group (Figure 2-3 A) without affecting the meal length during early lactation (Figure 2-3 B); however, the OMN group exhibited greater intermeal interval (1.22 vs 1.43 ± 0.04 h, for CON and OMN, respectively; Figure 2-3 C) and fewer meals/day (14.9 vs 13.3 ± 0.48 /d, for CON and OMN, respectively; Figure 2-3 D) in comparison with CON.

Cows fed OMN had lesser milk yields over the first 49 DIM compared with CON group (51.8±1.19 vs. 53.3±1.22 kg/d; Figure 2-4 A). However, OMN effects on milk yield were influenced by mean THI experienced by cows; when average THI around parturition was greater than 66, OMN-treated cows produced more milk than CON cows (Figure 2-4 B). We also tested daily THI (linear and quadratic terms) and its interaction with treatment in the model, but no effects were detected (P > 0.72). The ECM yield and milk lactose percentage decreased ($P \leq 0.03$) in cows fed OMN (Table 2-3). Milk fat production tended to decrease (P = 0.084) when feeding OMN. Treatment by THI interactions for ECM had similar pattern as milk yield (Figure 2-5). The interaction plot between THI and treatment revealed that cows fed OMN may present lower body temperature during lactation when mean transition THI is greater than 68 (Figure 2-6).

Blood metabolites

Cows fed OMN had lesser (P < 0.001) plasma glucose concentration during the pre-partum period and lesser plasma concentrations of FFA (P = 0.005) and BHB (P = 0.043) over early lactation compared with CON (Table 2-4). Interactions between treatment and THI for plasma Hp

and serum urea concentrations are shown in Figure 2-7. No treatment effects were observed on CBC, except for greater mean red blood cell volume ($P < 0.0001$) of cows fed OMN, however, values were within the reference interval (Error! Reference source not found.). OmniGen-AF increased ($P \leq 0.031$) plasma palmitic acid, linoleic acid, and stearic acid concentrations and tended to increase ($P = 0.08$) dihihomo- γ-linolenic acid compared with CON (Table 2-6). An interaction between treatment and time was observed for plasma palmitic acid concentration, as greater concentrations of palmitic acid were observed on d 1 and 7 of lactation in OMN cows. Interactions between THI and treatments for plasma FA are shown in Figure 2-8. Cows fed OMN had minor differences in oxylipid profile relative to CON group such as a greater plasma 6-ketoprostaglandin F_1 α concentration (Table 2-7). Interactions between treatment and time were observed for thromboxane B_2 (TXB_2) and 12-HHTr-E, where OMN increased plasma concentrations of TXB_2 and 12-HHTr-E on d -14 relative to parturition (Figure 2-9).

Endometrial PMN infiltration, oxidative burst potential, and uterine bacteria quantification

No treatment effects were observed either for uterine PMN% in cytology or granulocyte oxidative burst (Table 2-8). As expected, PMN% in uterine cytology decreased throughout the first 38 DIM (Figure 2-10). An interaction of treatment and time was observed for the delta MFI of uterine granulocyte oxidative burst, where OMN cows had lesser oxidative burst response on day 10 and greater oxidative burst response on day 38 of lactation in comparison with CON (Figure 2-11). Interaction between treatment and transition THI was detected for uterine granulocyte (% leukocytes), shown in Figure 2-12. OmniGen-AF numerically decreased the proportion of cows with detectable virulence factors from pathogenic bacteria in uterus of early lactating cows (Table

2-9), but no differences were observed for treatments on risk of uterine contamination by pathogenic bacteria (Table 2-10).

DISCUSSION

To date, the literature has described either no effects (Leiva et al., 2017a; Hall et al., 2018) or improved milk yield (Brandão et al., 2016; Casarotto et al., 2020) with OMN treatment during the dry period and early lactation. However, OMN had a negative effect on milk yield under the conditions of this study. It is worth mentioning that positive effects of OMN on milk yield have been observed in cows with significantly lower plasma concentrations of haptoglobin (Brandão et al., 2016), significantly lesser milk yield (Brandão et al., 2016; Gandra et al., 2019), or in cows under a relatively high THI environment (>70; Fabris et al., 2017a; Gandra et al., 20129) in comparison with the current study. Furthermore, previous studies have not mentioned whether cows with health disorders were replaced during the experiment or only the data from healthy cows were used (Brandão et al., 2016; Fabris et al., 2017b; Leiva et al., 2017b). We observed a strong interaction between treatment and transition THI values for milk yield, where results indicated that OMN may improve milk production in cows exposed to a mean THI greater than 68 during the 4-wk transition period. On the other hand, no effects of daily THI (dTHI) were observed when adding either linear or quadratic terms in the statistical model instead of THI average values around parturition (dTHI $P = 0.73$ and $dTHI^2$ $P = 0.74$), suggesting that THI around parturition has a greater impact on milk yield responses to OMN compared with the THI over lactation. One can ask whether the different lactating diets (Table 2-1) influenced the study outcomes, but no interaction between diets used during lactation and treatments was detected in the statistical model

(P = 0.47). Changes in lactation diet were made because: 1) the experiment was longer than expected due to cow replacements, and 2) shortage of triticale silage during the study.

Agreeing with the concept that OMN has a greater effectiveness for reducing the impacts of high THI on milk yield, Fabris et al. (2017b) did not report differences in milk yield of transition cows fed OMN when they were housed in pens with active cooling systems during the dry period. However, when cows were housed in pens without active cooling systems during the dry period, OMN cows had similar milk yield compared with those under cooling and exhibited greater milk yield in comparison with controls in the same environment (Fabris et al., 2017b). A study with finishing cattle under heat stress conditions demonstrated that OMN supplementation can ameliorate hyperthermia but did not affect productive responses of heifers (Colombo et al., 2019a). Aligning with reports in the literature, the numerous treatment interactions with transition THI in this study support the concept that OMN substantially modulates the physiological responses to heat stress. Nevertheless, the reasons/mechanisms by which OMN improves cows' adaptation to high THI environments have not been fully elucidated. Gandra et al. (2019) suggested that OMN improves heat dissipation by altering skin blood perfusion. Although no interactions between treatment and time were detected for feed intake and milk yield, cows had a marginal decrease in DM intake around d 10 to d 15 of lactation. We performed cytobrush sampling around d 10, which may have altered milk yield of cows under OMN treatment. To study whether the body temperature was different between groups, we analyzed rectal temperatures over the 4 d after the cytobrush was performed and no effects were detected (P = 0.62, 38.79 ± 0.084 vs 38.85 ± 0.093 °C for CON and OMN, respectively), providing no evidence of uterine injury or infections after the procedure.

Although this experiment did not detect differences in DM intake either during the dry period or early lactation, treatments altered the feeding behavior of cows, which might partially

explain milk yield and composition outcomes. Cows fed OMN had greater average meal weights, but at the same time, cows fed OMN exhibited an increase in intermeal interval and a decrease in meal count compared with controls. Larger meals may cause sharper post-meal drops in rumen pH (Allen, 1997); on the other hand, ingesting smaller meals in a more consistent pattern across the day would lead to a more stable rumen environment, reducing the risk of sub-acute acidosis and improving milk fat production (Krause and Oetzel, 2006; DeVries and Chevaux, 2014). Supporting the data of meal frequency (meals/d) and milk yield observed in this experiment, authors predicted an increase of 0.3 kg/d milk yield for every extra meal per day in mid-lactation cows (Johnston and DeVries, 2018). However, data used in the latter study were retrieved from cows housed in free-stalls. In the current experiment, OMN cows consumed, on average, 1.6 fewer meals/d in comparison with CON cows, which would predict a decrease in milk production of 0.48 kg/d. It is worth mentioning that there is limited literature about OMN effects on feeding behavior in lactating cows.

Blood concentrations of FFA and BHB decreased with OMN treatment, but this may have been related to lesser milk production observed in cows on OMN. Regardless of effects on milk, OMN has not affected blood FFA concentrations in most studies with dairy cows (Brandão et al., 2016; Leiva et al., 2017a; Hall et al., 2018). However, one study with transition cows and studies with beef cattle have shown evidence that OMN may affect adipose tissue mobilization. Mezzetti et al. (2019) found lower FFA and BHB concentrations in blood of early lactating cows fed OMN compared with controls, with no treatment effects detected for milk production. These authors suggested that niacin and choline contained in OMN reduced blood concentrations of FFA and BHB as previously reported in other studies with transition cows (Morey et al., 2011; Zhou et al., 2016). The antilipolytic effects of niacin is described elsewhere (Wise et al., 2003; Pires and

Grummer, 2007) and choline is an essential substrate for hepatic VLDL synthesis, triglyceride transport, and thus liver function (Vance, 2002). Although not explored in this experiment, OMN has increased insulin release in a glucose tolerance test while reducing FFA concentrations in blood of beef heifers (Burdick Sanchez et al., 2019). In addition, these authors reported that OMN reduced blood FFA concentrations before the glucose tolerance test (Burdick Sanchez et al., 2019). Similarly, beef steers fed OMN had lower blood FFA concentrations compared with controls during the transition to feedlot and during the finishing phase (Armstrong et al., 2018). Because FFA and BHB are required for milk production and fat synthesis in mammary gland, it is somewhat expected that lower blood concentrations of these metabolites negatively affected performance of cows in this study.

Because the association between exacerbated/chronic inflammation and periparturient diseases is well established (Ohtsuka et al., 2001; Ametaj et al., 2005) and there is little data regarding the effects of OMN on inflammation status of transition cows, we evaluated mediators of inflammation in blood, especially acute phase proteins (Hp and AGP) and oxylipids. An increase in peripartum inflammation signaling across several species is supported by a scientific consensus, and an acute-phase response in post-partum dairy cows is well established (Bradford et al., 2015). In the current study, both Hp and AGP increased after parturition, notably during the first week of lactation. Several studies have indicated an elevation in inflammatory and positive acute-phase mediators in the days after parturition, even in the absence of disease (Bionaz et al., 2007; Mullins et al., 2012; Akbar et al., 2015). Responses in inflammatory signaling when feeding OMN have been inconsistent. Recently, Marins et al. (2020) found an increase in IL (interleukin)-10 secretion after LPS challenge in cows fed OMN; IL-10 is a cytokine with anti-inflammatory properties. In the current experiment, we evaluated acute phase proteins (which are elevated under

a pro-inflammatory status), however, no evidence for treatment effects were detected. OmniGen-AF had no effect on Hp when cows were not challenged, but OMN increased blood Hp concentration after LPS stimulation in a previous study (Brandão et al., 2016). Similarly, other authors found no differences in blood Hp either pre- or post-partum in dairy cows fed OMN (Wu et al., 2019).

OmniGen-AF increased plasma concentrations of palmitic, stearic, and linoleic acids; note that these concentrations include fatty acids incorporated in triglycerides, cholesterol esters, and phospholipids in addition to FFA. Fatty acids are substrates for the biosynthesis of lipid hormones, and variation in their concentration in plasma lipid fractions and immune cell membranes may affect the production of inflammation signaling molecules (Calder, 2006). Palmitic acid and stearic acid are known for their proinflammatory potential because they can activate nuclear factor-kB and mitogen-activated protein kinase. These fatty acids may also enhance the production of inflammatory cytokines such as tumor necrosis factor-α and interleukin (IL)-1β in monocytes (Calder and Yaqoob, 2007; Serhan et al., 2008). Linoleic acid, meanwhile, is an essential n-6 fatty acid that can be elongated and desaturated to form arachidonic acid (a major precursor for the synthesis of oxylipids).

Oxylipids are potent mediators of inflammation derived from the hydrolysis of polyunsaturated fatty acids present in membrane phospholipids of cells and further oxidation reactions (enzymatic or non-enzymatic). The hydrolysis of esterified polyunsaturated fatty acids is promoted by phospholipase A_2 (Burke and Dennis, 2009), and this enzyme is mobilized as a consequence of pathogen exposure or tissue damage. Oxylipids can either promote or resolve inflammatory processes, depending on the fatty acid substrate and oxygenation pathway (Serhan and Chiang, 2008; Mattmiller et al., 2013). Interestingly, oxylipids affected by OMN treatment are

derived from arachidonic acid and the downstream cyclooxygenase pathway (Sordillo, 2018). Cows fed OMN had greater 6-keto $PGF_{1\alpha}$, TXB_2, and 12-HHTrE concentrations in comparison with CON. 6-Keto-$PGF_{1\alpha}$ and TXB_2 are physiologically active and stable metabolites of prostacyclin and TXA_2, respectively. Prostacyclin is unstable at physiological pH and, thus, has a very short half-life in vivo (< 2 min), rapidly forming the hydration product 6-keto-$PGF_{1\alpha}$ (Smyth and Fitzgerald, 2002). Indeed, the measurement of TXB_2 is considered an index of arachidonic acid metabolism efficiency in human platelets (Patrono et al., 1980; Defreyn et al., 1982). The question that remains to be answered is how OMN can affect a specific pathway in oxylipid metabolism?

Cyclooxygenase-derived metabolites are usually associated with the regulation of vascular tone and platelet function. For example, prostacyclin induces vasodilation and inhibits platelet aggregation; on the other hand, TXA_2 stimulates platelet aggregation and causes vasoconstriction. Therefore, the balanced metabolism of these oxylipids is critical for vascular health and immune capabilities (Ryman et al., 2015). One can speculate that the positive effects of OMN in controlling body temperature in ruminants (Leiva et al., 2017; Colombo et al., 2019b) might be related to vascular tone modification. Although much research has focused on platelets' role in hemostasis, platelets have non-hemostatic activities, including recognition of pathogen invasion and mobilization of immune and inflammatory cells to the injured site (Parvathenani et al., 1998; Li, 2008; Scull et al., 2010; Vieira-de-Abreu et al., 2012; Stephen et al., 2013; Pitchford et al., 2017). For example, platelet adhesion to the vascular endothelium facilitates neutrophil capture in a P-selectin dependent manner, guiding rolling and crawling of neutrophils to their site of transmigration (Zuchtriegel et al., 2016). Note that changes in oxylipid profiles occurred before parturition, and whether these changes are of biological significance is unknown.

One of the main objectives of this study was to evaluate whether OMN can have positive impacts on uterine immunity such as has been observed in peripheral blood of cows. Experiments to detect differences in disease incidence require a large number of experimental units, and to date, there is no strong evidence that OMN can reduce the incidence of uterine diseases (Nace et al., 2014a; Mammi et al., 2018; Wu et al., 2019; Casarotto et al., 2020). However, studies with animal models suggested that OMN may impact uterine health. For instance, a murine experiment demonstrated that OMN may hasten uterine involution, decrease uterine lesions (at a histological level), and promote fertility (Jorgensen-Muga et al., 2018). The average PMN percentage found in cytology throughout the experiment was below the cut-off for subclinical endometritis diagnosis established by Kasimanickam et al. (2004), where cows with >18% neutrophils in cytology between 20-33 DIM or >10% neutrophils in cytology at 34-47 DIM are considered cases of subclinical endometritis. However, 3 cows from the CON group and 3 cows from the OMN group had >18% neutrophils in cytology performed on d 10, and 1 cow from CON group had >10% neutrophils in cytology from d 38. Furthermore, OMN tended to reduce endometrial granulocyte oxidative burst potential on d 10 and tended to increase it on d 38 of lactation. After bacterial attachment to the cell membrane, phagocytosis, and vacuole formation, PMN produce reactive oxygen species (ROS; hydroxyl radicals, singlet oxygen, oxygen halides, and hydrogen peroxide) that have bactericidal properties (Paape et al., 2002). Few studies have evaluated oxidative burst potential of granulocytes isolated from cows fed OMN. Blood neutrophils from dairy heifers fed OMN showed greater ROS production after stimulation with PMA on d 30 of supplementation (d -30 relative to parturition), but this effect was not observed on day 60 of treatment (Ryman et al., 2013). On the other hand, heifers fed OMN from -60 d relative to the expected calving date until

30 d postpartum exhibited decreased oxidative burst (maximum generating capacity) in PMN on d -30 and d 1 relative to parturition (Nace et al., 2014b).

An initial screening of the OMN effects on the immune system showed that cows treated with OMN prepartum had greater transcript abundance of epidermal growth factor receptor (EGF-R) in neutrophils 15 h after parturition (Wang et al., 2004). Authors have associated EGF-R with increased TNF-induced priming of neutrophils, secretion of IL-8, and mitochondrial ROS generation (Hamilton et al., 2003; Lewkowicz et al., 2005). A study with rats fed OMN for 28 d revealed greater abundance of transcripts involved with bacterial pattern recognition receptors [toll-like receptor (TLR) 1, TLR4, TLR6], and TLR and TNF-α signaling pathways (Branson et al., 2016). The reasons why OMN would affect ROS production potential in granulocytes from the endometrium but not in granulocytes from peripheral blood are unknown. This might be related to previous priming of granulocytes collected from endometrium by the presence of bacteria, such those detected by DNA analysis in this study. Priming of neutrophils following exposure to a ligand that does not itself induce ROS production promotes the activation of NADPH oxidase upon binding to a second ligand (El-Benna et al., 2008; El-Benna et al., 2016).

In order to limit the number of bacterial species to quantify in cytobrush samples, we selected primers specific for virulence factors found in bacteria associated with uterine diseases in dairy cows (Bicalho et al., 2012). However, despite differences in oxidative burst potential of endometrial granulocytes observed on d 10 and 38 of lactation, we did not detect a treatment effect on the risk for the presence of pathogenic bacteria. Cows have a uterine microbiome during the pregnancy that changes after parturition, becoming less diverse in metritic dairy cows, with increased abundance of *Bacteroides*, *Porphyromonas*, and *Fusobacterium* in particular (Pascottini and LeBlanc, 2020). It is important to state that we analyzed data only from cows without clinical

signs of metritis or endometritis. Collectively, OMN did not show major effects on CBC parameters (including immune cell concentrations) and inflammation blood markers (Hp and AGP) in apparently healthy cows. The lack of OMN effects on CBC has been reported in cows either under heat stress or not (Fabris, 2017). Contrasting with observations by Brandão et al. (2016), we did not observe treatment differences in PMN% in smears collected by cytobrush technique. The difference between studies might be related to different sampling dates, number of samplings, and number of cells counted under microscope, besides cows' milk production level. We sampled endometrial cells on days 10, 21, and 38 of lactation whereas Brandão and colleagues collected on d 30 and 46 in milk. Melcher et al. (2014) evaluated different counting techniques of endometrial cytology between 20 to 30 d of lactation and found that counting 100 cells under microscope either overestimated or underestimated the prevalence of subclinical endometritis, whereas counting 300 cells was suitable to evaluate the percentage of PMN in endometrial samples.

CONCLUSION

The hypothesis that an immunomodulatory feed additive fed during the dry period and early lactation would improve peripheral and uterine granulocyte activity, and as a consequence would improve overall performance of cows, was not confirmed in this study. OmniGen altered feeding behavior of early lactation cows by increasing the meal size (kg DM) which might partially explain the negative effects observed on performance of cows. OmniGen decreased blood concentrations of FFA and BHB which might have also negatively affected milk and fat synthesis in mammary gland. Indeed, OMN modulated the oxidative burst potential of endometrial granulocytes and the latter effect depended on the sampling date. Furthermore, OMN altered both

blood profiles of fatty acids and oxylipids where increased blood concentrations of eicosanoids derived from arachidonic acid metabolism by COX. Finally, this study did not reveal evidence for differences in the presence of pathogenic bacteria in the uterus of cows.

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Table 2-1. Ingredients and chemical composition of experimental diets.

Item	Dry period	Lactation 1[1]	Lactation 2[2]
Ingredient, %DM			
Corn silage	21.93	23.6	23.63
Triticale silage		9.35	4.49
Alfalfa hay		7.65	12.51
Prairie hay	40.01	1.53	1.53
Wet corn gluten feed[3]	18.59	22.44	22.44
Corn grain, ground, dry	8.17	2.55	2.55
Cottonseed		3.91	3.91
Soybean meal	5.81		
Dry cow grain mix[4]	5.51		
Lactation grain mix[5]		28.94	28.94
Chemical, % DM			
Dry matter, % as-fed	57.3±3.35	46.6±0.84	50.3±1.84
Crude protein	12.0±0.90	17.8±0.70	18.55±0.07
Acid detergent fiber	28.3±2.98	21.1±0.42	19.45±1.20
Neutral detergent fiber	45.9±2.63	35.6±1.97	31±3.11
NEL, MJ/kg	6.6±0.05	6.92±0.01	7.05±0.06

[1]Diet fed until 11/28/2018 – 17 cows were fed lactation 1 diet.
[2]Diet fed after 11/28/2018 – 19 cows were fed lactation 2 diet.
[3]Sweet Bran (Cargill, Dallas, TX).
[4]Composition (% diet DM): 0.42% Reashure, 0.37% vitamin E 20,000, 0.08% salt, 0.06% Zinpro 4-Plex, 0.05% selenium 0.06%, 0.04% vitamin A 30,0000, 0.02% Zinpro 120, 0.02% Rumensin, 0.02% vitamin D 8,8000, 0.01% biotin 100, 3.49% limestone, 0.93% Megalac R.
[5]Composition (% diet DM): 17.9% ground corn, 6.86% soy plus, 0.94% Kruse lactation premix, 1.06% limestone, 0.72% sodium bicarbonate, 0.72% Megalac R, 0.15% vitamin E 20,0000, 0.15% salt, 0.15% trace minerals, 0.25% magnesium oxide, 0.024 Zinpro 4Plex C, 0.012% Zinpro 120, 0.006% Rumensin.

Table 2-2. Primers used for quantitative real-time PCR detection of microbial species.

Species	Target gene	Forward Reverse	Reaction *E*	Reference
All bacteria	16S rRNA	ACTCCTACGGGAGGCAGCAGT TATTACCGCGGCTGCTGGC	1.76	Clifford et al. (2012)
T. pyogenes	fimA	CACTACGCTCACCATTCACAA G GCTGTAATCCGCTTTGTCTGTG	1.84	Silva et al. (2009)
F. necrophorum	IktA	GATTGGGGGATAGCGGTAAT GAGCCTCCACATTTAGTCGC	Undetermined	Cunha et al. (2018)
E. coli	fimH	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	1.58	Moreno et al. (2005)

Table 2-3. Energy-corrected milk (ECM) and milk solids yield, milk urea nitrogen (MUN), and somatic cell linear score (SCLS) of cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period and early lactation (LSMeans ± SE).

Item	Treatment		*P*-value		
	CON	OMN	Trt	Time	Trt × time
Milk yield, kg/d	53.3 ± 1.22	51.8 ± 1.19	0.023	<.0001	0.691
ECM, kg/d	58.0 ± 1.22	55.4±1.30	0.030	<.0001	0.205
Fat, kg/d	2.25 ± 0.051	2.15 ± 0.552	0.084	<.0001	0.298
Protein, kg/d	1.52 ± 0.028	1.48 ± 0.0308	0.211	0.072	0.342
Lactose, kg/d	2.66 ± 0.083	2.54 ± 0.089	0.248	<.0001	0.142
Fat, %	4.31 ± 0.075	4.24 ± 0.082	0.546	<.0001	0.388
Protein, %	2.95 ± 0.041	2.93 ± 0.045	0.697	<.0001	0.274
Lactose, %	4.91 ± 0.032	4.85 ± 0.033	0.028	<.0001	0.599
MUN, mg/dL	10.7 ± 0.454	11.5 ± 0.487	0.085	0.007	0.226
SCLS[1]	1.40 ± 0.266	1.47 ± 0.291	0.851	0.003	0.271
Body weight, kg	696 ± 5.9	670 ± 6.4	0.681	<.0001	0.530
Body condition score	2.84 ± 0.032	2.86 ± 0.035	0.717	<.0001	0.662
Body temperature, °C	38.72 ± 0.033	38.67±0.0322	0.111	<.0001	0.884

[1]Somatic cell linear score (SCLS) calculated as: $\log_2$(somatic cell count ÷ 100) + 3, according to Shook (1993).

Table 2-4. Blood plasma parameters of cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period and early lactation (LSMeans ± SE).

	Treatment		*P*-value		
Item	CON	OMN	Trt	Time	Trt × time
Dry period					
Urea, m*M*	1.76 ± 0.14	1.97 ± 0.15	0.179	0.022	0.395
Glucose, mg/dL	71.9 ± 1.33	63.9 ± 1.43	< 0.001	0.213	0.982
Free fatty acids, µ*M*	138 ± 22.6	125 ± 24.6	0.628	0.025	0.147
Beta-hydroxybutyrate, µ*M*	258 ± 19.9	247 ± 21.8	0.734	0.529	0.654
Alpha-1 acid glycoprotein, µg/mL	177 ± 15.9	160 ± 17.1	0.389	0.899	0.191
Haptoglobin, µg/mL	756 ± 120.9	544 ± 139.9	0.186	0.658	0.467
Early lactation					
Urea, m*M*	1.93 ± 0.100	2.08 ± 0.110	0.325	0.007	0.855
Glucose, mg/dL	51.3 ± 4.98	58.1 ± 1.58	0.195	<.0001	0.849
Free fatty acids, µ*M*	482 ± 51.9	331 ± 52.1	0.005	0.002	0.769
Beta-hydroxybutyrate, µ*M*	562 ± 41.1	464 ± 44.4	0.043	0.006	0.202
Alpha-1 acid glycoprotein, µg/mL	319 ± 27.8	297 ± 28.9	0.366	0.001	0.591
Haptoglobin, µg/mL	969 ± 163.2	1042 ± 183.7	0.693	0.275	0.801

Blood samples were collected at enrollment (for covariate adjustments) and on days -28, -14, -7, 1, 7, 14, 28, and 42 relative to parturition.

Table 2-5. Complete cell count of cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period and early lactation (LSMeans ± SE).

Item	Treatment		Ref. interval	*P*-value		
	CON	OMN		Trt	Time	Trt
Leukocyte count, K/µL	12.1 ± 0.55	12.4 ± 0.57	4.9 - 12.0	0.484	0.0001	0
Segmented neutrophil, K/µL	3.88 ± 0.21	4.21 ± 0.23	1.8 - 6.3	0.292	<.0001	0
Lymphocyte, K/µL	7.86 ± 0.422	7.54 ± 0.44	1.6 - 5.6	0.355	0.557	0
Monocyte, K/µL	0.489 ± 0.044	0.499 ± 0.048	0.0 - 0.8	0.886	0.220	0
Eosinophil, K/µL	0.166 ± 0.032	0.247 ± 0.036	0.0 - 0.9	0.111	0.007	0
Cellular hemoglobin, g/dL	10.7± 0.09	10.9 ± 0.11	No Ref. Int.	0.124	<.0001	0
Hemoglobin	10.8 ± 0.11	10.7 ± 0.18	8.5 - 12.2	0.747	<.0001	0
Erythrocyte concentration, M/µL	6.32 ± 0.081	6.32 ± 0.087	5.10 - 7.60	0.956	<.0001	0
Mean cell volume, fL	45.6 ± 0.59	47.9 ± 0.60	38.0 - 50.0	<.0001	<.0001	0
Mean cell hemoglobin, g/dL	37.0 ± 0.12	36.9 ± 0.13	36.0 - 39.0	0.914	0.152	0
RBC distribution width, %	18.2 ± 0.19	18.4 ± 0.20	15.5 - 19.7	0.396	0.003	0
Platelet, K/µL	387 ± 26.1	351 ± 28.9	193 - 637	0.240	<.0001	0
Hematocrit, %	30.4 ± 0.38	31.1 ± 0.42	22 - 33	0.125	<.0001	0
Plasma protein, g/dL	7.29 ± 0.085	7.23 ± 0.089	7.0 - 9.0	0.481	<.0001	0
Fibrinogen, mg/dL	367 ± 14.5	403 ± 15.8	300 - 700	0.102	0.134	0

Blood samples were collected at enrollment (for covariate adjustments) and on days -14, 1, 7, 14, 28, and 42 relative to parturition.

Table 2-6. Plasma fatty acid concentrations of cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period and early lactation (LSMeans±SE).

Fatty acid, n*M*	Treatment		*P*-value		
	CON	OMN	Trt	Time	Trt × time
Lauric acid	0.992±0.0869	0.991±0.102	0.107	0.001	0.904
Eicosapentaenoic acid	4.07±0.438	4.71±0.495	0.323	0.047	0.911
Arachidonic acid	25.7±4.79	24.7±5.25	0.827	0.165	0.529
Dihomo-γ-linolenic acid	10.7±2.19	14.9±2.46	0.080	0.059	0.473
Docosahexaenoic acid	0.789±0.133	0.909±0.146	0.405	0.002	0.629
Myristic acid	493±67.4	516±76.9	0.790	<.0001	0.328
Docosapentaenoic acid	16.5±2.52	17.4±2.81	0.744	0.016	0.579
Adrenic acid	12.3±1.52	13.99±1.75	0.342	<.001	0.278
Palmitoleic acid	816±125.9	1040±143.2	0.193	<.0001	0.418
Palmitic acid	23,019±6,191	41,788±7,254	0.031	0.037	0.021
Alpha-linolenic acid	73.2±9.45	89.5±10.59	0.144	<.0001	0.447
Linoleic acid	310±58.3	467±63.6	0.013	<.0001	0.105
Oleic acid	74,392±9,627	80,094±11,330	0.670	<.0001	0.733
Stearic acid	10,460±1,279.1	14,651±1481.2	0.017	0.191	0.125

Plasma samples were collected on days -14, 1, 7, and 14 relative to parturition.

Table 2-7. Plasma oxylipid concentrations of cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period and early lactation (LSMeans±SE).

Oxylipid[1] (n*M*)	Biosynthesis pathway[2]	Treatment		*P*-value		
		CON	OMN	Trt	Time	Trt×time
Arachidonic acid derived						
6ketoPGF1alpha	COX	0.397 ± 0.082	0.695 ± 0.094	0.018	0.023	0.239
PGD2		0.496 ± 0.052	0.429 ± 0.059	0.406	0.001	0.190
TXB2		2.51 ± 0.34	2.33 ± 0.392	0.728	0.050	0.046
12-HHTrE	CyP450	2.29 ± 0.382	2.56 ± 0.44	0.610	0.097	0.048
8,9-EET		1.07 ± 0.179	0.877 ± 0.206	0.448	<.0001	0.748
11,12-EET		0.354 ± 0.082	0.356 ± 0.095	0.981	0.141	0.187
14,15-EET		0.642 ± 0.068	0.585 ± 0.076	0.452	0.002	0.516
20-HETE		13.4 ± 1.41	11.5 ± 1.631	0.386	<.0001	0.492
8,9-DHET		1.65 ± 0.100	1.46 ± 0.116	0.233	<.0001	0.19
11,12-DHET		5.37 ± 0.335	4.84 ± 0.37	0.170	<.0001	0.466
14,15-DiHETE		7.39 ± 0.446	6.54 ± 0.521	0.214	0.001	0.726
14,15-DHET		6.28 ± 0.369	5.89 ± 0.413	0.428	0.002	0.986
17,18-DiHETE		92.8 ± 6.837	79.3 ± 7.714	0.169	0.012	0.396
5-HETE	LOX	931 ± 92.243	989 ± 106.44	0.676	<.0001	0.679
5-oxoETE		35.3 ± 4.72	39.8 ± 5.428	0.511	<.0001	0.564
9-HETE		25.7 ± 3.626	20.5 ± 4.178	0.054	<.0001	0.175
15-HETE		29.5 ± 4.615	28.1 ± 5.248	0.810	<.0001	0.218
15-oxoETE		4.05 ± 0.381	3.84 ± 0.433	0.679	<.0001	0.242
13-(S)-HOTrE		149 ± 15.109	153 ± 16.927	0.818	0.995	0.124
17-HDoHE		8.13 ± 0.721	8.06 ± 0.775	0.924	0.009	0.414
11-HETE		27.4 ± 3.00	23.6 ± 3.448	0.414	<.0001	0.286
5-iPF2alpha-VI	NE	1.27 ± 0.099	1.19 ± 0.116	0.579	0.001	0.963
8-iso-PGF2alpha		0.163 ± 0.051	0.19 ± 0.059	0.731	0.98	0.22
8-iso-PGE-2		0.684 ± 0.187	0.913 ± 0.217	0.433	0.914	0.131
8-iso-PGA-1		0.207 ± 0.025	0.193 ± 0.029	0.716	0.053	0.094
8,12-iso-iPF2alpha-VI		1.82 ± 0.145	1.92 ± 0.17	0.663	<.0001	0.627
Linoleic acid derived						
9-HODE	LOX/NE	628 ± 77.673	587 ± 89.982	0.735	<.0001	0.616
9-oxoODE		36.0 ± 3.8	33.1 ± 4.24	0.497	<.0001	0.655
13-HODE		422 ± 40.4	426 ± 44.079	0.925	0.001	0.698
13-oxoODE		34.0 ± 3.57	30.6 ± 4.119	0.471	<.0001	0.811
9,10-EpOME	CyP450	19.5 ± 2.34	17.8 ± 2.538	0.458	<.0001	0.591
9,10-DiHOME		100 ± 7.7	98.3 ± 9.018	0.883	<.0001	0.058
12,13-EpOME		28.0 ± 3.28	25.3 ± 3.682	0.482	<.0001	0.566

Plasma samples were collected on days -14, 1, 7, and 14 relative to parturition.

[1]Abbreviations are described in Putman et al. (2019).

[2]Cyclooxygenase (COX), cytochrome P450 (CyP450), lipoxygenase (LOX), and non-enzymatic oxidation (NE).

Table 2-8. Polymorphonuclear cells (PMN) in uterine cytology and median fluoresce intensity (MFI) of uterine and blood granulocytes oxidative burst stimulated or not with phorbol myristate acetate from cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period and early lactation (LS means ± SE).

Item	Treatment		*P*-value		
	CON	OMN	Trt	Time	Trt × time
Smear PMN, %	10.7 ± 2.71	9.30 ± 2.867	0.676	0.011	0.621
Blood[1]					
Granulocyte, % leukocytes	26.6 ± 1.97	21.9 ± 2.08	0.111	0.141	0.687
Granulocyte non-stimulated, MFI[2]	1,134 ± 71.3	1,083 ±72.3	0.617	0.003	0.133
Granulocyte stimulated, MFI[2]	8,226 ± 1187	8,061 ± 1000	0.905	0.006	0.532
Delta stimulated - non-stimulated, MFI[2]	6,820 ± 984.6	6,447 ± 1010	0.793	0.029	0.622
Endometrium[1]					
Granulocyte, % leukocytes	32.5 ± 2.13	30.9 ± 2.37	0.618	0.664	0.877
Granulocyte non-stimulated, MFI[2]	2,084 ± 564.9	1,991 ± 599	0.909	0.311	0.720
Granulocyte stimulated, MFI[2]	3,912 ± 1032	3,903 ± 1176	0.995	0.069	0.889
Delta stimulated - non-stimulated, MFI[2]	1,199 ± 431.2	968.5 ± 381.6	0.665	0.126	0.040

[1]Endometrial and blood samples were collected on d 10, 21, and 38 of lactation.

[2]Data were $\log_{10}$-transformed for analysis, and reported means and SE are back-transformed.

Table 2-9. Occurrence of virulence factors associated with bacterial uterine diseases in apparently healthy cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period and early lactation.

	Treatment	
Item	CON	OMN
Day10		
fimH	11 (84.6%)	11 (73.3%)
IkTA	4 (30.8%)	6 (40.0%)
fimA	2 (15.4%)	1 (7.14%)
None	1 (7.70%)	2 (13.3%)
Day 21		
fimH	7 (53.8%)	6 (46.2%)
IkTA	3 (23.1%)	2 (15.4%)
fimA	3 (23.1%)	0 (0.0%)
None	3 (23.1%)	6 (46.2%)
Day 38		
fimH	8 (66.7%)	4 (57.1%)
IkTA	3 (25.0%)	2 (28.6%)
fimA	3 (25.0%)	2 (28.6%)
None	3 (25.0%)	3 (43.0%)
Total		
fimH	26 (68.4%)	21 (55.3%)
IkTA	10 (26.3%)	10 (26.3%)
fimA	8 (21.1%)	3 (7.90%)

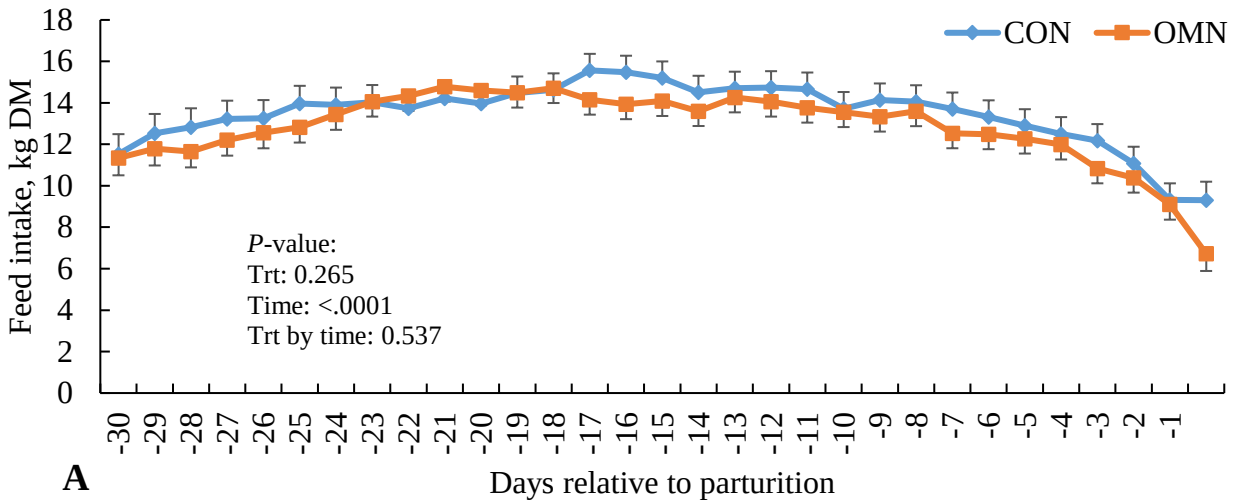
Virulence factors of pathogenic bacteria: fimH (*Escherichia coli*), IkTA (*Fusobacterium necrophorum*), and fimA (*Trueperella pyogenes*).

Table 2-10. Association between the presence of pathogenic bacteria in the uterus of cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period and early lactation.

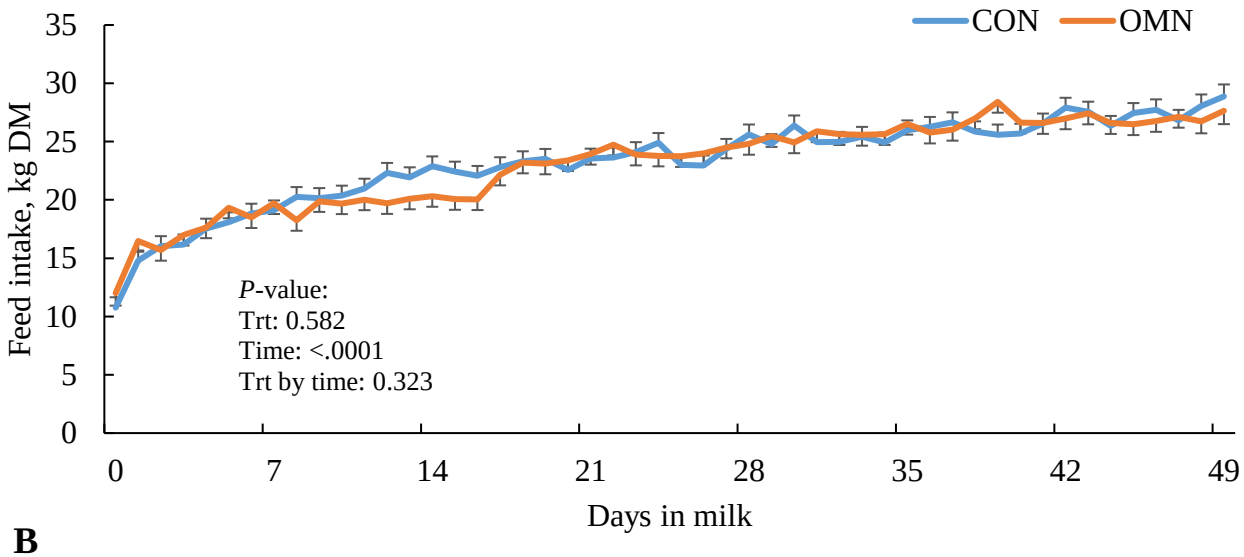
Gene*	Microbe	Odds ratio (OMN/CON)	95% CI	Trt *P*-value
IkTA	*Fusobacterium necrophorum*	0.90	0.30-2.79	0.871
FimH	*Trueperella pyogenes*	2.58	0.73-9.10	0.140

*The logistic model was unstable to analyze FimA because only 11 samples were positive. *P*-values for two-tail Fisher's exact test to analyze presence or absence of pathogens were fimH = 0.655, IkTA = 0.706, and FimA = 0.583.

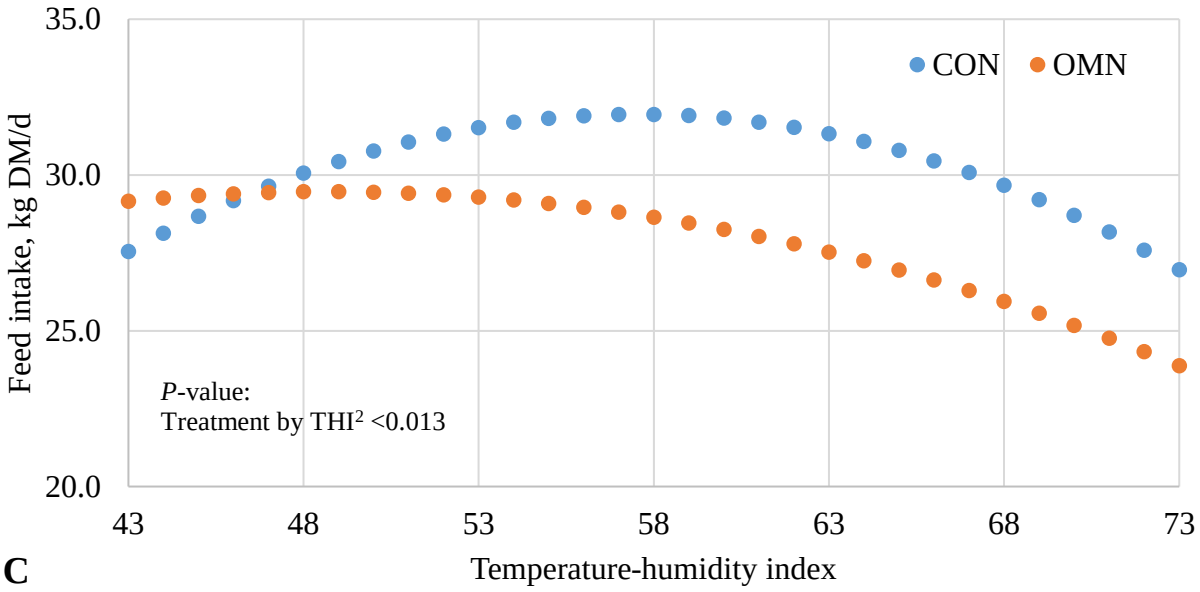
Figure 2-1. Daily feed intake of cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period (A) and early lactation (Panel B). Association between feed intake during lactation and temperature-humidity index (THI; Panel C). Values are LS means and error bars are SE.



A

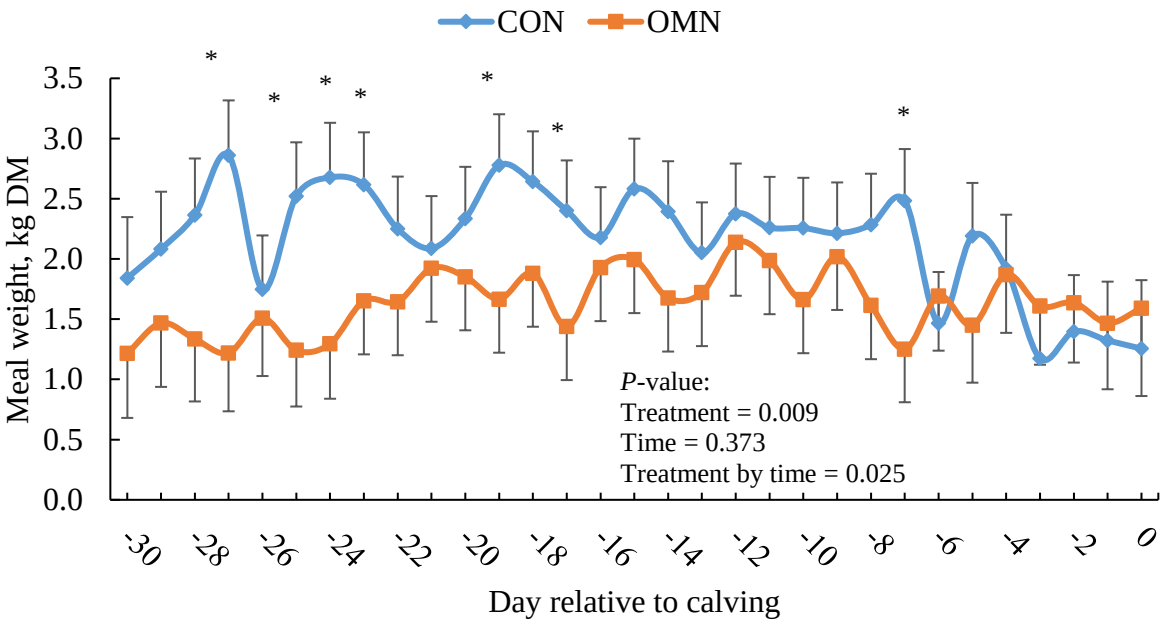


B

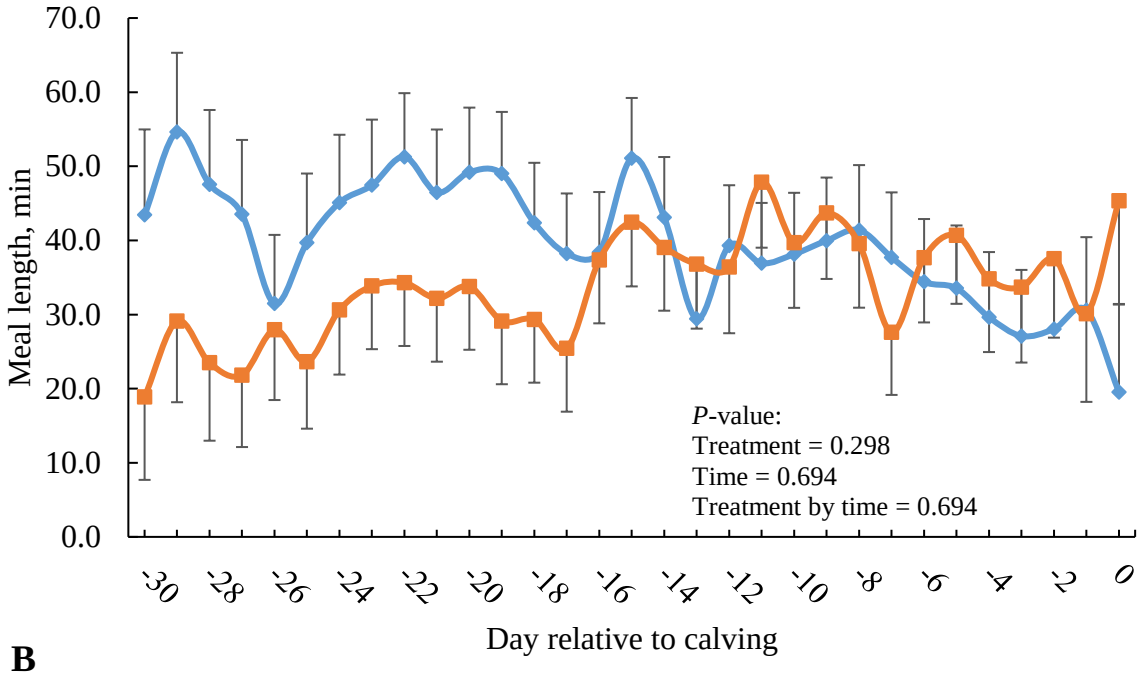

35.0
30.0
25.0
20.0
Feed intake, kg DM/d
CON
OMN
P-value:
Treatment by THI² <0.013
43
48
53
58
63
68
73
Temperature-humidity index

C

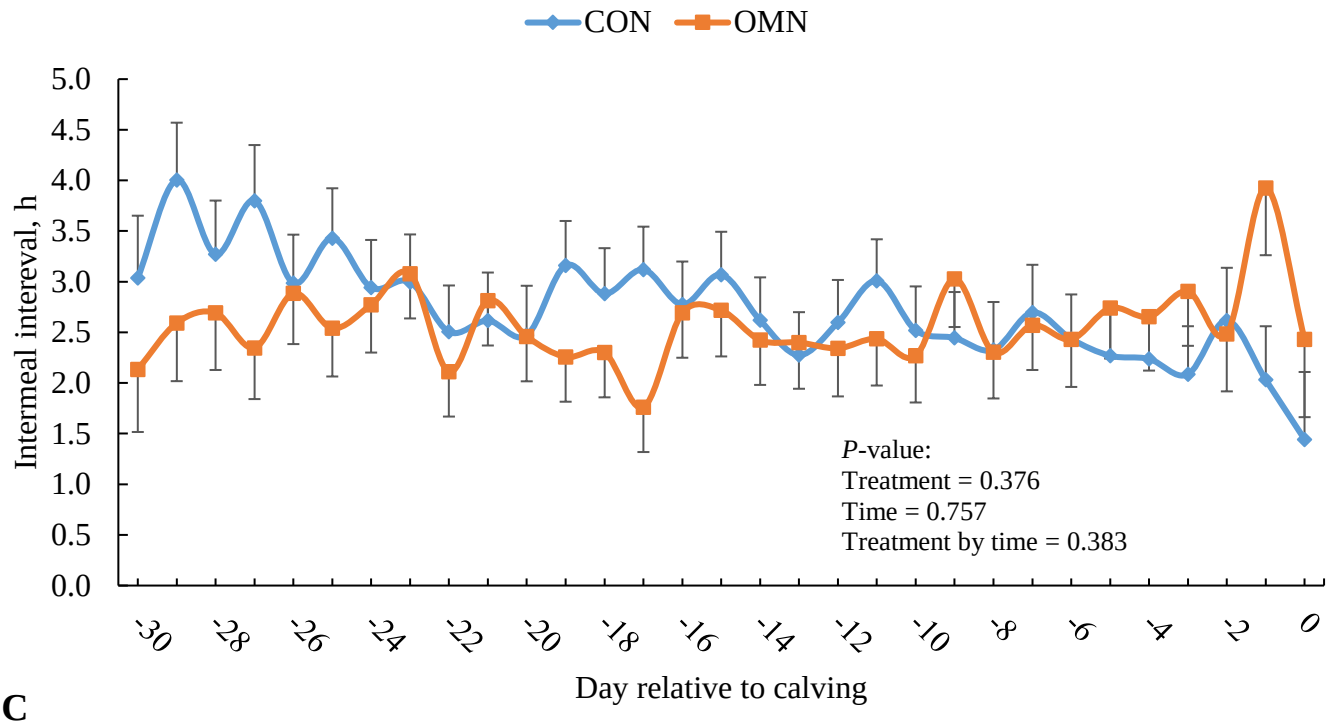
Figure 2-2. Feeding behavior during the dry-period of cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period and early lactation. Meal weight (Panel A), meal length (Panel B), intermeal interval (Panel C), and meal count (Panel D). Values are LS means and error bars are SE.



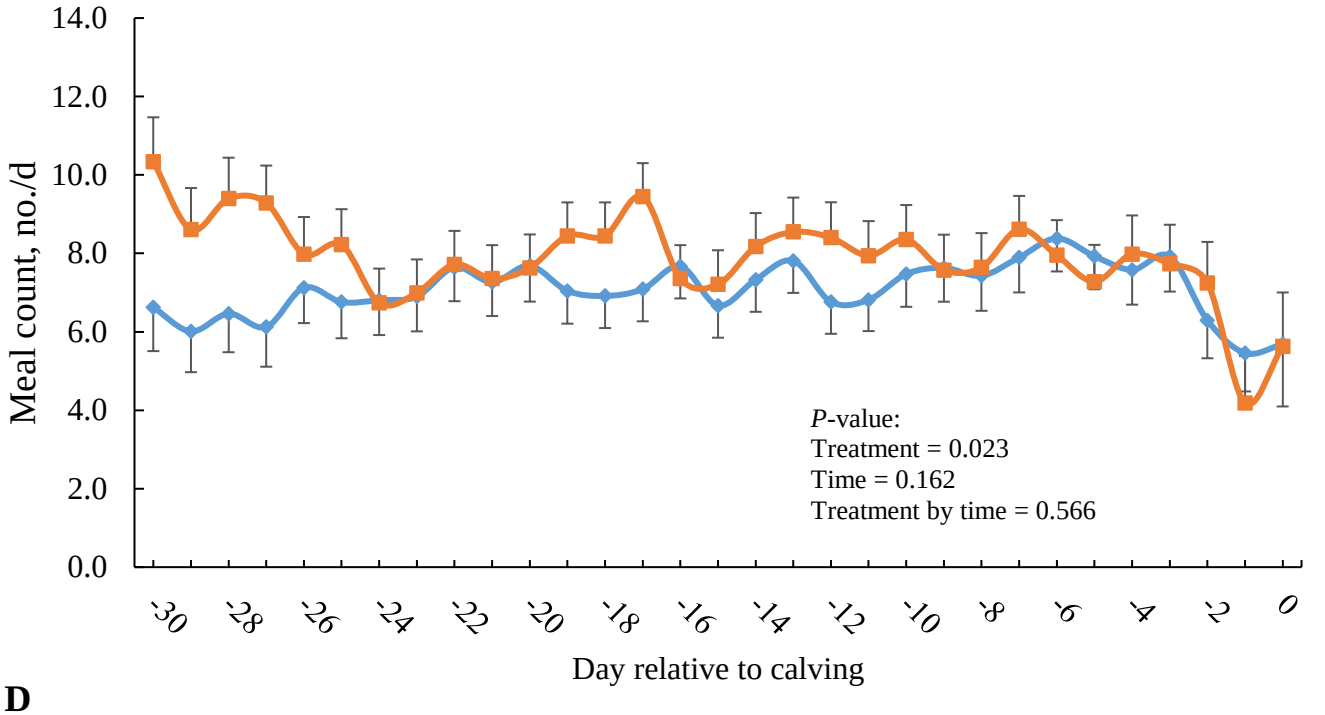
A



B

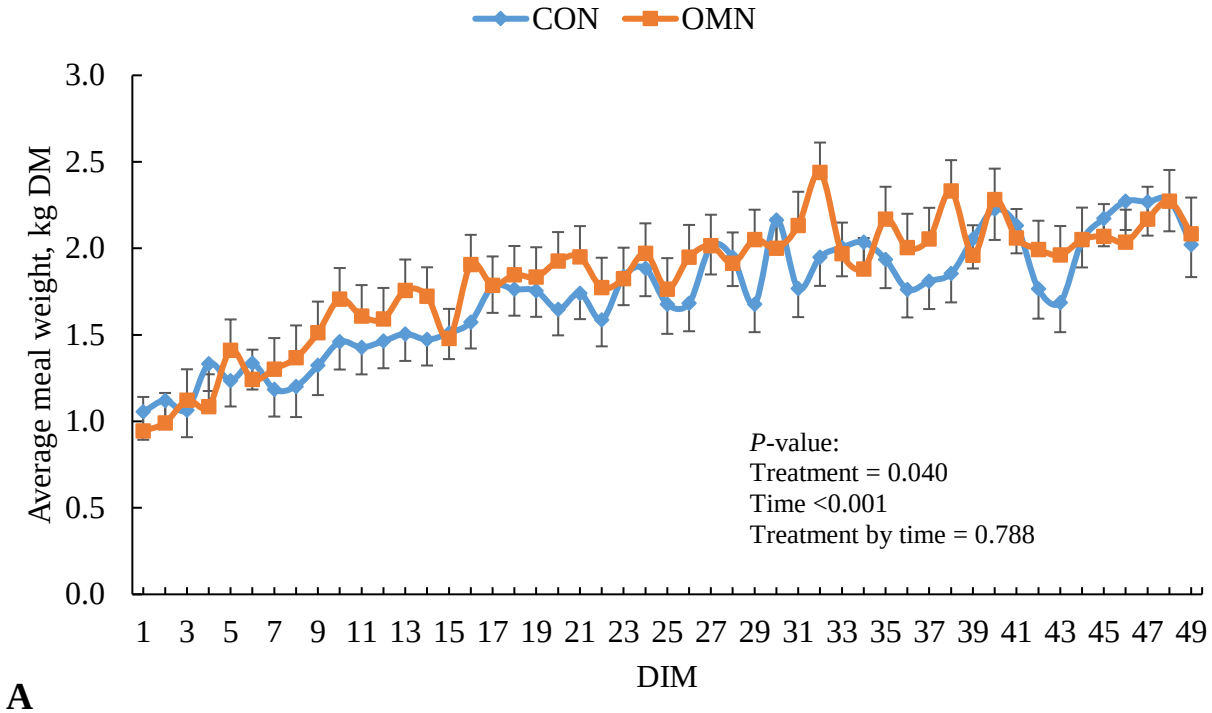

CON
OMN
Intermeal interval, h
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-30
-28
-26
-24
-22
-20
-18
-16
-14
-12
-10
-8
-6
-4
-2
0
Day relative to calving
P-value:
Treatment = 0.376
Time = 0.757
Treatment by time = 0.383

C

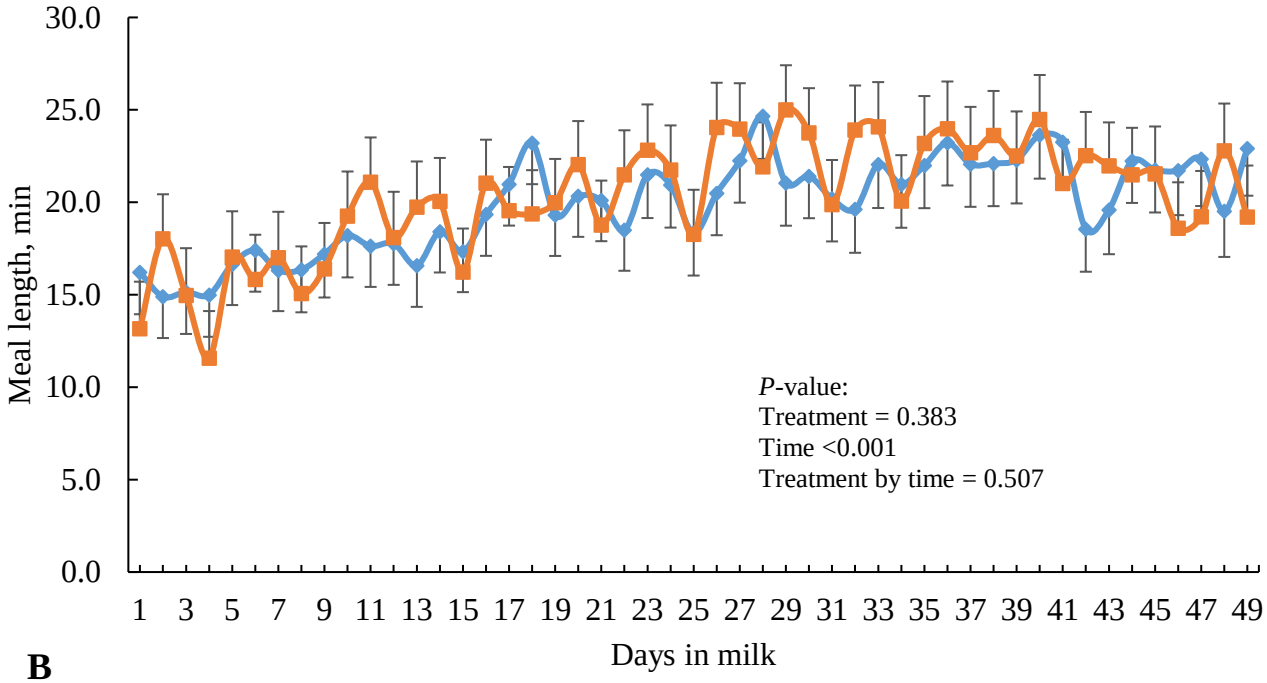

Meal count, no./d
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
-30
-28
-26
-24
-22
-20
-18
-16
-14
-12
-10
-8
-6
-4
-2
0
Day relative to calving
P-value:
Treatment = 0.023
Time = 0.162
Treatment by time = 0.566

D

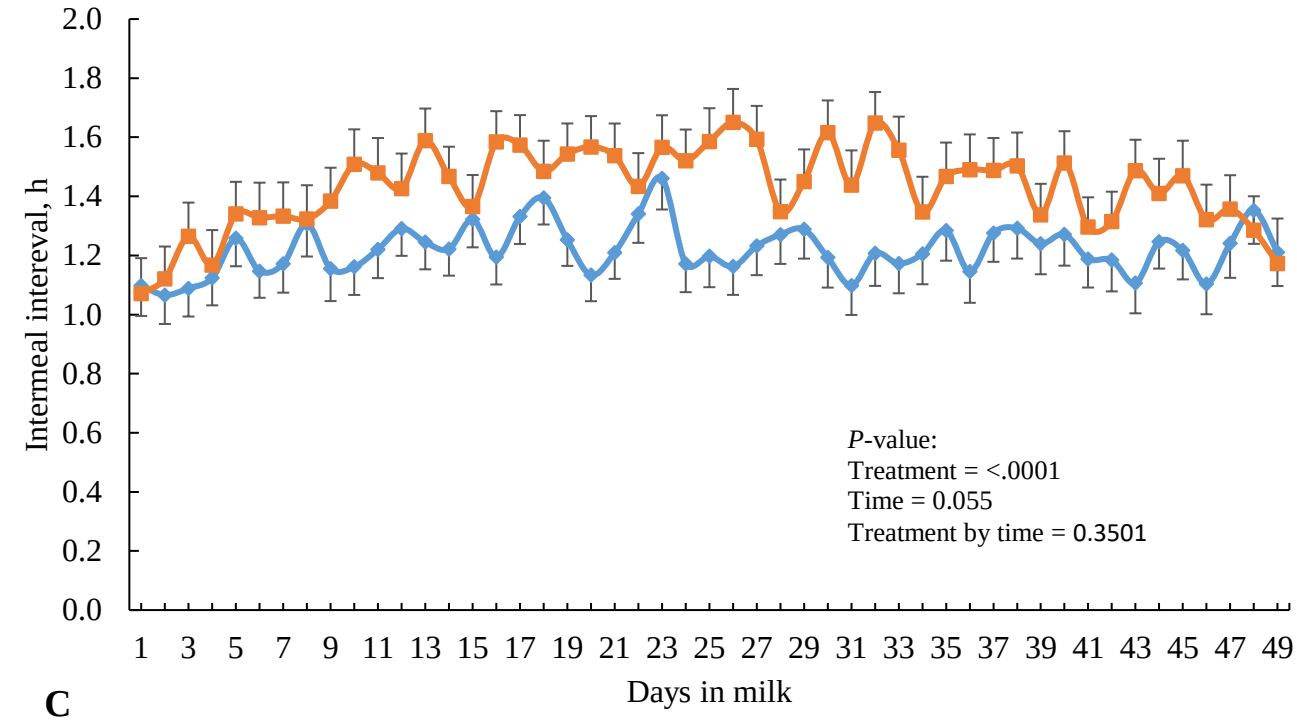
Figure 2-3. Feeding behavior during early lactation of cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period and early lactation. Average meal weight (Panel A), meal length (Panel B), intermeal interval (Panel C), and meal count (Panel D). Values are LS means and error bars are SE.



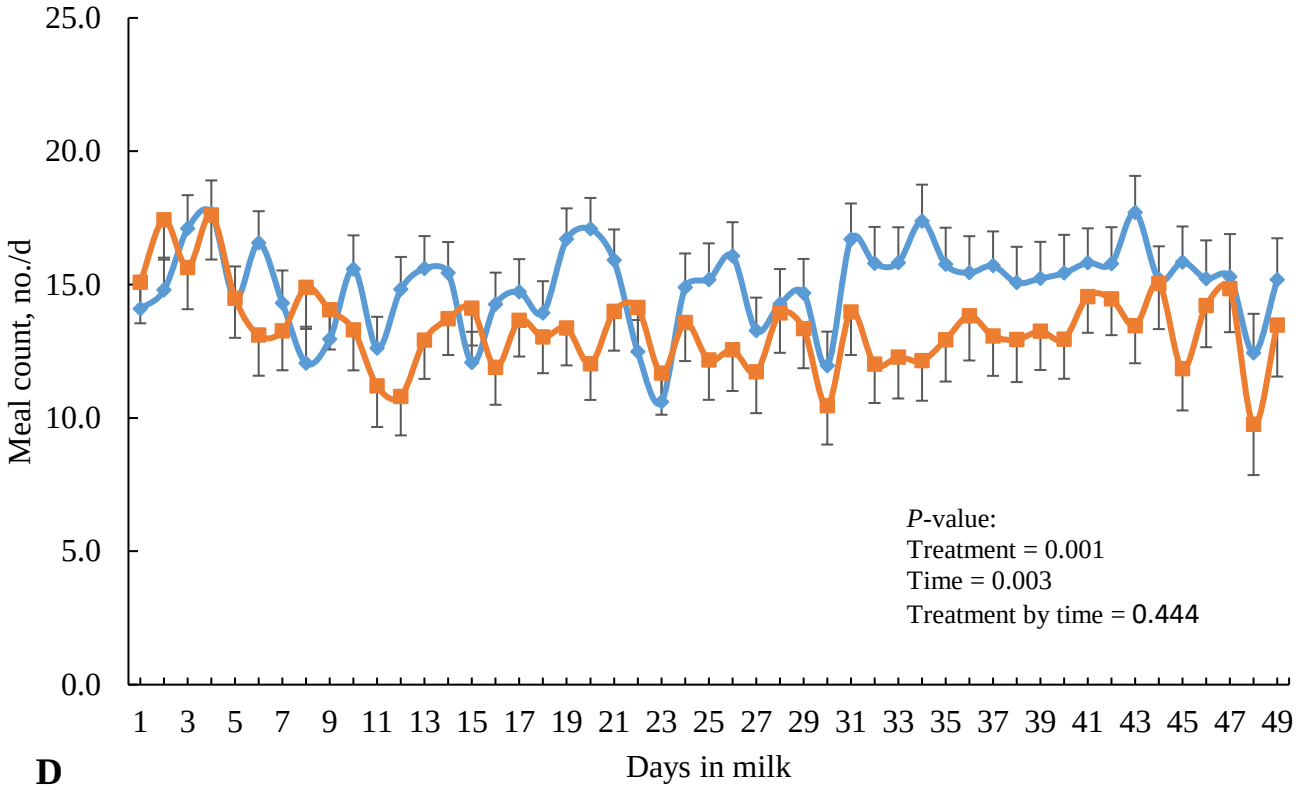
A



B

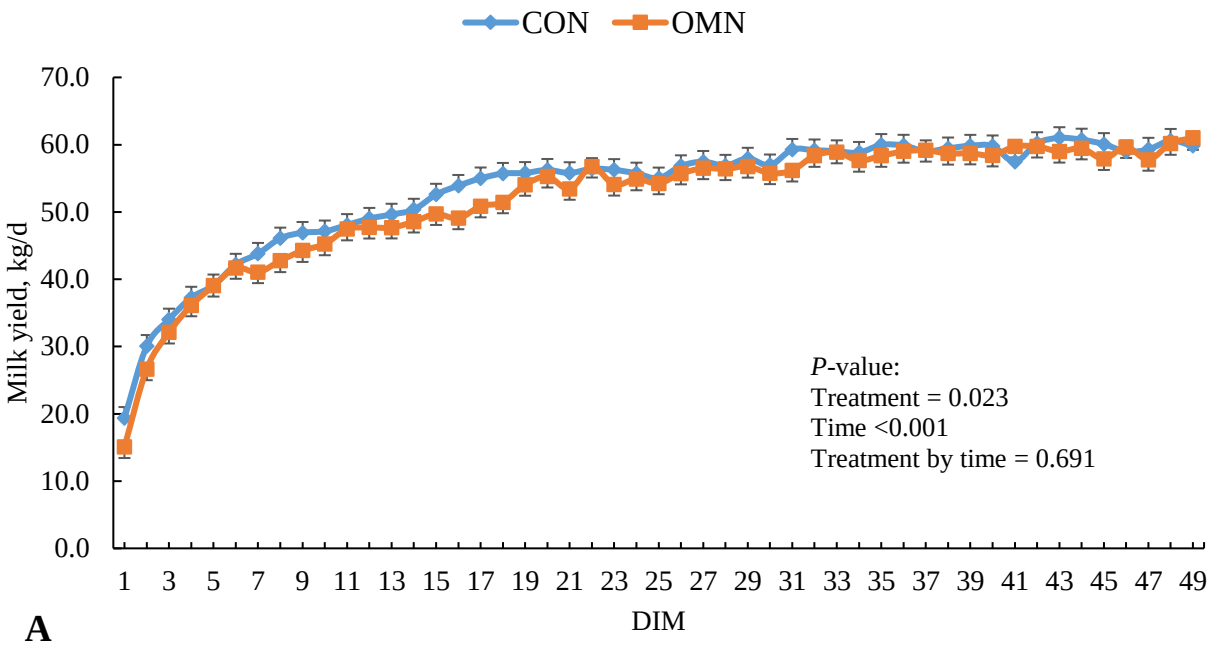

Intermeal intereval, h
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49
Days in milk
P-value:
Treatment = <.0001
Time = 0.055
Treatment by time = 0.3501

C


Meal count, no./d
25.0
20.0
15.0
10.0
5.0
0.0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49
Days in milk
P-value:
Treatment = 0.001
Time = 0.003
Treatment by time = 0.444

D

Figure 2-4. Daily milk yield of cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period and early lactation (Panel A). Interaction between treatment and transition temperature-humidity index (THI) observed in the study (Panel A). Interaction between treatment and transition THI around observed in the study (Panel B).



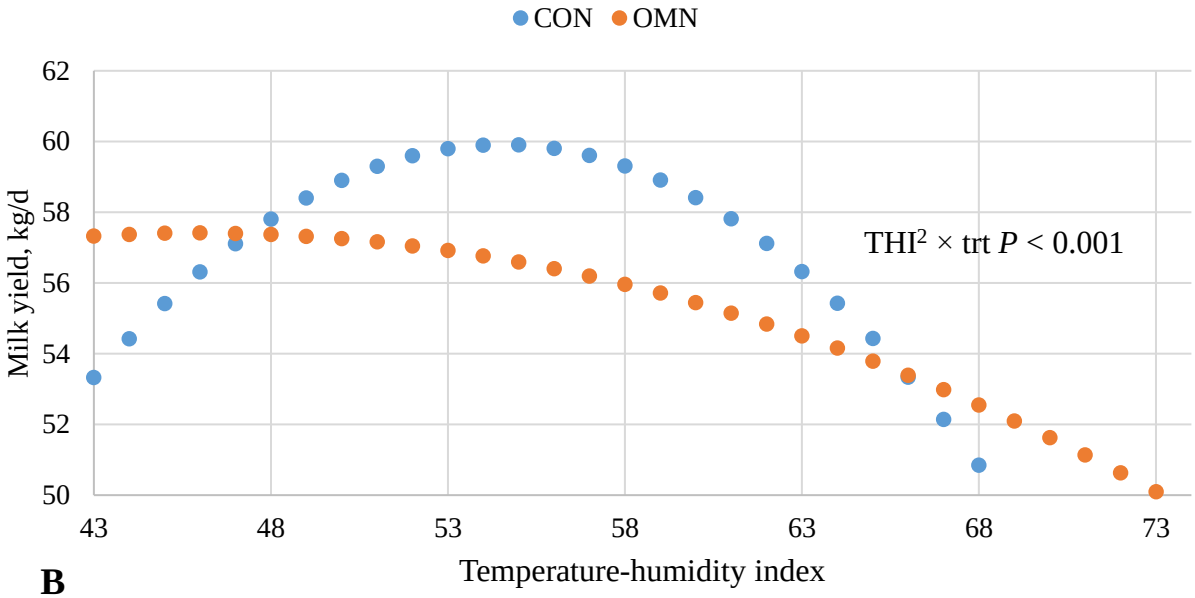
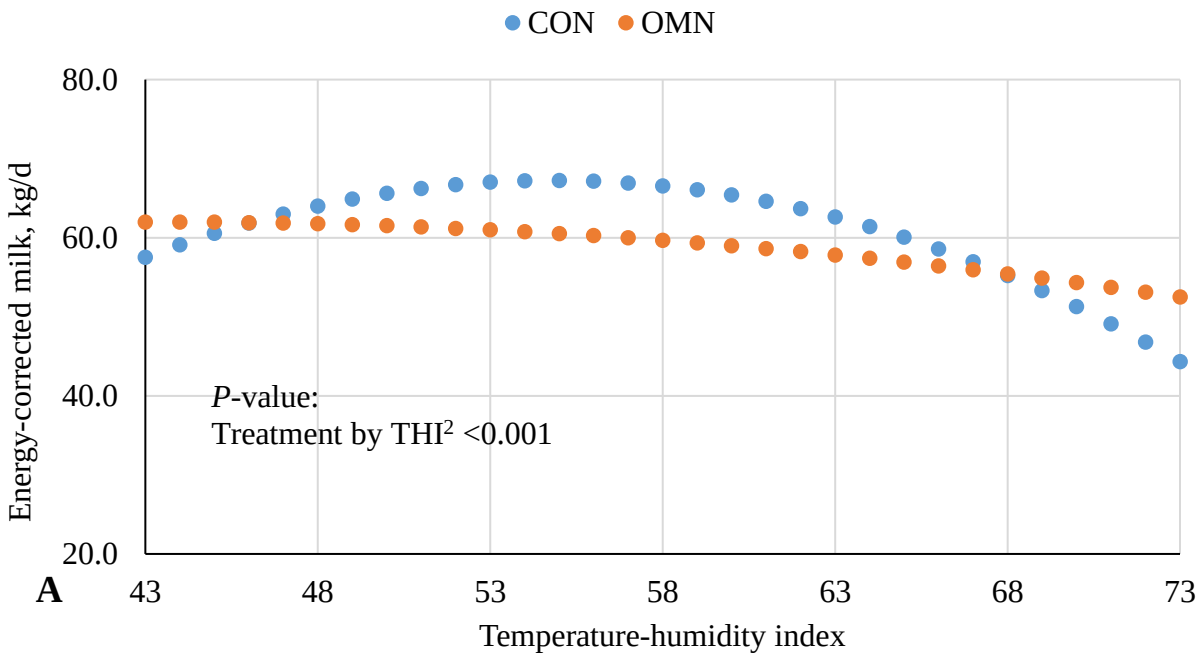


Figure 2-5. Interactions between treatment and transition temperature-humidity index (THI) for energy-corrected milk (A) and between treatment and parity (B) for milk lactose (%) observed in this study.



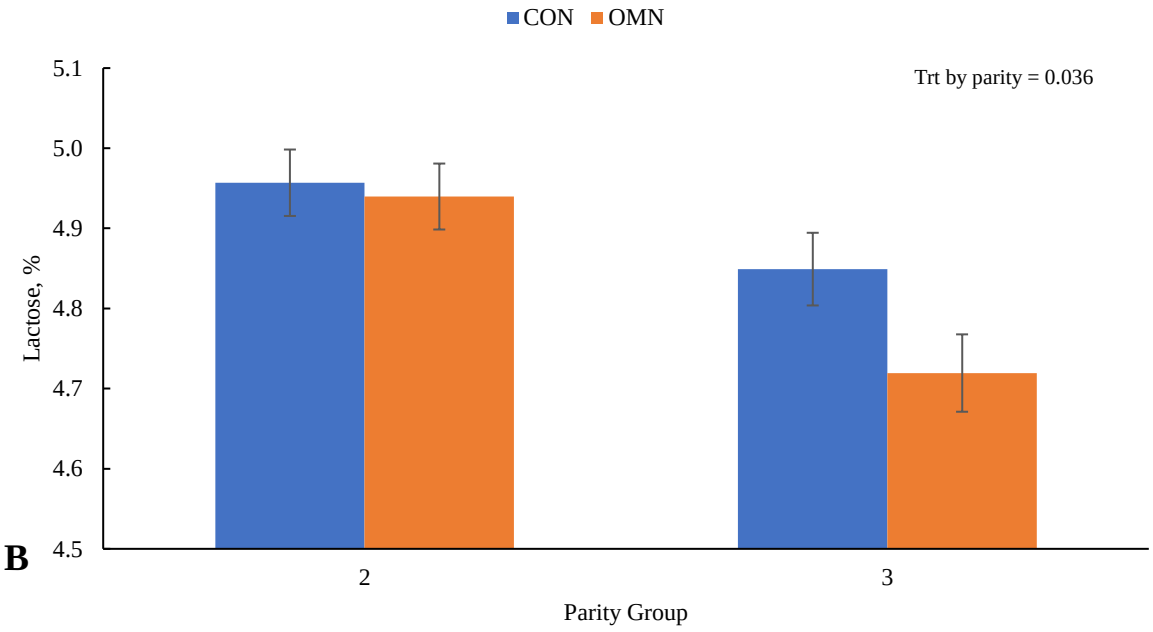


Figure 2-6. Interactions between treatment and average temperature-humidity index (THI) around parturition for rectal temperature of cows during lactation observed in this study.

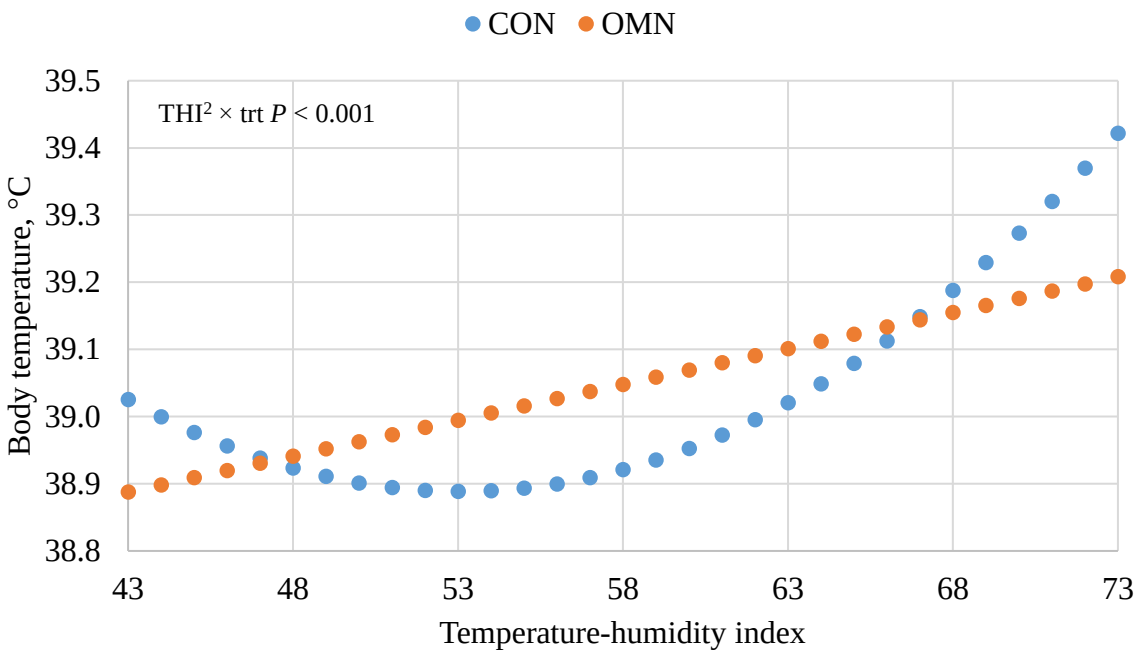
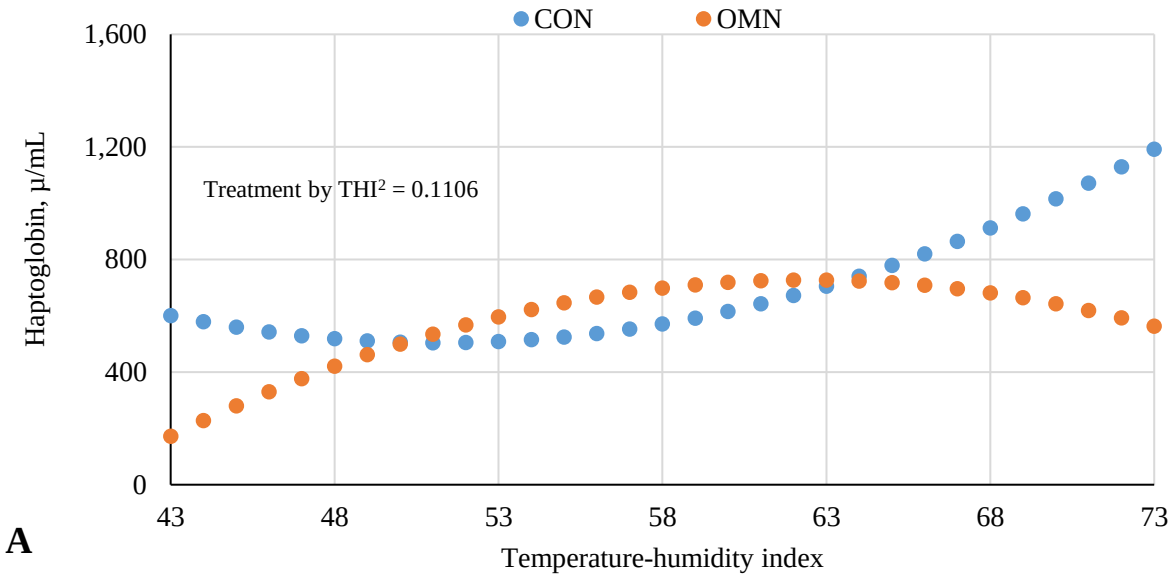
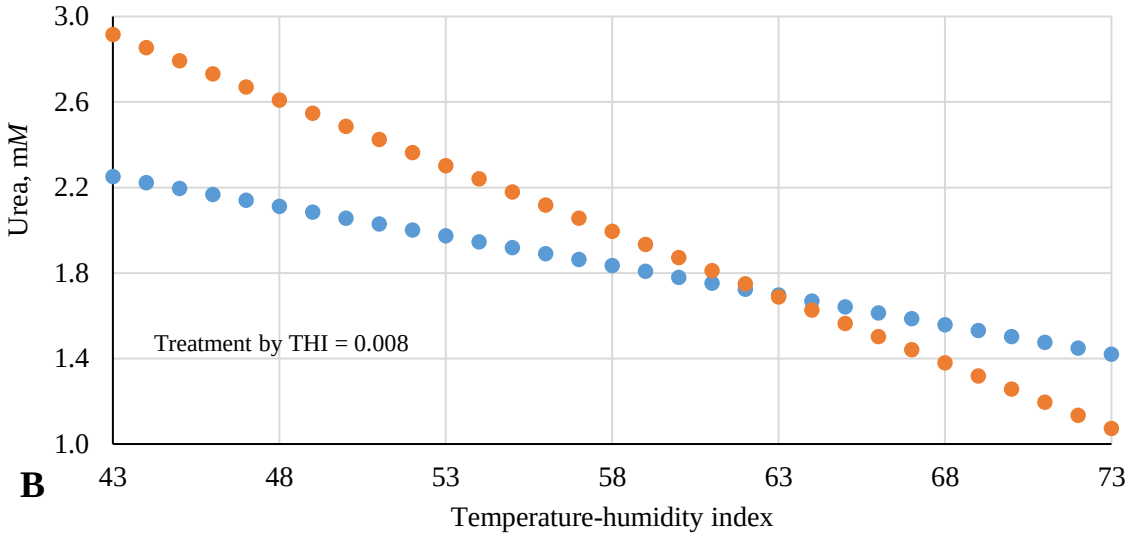


Figure 2-7. Interactions between treatment and transition temperature-humidity index (THI) for (A) plasma haptoglobin concentration and (B) plasma urea concentration observed in this study.



A



B

Figure 2-8. Associations between treatment and transition temperature-humidity index (THI) for plasma fatty acid concentrations observed in this study.

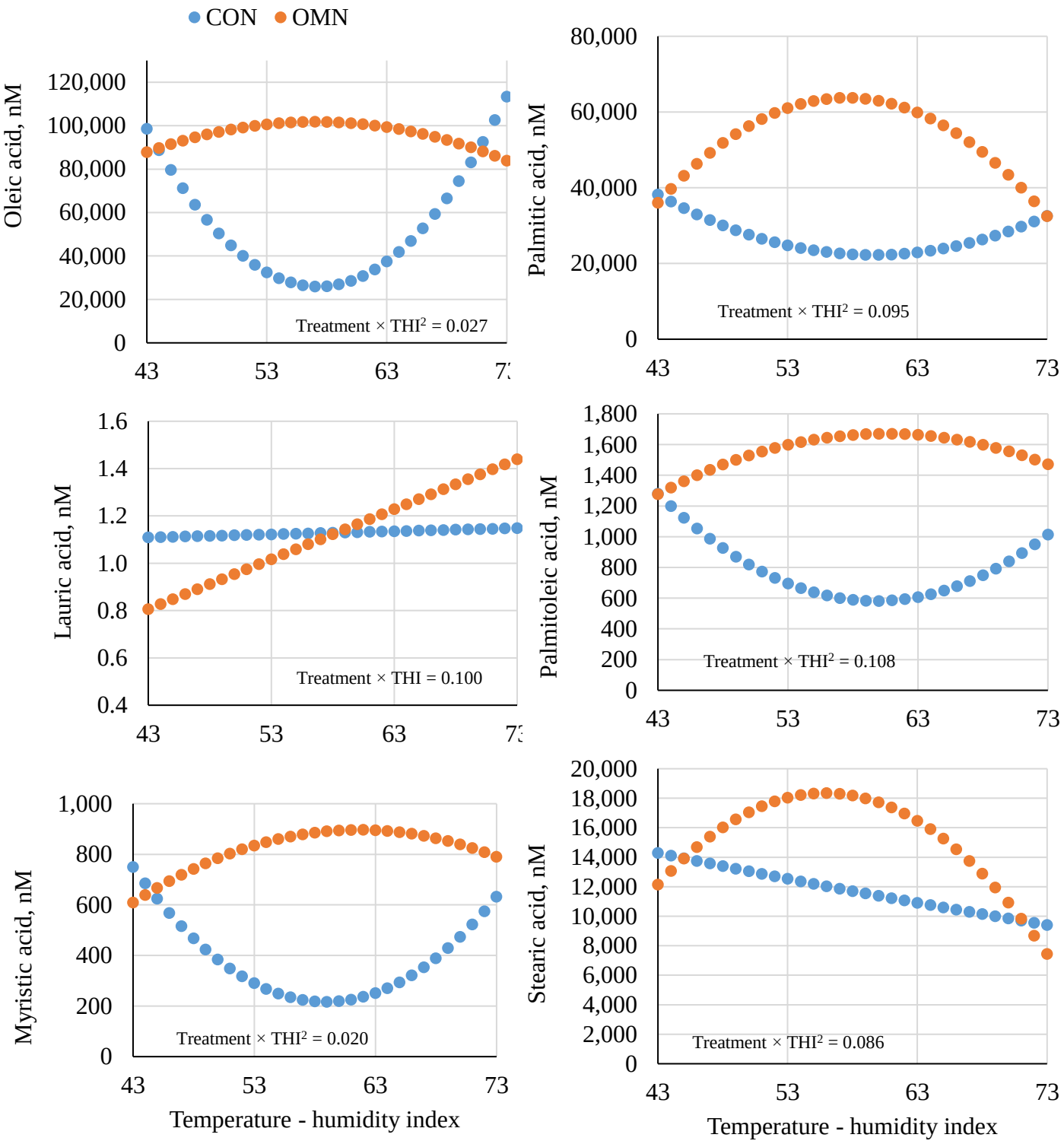


Figure 2-9. Plasma oxylipid profiles from cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period and early lactation (LS means ± SE). Interactions between treatment and time for thromboxane B_2 (Panel A) and for 12-HHTr-E (Panel B). (*) $P < 0.05$ and (§) $P < 0.10$.

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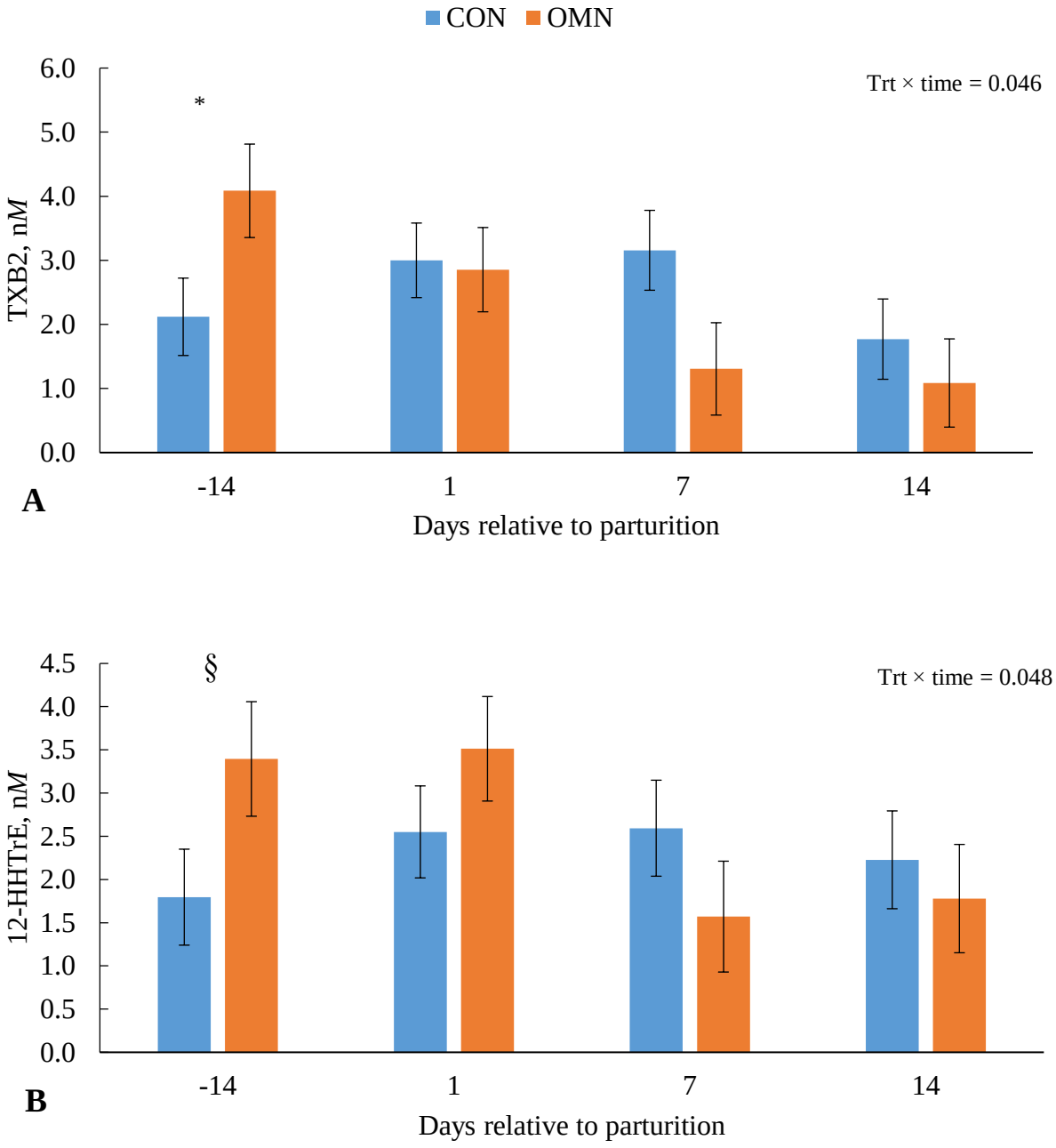


Figure 2-10. Polymorphonuclear cells percentage found in uterine cytobrush of cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period and early lactation (LS means ± SE).

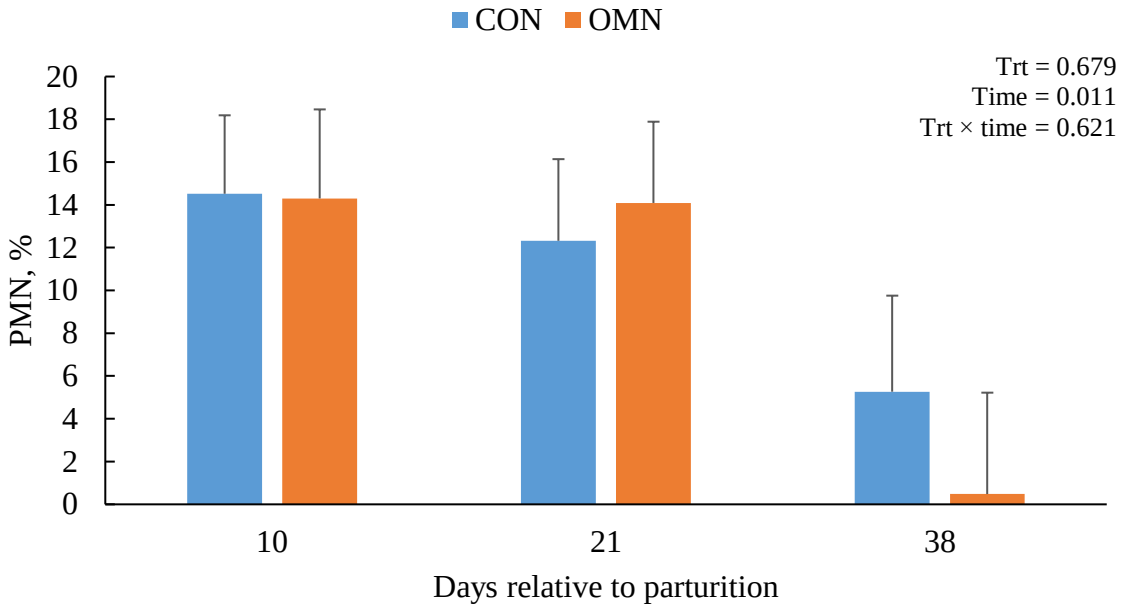


Figure 2-11. Oxidative burst potential of uterine granulocytes from cows fed OmniGen-AF (Phibro Animal Health, Teaneck, NJ; top-dressed 56 g/d) or control (CON) during the dry period and early lactation (LS means ± SE). Data were log_{10}-transformed for analysis and reported means and SE were back-transformed.

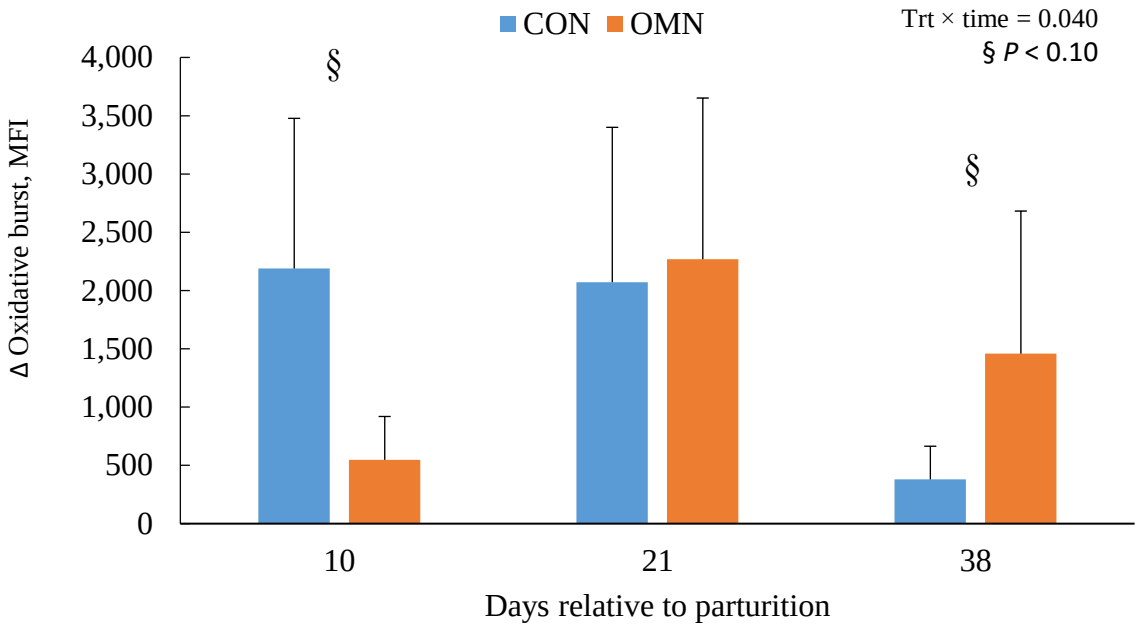
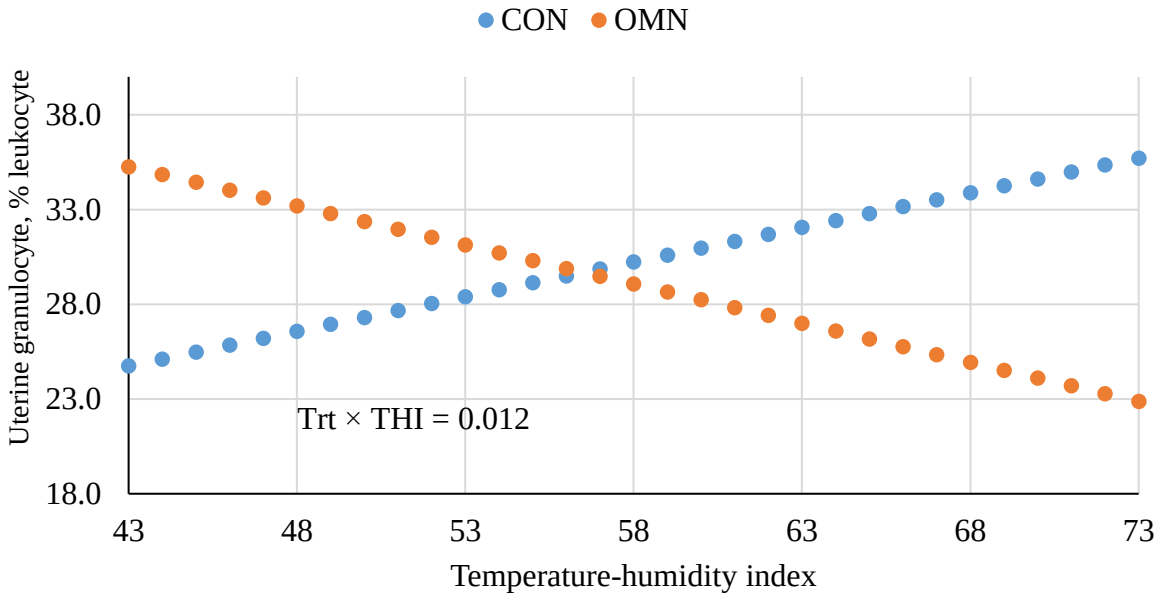


Figure 2-12. Interactions between treatment and transition temperature-humidity index (THI) for uterine granulocyte percentage observed in this study.



Chapter 3 - Proteomic analysis reveals greater abundance of complement and inflammatory proteins in subcutaneous adipose tissue from postpartum cows treated with sodium salicylate

C. S. Takiya, S. R. Montgomery, L. K. Mamedova, G. Kra, N. Nemes-Navon, Y. Levin, S. D. Fleming, B. J. Bradford, and M. Zachut

2019. J. Proteomics. 204: Article 103399

ABSTRACT

This study aimed to investigate sodium salicylate (SS) treatment effects on the proteome of adipose tissue (AT) in postpartum cows. Twenty Holstein cows were assigned to control (CON, n = 10) or SS (n = 10) provided via drinking water (2.3 g/L) during the first 7 d of lactation. Subcutaneous AT was collected on d 7 of treatment and label-free quantitative shotgun proteomics and immunoblotting were analyzed in a subset of 5 AT per group. Eighty out of 1,422 proteins (5.6%) were differentially abundant between CON and SS [fold change ± 1.5, $P < 0.05$]. Top canonical pathways differing between CON and SS (Ingenuity) were complement system, interleukin-10 signaling, and acute phase response signaling. The abundances of complement C1r, C1qC, C1qB and C6 were greater in SS than CON. Regarding IL-10 signaling, the abundances of BLVRB, STAT3, and lipopolysaccharide binding protein (LBP) were greater in SS AT compared to CON. Immunoblots revealed increased abundance of paraoxanase-1 and tumor necrosis factor-alpha, as well as a tendency for greater abundance of cluster differentiation 172a in SS AT, which may indicate increased macrophage infiltration. Sodium salicylate treatment postpartum likely promotes inflammatory signaling in AT of dairy cows, perhaps due to immune cell recruitment.

INTRODUCTION

Beyond its role in energy storage, adipose tissue (AT) is an active endocrine organ that has an important metabolic role and is also able to produce cytokines which modulate inflammatory responses and further influence the metabolism of other tissues (Rosen and Spiegelman, 2006; Desruisseaux et al., 2007; Han et al., 2017). During the transition period (-3 wk to +3 wk relative to parturition), the dairy cow AT is remodeled due to intense lipid mobilization associated with an increase in energy requirements for colostrum and milk synthesis. This lipid mobilization leads to a reduction of AT mass and changes in cytokine secretion that promote an inflammatory response with infiltration of immune cells (Contreras et al., 2017). In human AT, the populations of immune cells are diverse and include mononuclear cells (macrophages, mast cells, lymphocytes, dendritic cells, and natural killer cells) as well as polymorphonuclear cells [neutrophils, eosinophils and basophils;(Mathis, 2013)]. Among immune cells, macrophages are the most predominant immune cell type in AT of cattle (Ampem et al., 2016). During lipolysis there is evidence for increased trafficking of macrophages into AT of dairy cows (Contreras et al., 2018).

A certain degree of AT inflammation may be adaptive as a mechanism to cause a transient insulin resistance that favors lipid mobilization during this period of energy deficit; however, when inflammation and insulin resistance are exacerbated and prolonged, cows are prone to metabolic and inflammatory diseases (Faulkner and Pollock, 1990; De Koster and Opsomer, 2013). In fact, we have previously demonstrated that cows with extreme rates of lipolysis had insulin resistant AT (Zachut et al., 2013) and proteomic analysis showed enriched inflammatory functions in their AT (Zachut, 2015).

One approach that has been attempted to reduce the degree of inflammation during the transition period is the use of nonsteroidal anti-inflammatory drugs such as sodium salicylate (SS).

In previous studies with dairy cows we have demonstrated that SS treatment postpartum increased whole-lactation milk production (Farney et al., 2013a), and altered glucose metabolism (Farney et al., 2013b). Studies reported different effects of SS on inflammatory markers. Carpenter et al. (Carpenter et al., 2016) reported greater plasma haptoglobin concentration in cows that consumed SS, whereas Farney et al. (2013a) found lower liver abundance and plasma concentration of tumor necrosis factor-alpha (TNF-α). Carpenter et al. (Carpenter et al., 2018) reported no difference in blood haptoglobin concentration with SS treatment.

Recently, we have shown that SS treatment tended to decrease glucose turnover rate by 25% despite a significant decrease in the insulin:glucagon molar ratio (Montgomery et al., 2019). In that study, SS tended to increase phosphorylation of AMP-activated protein kinase and decrease protein kinase B phosphorylation in AT (Montgomery et al., 2019). Evidence suggests that salicylate accumulates specifically in inflamed tissue (Brune et al., 1980; Rainsford et al., 1980) which may cause a different response in AT compared to other organs. Based on our recent findings, we aimed to further investigate the specific effects of SS treatment on AT protein profiles in postpartum dairy cows by proteomic analysis.

MATERIALS AND METHODS

The experiment was carried out after the approval by the Kansas State University Institutional Animal Care and Use Committee (protocol 3182). Full details on animal management and handling are provided in the companion paper (Montgomery et al., 2019).

Animals and design

Twenty female Holstein (≥ 3 parities) were enrolled after parturition (4 to 36 h) and were assigned to either control (CON) or SS (2.3 g/L in drinking water; Wintersun Chemical, Ontario, CA) treatments during the first 7 d of lactation. Treatment was randomly assigned for the first cow enrolled and then alternated as additional cows were enrolled over the course of 209 d. Cows in both treatment groups also received molasses at 0.15 g/L in drinking water to mask SS flavor. Mean water intake of cows did not differ between treatments (77.9 vs. 73.9 ± 5.6 L/d for CON vs. SS, $P = 0.61$). At enrollment, body condition score (1 = thin, 5 = fat) was 3.6 and 3.5 ± 0.17 ($P = 0.76$), whereas body weight was 776 and 777 ± 22 kg ($P = 0.97$) for CON vs. SS, respectively. Body condition score is tightly correlated with mean subcutaneous adipocyte volume (Yuan et al., 2014).

Cows enrolled in the experiment had singleton births, did not experience dystocia, and showed no signs of clinical diseases (i.e. metritis, mastitis, lameness) during the treatment period. Cows were fed *ad libitum* twice daily (0630 h and 1600 h) and milked thrice daily at 8-h intervals. The diet contained 50.1% dry matter, 17.4% crude protein, 38% neutral detergent fiber, and 23.6% acid detergent fiber (Montgomery et al., 2019). Data on the performance and metabolic responses of the cows that were enrolled in the present study were recently published (Montgomery et al., 2019); SS did not affect dry matter intake or milk component production of cows during the first 7 d of lactation. Regarding endocrine and metabolic responses, SS treatment had no influence on plasma concentrations of adiponectin, beta hydroxy-butyrate, free fatty acids, or inflammation markers (haptoglobin and TNF-α (Montgomery et al., 2019). When these variables were analyzed with only the 10 cows utilized for proteomic analysis, we likewise found no treatment effects.

Adipose tissue biopsy

Subcutaneous adipose tissue was collected from all cows on d 7 postpartum. The area between the tailhead and pin bone was clipped, surgically scrubbed (70% ethanol and betadine), and locally anesthetized with 2% lidocaine hydrochloride (6 mL; Agri Laboratories Ltd., St. Joseph, MO). A 3 cm-long incision was made with a sterile surgical scalpel and subcutaneous adipose tissue (~ 5 g) was collected with the aid of sterile forceps and surgical scissors. Adipose tissue specimens were snap-frozen in liquid nitrogen and stored at -80°C. After collection of AT, the incision site was closed with surgical staples. The entire collection procedure (asepsis, incision, sampling/snap freezing, and suture) lasted 10 to 15 min.

Sample preparation for proteomic analysis

Protein concentration in each sample was determined using the bicinchoninic acid assay, and samples from 10 cows (5 per treatment) were selected for proteomics analysis based on protein yield. Sample size for proteomic analysis was based on previous studies with similar design in dairy cows (Zachut, 2015; Zachut et al., 2016). Samples were subjected to tryptic digestion using a modified filter-aided sample preparation protocol. Samples were lysed in 1 ml SDT lysis buffer (4% SDS, 100 m*M* Tris ph7.6, DTT 100 m*M*) for 6 min at 95 °C, and cell debris was removed by centrifugation (16,000 × *g*, 10 min). Fifty micrograms were taken from the supernatant and mixed with 200 µL urea buffer I (8.0 *M* urea in 0.1 *M* Tris–HCl pH 8.0), loaded onto a 30-kDa molecular-weight-cutoff filter (vivacon 500, VN01H22, Sartorius, Göettingen, Germany) and centrifuged for 30 min at 14,000 *g*, followed by one wash with urea buffer I and centrifuged 30 min at 14,000 *g*. Iodoacetamide then added on the filter, incubated for 10 min and centrifuged 20 min at 14,000 *g*. Two washes were made using 200 µL ammonium bicarbonate. Trypsin (1 µg) in 40 µL ammonium

bicarbonate was added, and samples were incubated at 37 °C overnight. Digested proteins were centrifuged, acidified with trifluoroacetic acid, and desalted in a solid-phase extraction column (Oasis HLB, Waters, Milford, MS, USA). Samples were stored at -80 °C until further analysis.

Liquid chromatography

Ultra LC–MS-grade solvents were used for all chromatographic steps (Bio-Lab, Jerusalem, Israel). Each sample was subjected to split-less nano ultra-performance liquid chromatography (UPLC; 10K psi nanoAcquity, Waters). The mobile phases were: (A) H_2O + 0.1% (v/v) formic acid and (B) acetonitrile + 0.1% formic acid. Samples were desalted online using a reverse-phase C18 trapping column (180-µm internal diameter, 20-mm length, 5-µm particle size; Waters). The peptides were then separated using an HSS T3 nano-column (75-µm internal diameter, 250-mm length, 1.8-µm particle size; Waters) at 0.35 µL/min. Peptides were eluted from the column into the mass spectrometer using the following gradient: 4% to 35% solution B in 150 min, 35% to 90% B in 5 min, maintained at 95% B for 5 min, and then back to initial conditions.

Mass spectrometry

The nano-UPLC was coupled online through a nano-ESI emitter (10-µm tip; New Objective, Woburn, MA) to a quadrupole orbitrap mass spectrometer (Q Exactive Plus, Thermo Scientific, Watham, MA) using a FlexIon nanospray apparatus (Thermo Scientific). Data were acquired in data-dependent acquisition mode using a Top20 method. The quadrupole isolation window was set to 1.6 mass units, MS1 resolution was set to 70,000 (at 400 m/z) with an automatic gain control (AGC) target of 3e6, and maximum injection time was set to 120 ms. MS2 resolution was set to 17,500 with an AGC target of 1e6 and a maximum injection time of 60 ms, and

normalized collision energy was set to 26. Singly charged ions were excluded and dynamic exclusion was set to 60 s.

Data processing and analysis

Proteins were quantified by intensity-based label-free proteomics as described in Shalit et al. (2015). Raw data were processed as reported by (Shalit et al., 2015) briefly, raw data were imported into Expressionist® software version 9.2.4 (Genedata, Basal, Switzerland). Data were filtered, smoothed, and aligned in retention time. This was followed by feature detection based on peak volume in retention time, m/z and intensity space, as well as isotopic clustering. A master peak list was generated from all MS/MS events and sent for database search using Mascot v2.5.1 (Matrix Sciences, Boston, MA). Data were searched against the *Bos taurus* sequences in UniprotKB (http://www.uniprot.org/), version 2015_07, appended with 125 common laboratory-contaminating proteins for a total of 23,970 entries. Fixed modification was set to carbamidomethylation of cysteines and variable modification was set to oxidation of methionines. Search results were then imported into Scaffold version 3.5 (Proteome software) for filtering. False discovery rate was set to a maximum 1% at the protein level using the embedded ProteinProphet algorithm. Identifications were then imported to Expressionist (Genedata, Basal, Switzerland). Protein grouping and quantification were conducted using an in-house script (Shalit et al., 2015). Protein quantification was based on the three most abundant peptides per protein, unless the protein was detected with one or two peptides. Data were normalized based on the total ion current. Protein abundance was obtained by the iBAQ method (sum of all peptide intensities per protein divided by the theoretical number of tryptic peptides for that particular protein). Principal component analysis was used to assess the global integrity of the data and search for outlier samples.

Bioinformatic analysis

Proteins that were differentially abundant at $P < 0.05$ and fold change (FC) ± 1.5 were analyzed by Qiagen's Ingenuity® Pathway Analysis (IPA®, Qiagen Redwood City, CA; www.qiagen.com/ingenuity) to determine the most relevant pathways altered by treatment.

Western blot analysis and blood parameters

To validate the results of the proteomic data and to examine additional proteins that are related to inflammation in AT, complementary immunoblots were performed from AT lysates. Protein concentrations in lysates were determined by Bradford assay as well as by BCA assay. From each sample, 20 µg of sample in Laemmli loading buffer was resolved by SDS-PAGE under reducing conditions, and transferred onto a nitrocellulose membrane with antibodies targeting the following proteins: for validation of proteomic data - complement C1r (1 µg/mL, ab71652, Abcam Biotech, Cambridge, MA), lipopolysaccharide binding protein (LBP; 1 µg/mL, ARP41546_P050, Aviva System Biology, San Diego, CA), and complement C1q B chain (C1QB; 1 µg/mL, ab90181, Abcam Biotech). To add information on inflammatory proteins in AT, we also examined the abundance of TNF-α (1:1000, OACAO4183, Aviva System Biology), paraoxanase 1 (PON1; 1 µg/mL, ab123558, Abcam Biotech), macrophage inhibitor factor (MIF; 1:1000, ab176565, Abcam Biotech), and CD172a (1:1000, Washington State University – Mononuclear Antibody Center, WA). The abundance of tubulin (1:1000, 2125S, Cell Signaling Technology, Danvers, MA) or β-actin (1:1000, ab46805, Abcam Biotech) was used as a loading reference.

Goat anti-rabbit horseradish peroxidase conjugated secondary antibody (111-035-003, Jackson Immunoresearch Laboratories, Inc., West Grove, PA) at a dilution of 1:10,000 was used

for an enhanced chemiluminescence reaction for protein detection. Data were processed and analyzed by densitometry using ImageJ software (NIH, Bethesda, MD). To ensure that quantitative data were obtained, chemiluminescent signals were measured after at least 5 consecutive exposure times to determine the linear range of the signal intensity of each antibody. Specific band signals were normalized to tubulin or β-actin. Blood samples from cows used in this study were collected and analyzed for haptoglobin and TNF-α as described in Montgomery et al. (2019).

Statistical analysis

Principle component analysis of the proteomic data showed 2 distinct clusters of AT samples. Cluster assignment was not associated with treatment ($P = 0.99$) but was associated with protein concentration of the initial AT lysate ($P < 0.01$); therefore, the effect of cluster was added to the statistical analysis. Proteomics data, after logarithmic transformation, were analyzed by 2-way ANOVA (Statmodel of Python, version 3.6.4) to determine the effects of cluster, treatment, and the interaction between treatment and cluster. Differentially abundant proteins for each effect were determined by $P < 0.05$ and an absolute fold change (FC) ± 1.5. The data analysis was based on the strategy of Feise (2002), calculating the ratios of the observed means as well as identifying outlying measurements that would skew the analysis, specifically for every significant protein. Abundance of proteins determined by immunoblot were analyzed using student's t-test. Blood parameter data were analyzed as described in Montgomery et al. (2019).

RESULTS

The present proteomics analysis discovered a total of 1,422 proteins in the subcutaneous AT of early lactating cows. The abundance of 80 proteins (5.6%) was different (FC ± 1.5, $P <$

0.05) in AT of SS compared to CON cows (Table 3-1). From the list of the differentially abundant proteins, 62 proteins were categorized based on cellular location and function using IPA software **(**Figure 3-1**)**. Many of the differentially-abundant proteins are located in the cytoplasm (n = 31) and function as enzymes (n = 20).

The top 10 canonical pathways influenced by SS treatment according to IPA **(**Figure 3-2**)** were the complement system, interleukin (IL)-10 signaling, and acute phase response signaling, as well as heme degradation, D-glucuronate degradation, glucocorticoid biosynthesis, mineralocorticoid biosynthesis, androgen biosynthesis, the coagulation system, and cell cycle regulation by the BTG family proteins.

The complement system is an integral part of the innate immune response and acts as a bridge between innate and acquired immunity; there are three known pathways for complement activation: classical, alternative and lectin pathway (CHAPTER 1 - Chapter 1 -Appendix A -). In our proteomic data, four proteins associated with the classical pathway of the complement system were greater in cows treated with SS compared to CON counterparts **(**Figure 3-3**)**: C1r (FC = 1.65, $P = 0.03$), C1qC (FC = 1.59, $P = 0.03$), C1qB (FC = 360, $P < 0.01$), and C6 (FC = 1.5, $P < 0.04$). Although did not reach the cut-off of fold change 1.5, other complement proteins were increased by SS treatment: C2 (FC = 1.4, $P = 0.014$), C8a (FC = 1.4, $P = 0.02$), and C4b-binding protein alpha chain (C4BPA, FC =1.42, $P = 0.02$). Differences in abundance of C1r and C1qB were validated through immunoblots; treatment means were in line with proteomic data, although results were not statistically significant (Figure 3-4).

IL-10 is one of the most-studied anti-inflammatory cytokines, secreted by nearly all leukocytes. We found that three proteins related to IL-10 signaling were differentially abundant in SS compared to CON AT (Figure 3-3): BLVRB (FC = 1.62, $P = 0.04$), STAT3 (FC = 2.22, $P =$

0.04), and LBP (FC = 2.31, P = 0.01). Differences in abundance of LBP between cows treated with SS and CON was further confirmed by immunoblotting, showing that the abundance of LBP was 9.7-fold greater in SS cows compared to CON (P = 0.01; Figure 3-4). For acute phase response signaling, proteomics analysis revealed decreased abundance of ferritin light chain (FTL; FC = -1.72, P = 0.02; Figure 3-3) for SS vs. CON, in contrast to effects on C1R, STAT3, and LBP (described earlier). For some of these proteins, the effect of cluster was also significant: C1R (P = 0.02), C1qC (P = 0.003), C6 (P = 0.003), C2 (P = 0.04), C8a (P = 0.002), C4BPA (P = 0.002), BLVRB (P = 0.03), and STAT3 (P = 0.01) all differed by cluster. However, the interactions of treatment and cluster were not significant for any of these proteins (Table 3-1), suggesting that sample differences identified by principle component analysis did not influence ability to detect treatment effects for these proteins.

Additional immunoblots were carried out to evaluate evidence for an increase in inflammation and in immune cell populations in SS AT. These analyses showed that the abundance of TNF-α was 18-fold greater (P = 0.03) and the abundance of CD172a tended to be greater (P = 0.06) in AT of SS cows compared to CON (Figure 3-5). The abundance of PON1 tended to be greater in SS than in CON AT ($P < 0.10$), whereas the abundance of MIF did not differ between groups (Figure 3-5). Neither treatment ($P \geq 0.66$) nor treatment × time interaction ($P \geq 0.44$) effects were observed for plasma concentrations of TNF-α and haptoglobin. Least square mean (± SE) plasma concentrations of TNF-α were 47.6 ± 10.5 vs. 95.3 ± 8.8 pg/mL, and of haptoglobin were 400 ± 139 vs. 821 ± 300 μg/mL for CON vs. SS groups, respectively.

DISCUSSION

Results from proteomics analysis and immunoblots suggest that oral SS treatment during the first 7 days of lactation increases the AT abundance of proteins related to complement system as well as inflammatory response proteins such as TNF-α and LBP. It is worth mentioning that AT proteins include those from several non-adipocyte cells such as immune cells (primarily macrophages), endothelial cells, and mesenchymal cells that reside in this tissue. The literature on the complement system and specifically on complement proteins in AT in cattle are scarce. Examining raw data from our previous proteomic analyses of AT that was obtained from peripartum cows showed that in the first study that identified 586 proteins in AT, the abundances of 6 complement proteins were found [C5, CFH, C6, C7, C1R, and C3; (Zachut, 2015)]. In two other studies, 13 complement proteins were found in transition cows' AT [C1QA, C1QB, C2, C7, CFB, CFD, C9, C1S, C3, C1R, C6, C5, CFH; in Zachut et al. (2017); and CFH, C5, C1R, CFD, C1QC, C1S, C8A, C3, C1QB, C9, C1QA, C7, C6; in Zachut et al. (2018)]. In the present proteomic analysis, 14 complement proteins were identified in AT (Supplementary Table 1). In humans, functional profiling and transcript analysis demonstrated that complement proteins are expressed in the adipocyte subfraction of AT and that expression of complement proteins is positively correlated with plasma triglyceride levels and measures of local insulin resistance in adipocytes (van Greevenbroek et al., 2012). Transcripts of C1 complement subcomponents were also detected in adipose cells from mice (Zhang et al., 2007). In fact, one of the first adipokines identified was a complement factor (Cook et al., 1987), and since then studies have demonstrated the interactions between the complement system and adipose tissue (Vlaicu et al., 2016a).Taken together, and based on our current findings, we can conclude that the AT of dairy cows is abundant in

complement proteins, and further work is required to elucidate their roles in the inflammatory process.

We found that SS treatment increased the abundance of C1r, C1qB, and C1qC proteins, although it is not clear if this increase in complement protein abundance was caused by macrophage AT infiltration and increased complement protein production in AT, if this increase was related to an inhibition of complement cascade (i.e. substrate accumulation), or both. Early studies demonstrated that both monocytes and macrophages produce and secrete C1 proteins (C1q and C1s) (Colten et al., 1979; Rabs et al., 1986). C1 is a protein complex (790 kDa) composed of a recognition protein, C1q, and two copies of C1r and C1s proteases. C1q is a protein with a globular head with 3 subunits (A, B, and C). The subunit B lies on the external part of the molecule, whereas the A and C subunits are positioned inside. After recognition and binding of C1q to a target cell or molecule, C1r is activated which in turn converts proenzyme C1 into a specific protease that cleaves C4 and C2, forming C4b2a, that can activate C3 protein (Gaboriaud et al., 2004; Vlaicu et al., 2016)(Gaboriaud et al., 2004; Vlaicu et al., 2016a). In the presence of C3, the C4b2a can be activated to C4b2a3b which can activate C5 that subsequently forms the membrane attack complex (C5b-9) (Vlaicu et al., 2016). Besides increased abundance of C1 proteins, we found that the abundance of C6 was greater in SS AT, and other complement proteins were increased but did not reach the fold change cut-off of 1.5, including C2 (FC = 1.4, $P = 0.014$), C8a (FC = 1.4, $P = 0.02$), and C4b-binding protein alpha chain (FC =1.42, $P = 0.02$), thus supporting the findings of the bioinformatics analysis that complement was the top pathway influenced by SS administration.

The increase in complement protein abundance in AT following SS treatment suggests an inflammatory effect of SS on AT, which might have been mediated by macrophage infiltration in

AT after 4 d of SS treatment. As mentioned above, macrophages are the predominant immune cell type in AT of cattle (Ampem et al., 2016). In the current study we observed a tendency for an increased abundance of CD172a, a surface marker of monocytes (Contreras et al., 2015), and increased abundance of TNF-α in AT of SS-treated cows. Two subsets of AT macrophages have been well characterized: M1 macrophages yield pro-inflammatory cytokines such as IL-6 and TNF-α, whereas M2 macrophages produce resolving signals arginase-1, IL-10, and chitinase 3-like-3 (Biswas and Mantovani, 2012; Sica and Mantovani, 2012; Zhuang et al., 2012). TNF-α stimulates several enzymes related with lipolysis (i.e. hormone sensitive lipase, adipose triglyceride lipase, and monoacylglycerol lipase (Contreras and Sordillo, 2011b)) and initiates and amplifies inflammatory cascades through chemokine regulation and cytokine release, recruitment of immune cells and adhesion molecules, apoptosis, etc. Although an increase in TNF-α abundance and possibly a greater macrophage infiltration was observed in AT, blood free fatty acid concentration was not affected by treatment (Montgomery et al., 2019). Fatty acid mobilization is positively associated with AT monocyte/macrophage infiltration, which further produces inflammatory mediators and exacerbates lipolysis (Contreras et al., 2017). Early studies reported a stimulation of rat peripheral blood leukocyte migration *in vitro* (Brown and Collins, 1977) when aspirin and salicylate were provided at lower concentrations (0.1 m*M*) than those that inhibit migration (10 m*M*). The mechanisms by which SS may possibly alter AT monocyte infiltration are unknown.

Although not significant, the adipokine complement factor D [CFD, also known as adipsin; (Lo et al., 2014)] was numerically 27% more abundant in SS AT. CFD production by AT accounts for almost all circulating CFD in mice (Wu et al., 2018), and it has a major role in the alternative pathway of complement activation by aiding C3 spontaneous activation (Vlaicu et al., 2016a). In

addition, CFD contributes to the maturation of preadipocytes into adipocytes (Wilkison et al., 1990). Interestingly, CFD transcript abundance is decreased when culturing adipocytes from rats either with insulin or glucose (Dani et al., 1989) and it increases 2-3 fold in rodents with insulin deficiency (Flier et al., 1989). It is possible that CFD production could be influenced by altered glucose turnover in cows treated with SS (Montgomery et al., 2019).

Depending on the dose, salicylates can either have an anti-inflammatory effect by reducing TNF-α production in macrophages (Vittimberga et al., 1999) or may lead to a rebound increase in IL-1β and TNF-α when administered in low doses (325 mg/d in human subjects; (Endres et al., 1996)). Endres et al. (Endres et al., 1996) observed greater cytokine synthesis by peripheral blood mononuclear cells stimulated by IL-1α after 2 weeks of oral aspirin treatment and 3 weeks after stopping the treatment. In typical human anti-inflammatory therapy, plasma concentrations of salicylate range from 150 to 300 µg/mL (Insel, 1996). In this study, SS treatment was designed to result in a plasma salicylate concentration of 34 µg/mL or 0.25 m*M* as reported by Farney et al. (2013). After Farney et al. (2013) applied this treatment for the first 7 d of lactation, a subsequent rebound increase in plasma concentrations of pro-inflammatory oxylipids pointed to an unexpected inflammatory response to SS, at least after treatment ended. Similar mechanisms may have been in play in the current study, although the reported impacts on AT proteins occurred while SS treatment was still being applied, and cows were not monitored after treatment ended. It is worth considering whether salicylate might have accumulated in AT, potentially leading to differential impacts in this tissue relative to others. A study on salicylate pharmacokinetics in human subjects, however, found that the volume of distribution for salicylate did not markedly change in obese subjects, leading authors to conclude that salicylate likely was not distributed into AT to any

meaningful extent (Greenblatt et al., 1986), which is not surprising for a water-soluble compound. Indeed, this suggests that SS may have had its primary impact on AT cells other than adipocytes.

Cows consuming SS exhibited greater STAT3 and LBP abundance in AT compared to controls. LBP is produced by different AT depots in cattle, including the subcutaneous tail head depot (Rahman et al., 2015). LBP binds to lipopolysaccharide (LPS) of gram-negative bacteria which allows LPS interaction with CD14. The latter complex is capable of stimulating macrophages through toll like receptor (TLR) 4 interaction, activating nuclear factor-κB with consequent pro-inflammatory cytokines production. The genes expressed in response to TLR activation encode pro-inflammatory cytokines such as IL-1, IL-12, and TNF-α (Fujiwara and Kobayashi, 2005). To avert the deleterious effects of excessive pro-inflammatory cytokines production, IL-10 is also produced by LPS-stimulated macrophages (Howard et al., 1993; Berg et al., 1995), leading to STAT3 phosphorylation. STAT3 phosphorylation has been correlated with attenuation of inflammatory response (Benkhart et al., 2000; Williams et al., 2004). STAT3 is a transcription factor that mediates anti-inflammatory effects of IL-10, and disrupting the STAT3 gene in macrophages results in exaggerated cytokine production (Takeda et al., 1999). However, STAT3 can also generate a pro-inflammatory response when stimulated by IL-6 (Braun et al., 2013). Thus, increased STAT3 abundance may have supported either a pro-inflammatory response (IL-6/STAT3 signaling pathway) or an anti-inflammatory response (IL-10/STAT3 signaling pathway) in AT. Interestingly, a study evaluating the IL-6/STAT3 pathway after a muscle injury demonstrated that IL-6 activates the gp130/STAT3 pathway in macrophages, promoting chemokine production (CCL2 and CCL3) and macrophage tissue infiltration (Zhang et al., 2013). Regardless of its pro- or anti-inflammatory role, the increase in STAT3 abundance may align with the marginal increase in AT abundance of CD172a and the proposed macrophage infiltration

(either M1 or M2) in response to SS. It should be noted that STAT3 also has a role in adipogenesis (Yuan et al., 2017), therefore its increased abundance can be related to AT metabolism, and not only to inflammatory pathways. The higher abundance of this protein as well as STAT3 and LBP in SS AT indicates enrichment of both the IL-10 and acute phase response signaling pathway in AT of cows treated by SS postpartum. BLVRB, biliverdin reductase B flavin reductase (NADPH), is an isoform of biliverdin reductase. Whereas biliverdin reductase A (BLVRA) modulates a wide range of signaling pathways (including insulin signaling), functionality of BLVRB in adult animals has been little explored (O'Brien et al., 2015). For instance, increased BLVRA expression on surface of macrophages and its phosphorylation leads to activation of Akt/PI3K pathway with enhanced production of IL-10 (Wegiel et al., 2009). Recently, researchers defined the first physiologically relevant function of BLVRB: aiding on the regulation of cellular redox in mature red blood cells (Wu et al., 2016), supporting the enrichment of heme degradation pathway of cows treated SS.

In support of a pro-inflammatory response, SS administration has increased the concentration of haptoglobin (Carpenter et al., 2016) in blood of periparturient cows. A marginal increase (treatment × time $P = 0.11$; (Montgomery et al., 2019)) in blood haptoglobin concentration was observed on d 7 of SS treatment in the larger study, coinciding with AT sample collection. Haptoglobin is a positive acute phase protein (like LBP) regulated by the nuclear factor-κB / STAT3 signaling pathway that regulates the maturation and activity of immune cells (Burgess-Beusse and Darlington, 1998). Blood haptoglobin concentration is positively associated with inflammatory response/dysfunction in transition cows (B.J. Bradford et al., 2015), which might indicate that the AT of the SS cows was in a pro-inflammatory state, although this was not

supported by levels of TNF-α. The pro- or anti- inflammatory effects of SS on AT requires further investigation.

Beyond changes in abundance of proteins involved in the immune system, proteomic analysis also revealed differences in abundance of proteins associated to iron metabolism, such as ferritin light chain (FTL) and flavin reductase. Ferritin is a stable protein capable of attracting iron ions, inducing mineralization, and accumulating in a cavity (Ford et al., 1984). Consumption of SS lead to a decrease in FTL abundance, suggesting lower capacity of AT accumulates iron. In mammals, the ferritin heavy chain assembles with FTL (which devoid ferroxidase activity) to form heteropolymers that are more efficient for mineralization and accumulating iron (Arosio et al., 2017). Although not significant ($P = 0.16$), ferritin heavy chain abundance had a FC = -3.6 in cows consuming SS. Altered iron metabolism can trigger AT dysfunction, since adipocyte differentiation occurs in a narrow range of iron concentrations (Moreno-Navarrete et al., 2013). For example, iron overload promotes adipocyte insulin resistance (Green et al., 2006; Gabrielsen et al., 2012). Similarly, strategies to decrease systemic iron levels (e.g., low-iron diets and chelation therapy) have improved insulin sensitivity in obese animal models and humans (Cooksey et al., 2010; Gabrielsen et al., 2012). Interestingly, we have demonstrated that the SS treated cows in the present study had a tendency for lower glucose turnover rate, which was claimed to be associated with greater hepatic insulin sensitivity (Montgomery et al., 2019). Furthermore, immunoblotting from AT revealed a tendency for greater phosphorylation of AMPK, whereas Akt phosphorylation tended to decrease in cows consuming SS (Montgomery et al., 2019). It can be suggested that the lower FTL abundance in the SS treated cows maybe related to the changes in the insulin sensitivity in their AT.

Aligning with lower capacity of AT to accumulate iron intracellularly, the second top pathway stimulated by SS treatment was heme degradation. Heme (iron protophophyrin IX) is essential for diverse biologic processes since it serves as the functional group of several proteins such as hemoglobin, nitric oxide synthase, myoglobin, and cytochromes (Beri and Chandra, 1993). Hemoglobin subunit β (HBB), the second most abundant protein detected in AT proteomics, was 2 times greater in SS group compared to CON (Table 1). Excess of intracellular heme is degraded by heme oxygenase releasing free iron (Arredondo et al., 2007). Increased iron, markers of iron accumulation, and heme oxygenase protein levels have been associated with AT dysfunction, including increased inflammation and decreased adipogenesis (Gabrielsen et al., 2012; Dongiovanni et al., 2013; Moreno-Navarrete et al., 2017). Another possible explanation to the increased abundance of HBB could be that SS treatment affected blood coagulation, although SS is only a weak inhibitor of thromboxane B_2 generation in clotting whole blood (Patrignani et al., 1997), and it does not inhibit vessel wall cyclooxygenase (Whittle et al., 1980; Dejana et al., 1981).

Taken together, the findings of the present proteomic analysis provide new insight on the effects of SS on the immune response of AT in postpartum cows.

CONCLUSION

In contrast to the expected anti-inflammatory effect of SS, this study revealed that consumption of SS during the first wk after parturition increases both pro- and anti-inflammatory signaling pathways and an immune response in AT of postpartum cows. SS treatment increased the abundance of complement proteins, STAT3, LBP, and TNF-α, and these outcomes may be related to greater monocyte infiltration and macrophage activation. Enrichment of IL-10 signaling is likely a response to dampen a pro-inflammatory signaling in AT of cows consuming SS. The

metabolic effects of SS observed in earlier studies might be associated with changes in abundance of proteins from complement system and iron metabolism in AT. Whether this inflammatory response in AT is transient and beneficial to periparturient cows, needs elucidation.

FUNDING

This project was supported by Agriculture and Food Research Initiative Competitive Grant no. 2013-01976 and Hatch project 1018048 from the USDA National Institute of Food and Agriculture (Washington, DC), and by Grant no. 362-0460 from the Israeli Dairy Board, Yehud, Israel.

ACKNOWLEDGMENTS

Contribution no. 19-255-J from the Kansas Agricultural Experiment Station (Manhattan). The authors thank Kansas State University graduate and undergraduate students for their assistance in sample collection and analysis. C. S. Takiya appreciates the scholarship (88881.170046/2018-01) provided by the Coordination for the Improvement of Higher Education Personnel (CAPES, Brasília, Brazil).

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Table 3-1. List of differentially abundant proteins in subcutaneous adipose of cows treated with sodium salicylate or not during the first week postpartum.

Protein Description	Uniprot Gene Name	Fold Change SS/CON	*P*-value		
			Treatment	Cluster	Treatment × Cluster
Trem-like transcript 1 protein	TREML1	0.00	0.004	0.103	0.088
Complement C1q subcomponent subunit B	C1QB	360	0.005	0.292	0.1
Plasma serine protease inhibitor	SERPINA5	-1.82	0.003	0.006	0.062
Lipopolysaccharide-binding protein	LBP	2.31	0.013	0.24	0.201
Proteasome activator complex subunit 2	PSME2	0.00	0.009	0.039	0.107
Ribonuclease 4	RNASE4	-11.11	0.019	0.13	0.232
Ribosomal protein L14	RPL14	0.00	0.01	0.024	0.089
Alpha-hemoglobin-stabilizing protein	AHSP	-1.69	0.024	0.093	0.205
Selenoprotein P	SEPP1	-4.00	0.015	0.039	0.1
Hemoglobin subunit beta	HBB	1.95	0.028	0.096	0.257
Complement C2	C2	1.43	0.014	0.041	0.062
Glutamate--cysteine ligase regulatory subunit	GCLM	2.37	0.022	0.092	0.111
Keratin, type II cytoskeletal 78	KRT78	-9.09	0.015	0.015	0.151
PSMD1 protein	PSMD1	2.37	0.02	0.055	0.106
Fumarate hydratase	FH	-3.45	0.02	0.022	0.182
Ferritin light chain	FTL	-1.72	0.047	0.19	0.325
Synaptosomal-associated protein	SNAP23	-12.50	0.035	0.166	0.193
Emopamil binding protein (Sterol isomerase)	EBP	-2.78	0.04	0.774	0.306
T-complex protein 1 subunit delta	CCT4	-12.50	0.037	0.095	0.291
Radixin	RDX	-50.00	0.035	0.093	0.221
Protein FAM49B	FAM49B	-2.00	0.029	0.239	0.097
TBC1 domain family member 24	TBC1D24	-14.29	0.039	0.634	0.208
MYO1B protein	MYO1B	-7.14	0.035	0.992	0.233
Laminin subunit alpha-3	LAMA3	-8.33	0.037	0.071	0.247
Alpha isoform of regulatory subunit A, protein phosphatase 2	PPP2R1A	-2.56	0.015	0.024	0.054
Zinc-alpha-2-glycoprotein	AZGP1	4.28	0.018	0.016	0.108
Alpha-crystallin B chain	CRYAB	-5.00	0.039	0.708	0.171
Beta-defensin 9	DEFB9	6.05	0.007	0.162	0.053
Proteasome inhibitor PI31 subunit	PSMF1	1.87	0.023	0.019	0.131
SH3 domain-binding glutamic acid-rich-like protein	SH3BGRL	-4.55	0.029	0.048	0.109
Fibromodulin	FMOD	-3.45	0.043	0.225	0.125
D-3-phosphoglycerate dehydrogenase	PHGDH	1.96	0.041	0.072	0.157
Complement component 1, r subcomponent	C1R	1.65	0.031	0.023	0.199
Heat shock 70 kDa protein 4	HSPA4	-50.0	0.011	0.004	0.08

40S ribosomal protein S9	RPS9	-2.33	0.035	0.032	0.217
Calcium regulated heat stable protein 1, 24kDa	CARHSP1	1.93	0.035	0.034	0.174
Phosphatidylinositol-glycan-specific phospholipase D	GPLD1	1.60	0.032	0.054	0.073
Ras-related C3 botulinum toxin substrate 1	RAC1	1.33	0.044	0.799	0.164
Hemoglobin, theta 1	HBQ1	2.11	0.011	0.003	0.075
Sterile alpha motif domain-containing protein 7	SAMD7	2.21	0.01	0.003	0.072
BIN2 protein	BIN2	-4.55	0.016	0.008	0.068
Phosphoserine phosphatase	PSPH	0.00	0.039	0.072	0.091
Histidine--tRNA ligase, cytoplasmic	HARS	-3.23	0.03	0.019	0.114
MGC159500 protein	MGC159500	1.58	0.042	0.032	0.202
Flavin reductase (NADPH)	BLVRB	1.62	0.042	0.027	0.222
Superoxide dismutase [Cu-Zn]	SOD1	1.36	0.02	0.007	0.123
Tropomyosin alpha-1 chain	TPM1	-3.03	0.024	0.01	0.121
L-xylulose reductase	DCXR	-3.45	0.039	0.034	0.125
Alpha-1B-glycoprotein	A1BG	1.37	0.035	0.014	0.175
Phosphoribosylformylglycinamidine synthase	PFAS	2.04	0.014	0.003	0.078
Basal cell adhesion molecule	BCAM	1.97	0.043	0.025	0.199
Carboxypeptidase B2	CPB2	2.30	0.048	0.03	0.209
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11, mitochondrial	NDUFB11	-3.03	0.031	0.017	0.098
Tricarboxylate transport protein, mitochondrial	SLC25A1	-2.70	0.04	0.099	0.051
Protein DDI1 homolog 2	DDI2	1.59	0.019	0.005	0.085
2,4-dienoyl-CoA reductase, mitochondrial	DECR1	-2.22	0.043	0.03	0.122
HMT1 hnRNP methyltransferase-like 2 isoform 3	HRMT1L2	-5.00	0.021	0.004	0.096
Procollagen galactosyltransferase 1	COLGALT1	-6.67	0.018	0.003	0.073
Signal peptidase complex subunit 2	SPCS2	1.77	0.045	0.014	0.151
Immunoglobulin lambda-like polypeptide 1	IGLL1	1.44	0.031	0.005	0.108
Elongation factor 2	EEF2	-2.08	0.019	0.002	0.076
Vitamin K-dependent protein S	PROS1	1.69	0.043	0.01	0.163
26S proteasome non-ATPase regulatory subunit 8	PSMD8	2.37	0.033	0.007	0.095
Uncharacterized protein	LOC515150	1.49	0.02	0.002	0.078
Signal transducer and activator of transcription 3	STAT3	2.22	0.038	0.012	0.062
Plasminogen	PLG	1.43	0.021	0.004	0.087
Complement component C8 alpha chain	C8A	1.37	0.02	0.002	0.072
C4b-binding protein alpha chain	C4BPA	1.42	0.021	0.002	0.073
CD34 molecule	CD34	2.23	0.022	0.002	0.071
C1QC protein	C1QC	1.59	0.029	0.003	0.092
SH3 domain-binding glutamic acid-rich-like protein 2	SH3BGRL2	2.25	0.03	0.003	0.093

Uncharacterized protein	CP	1.34	0.032	0.003	0.076
Syntaxin-7	STX7	1.86	0.022	0.002	0.054
Fetuin-B	FETUB	1.70	0.047	0.007	0.069
Karyopherin subunit beta 1	KPNB1	-2.17	0.039	0.003	0.094
Thyroxine-binding globulin	SERPINA7	2.34	0.042	0.003	0.074
Uncharacterized protein	-	1.37	0.037	0.002	0.079
Complement component C6	C6	1.53	0.039	0.003	0.09
Acidic leucine-rich nuclear phosphoprotein 32 family member A	ANP32A	-3.03	0.028	0.001	0.055
ERO1-like protein alpha	ERO1L	-2.70	0.046	0.002	0.066

Figure 3-1. Cellular locations (a) and functions (b) of differentially abundant proteins in subcutaneous adipose tissues of early lactating dairy cows treated with sodium salicylate (SS) or controls. Locations and functions of 62 out of 80 differentially abundant proteins were categorized through Ingenuity Pathway Analysis. The number of proteins in each category is provided in parentheses. Transmemb = transmembrane.

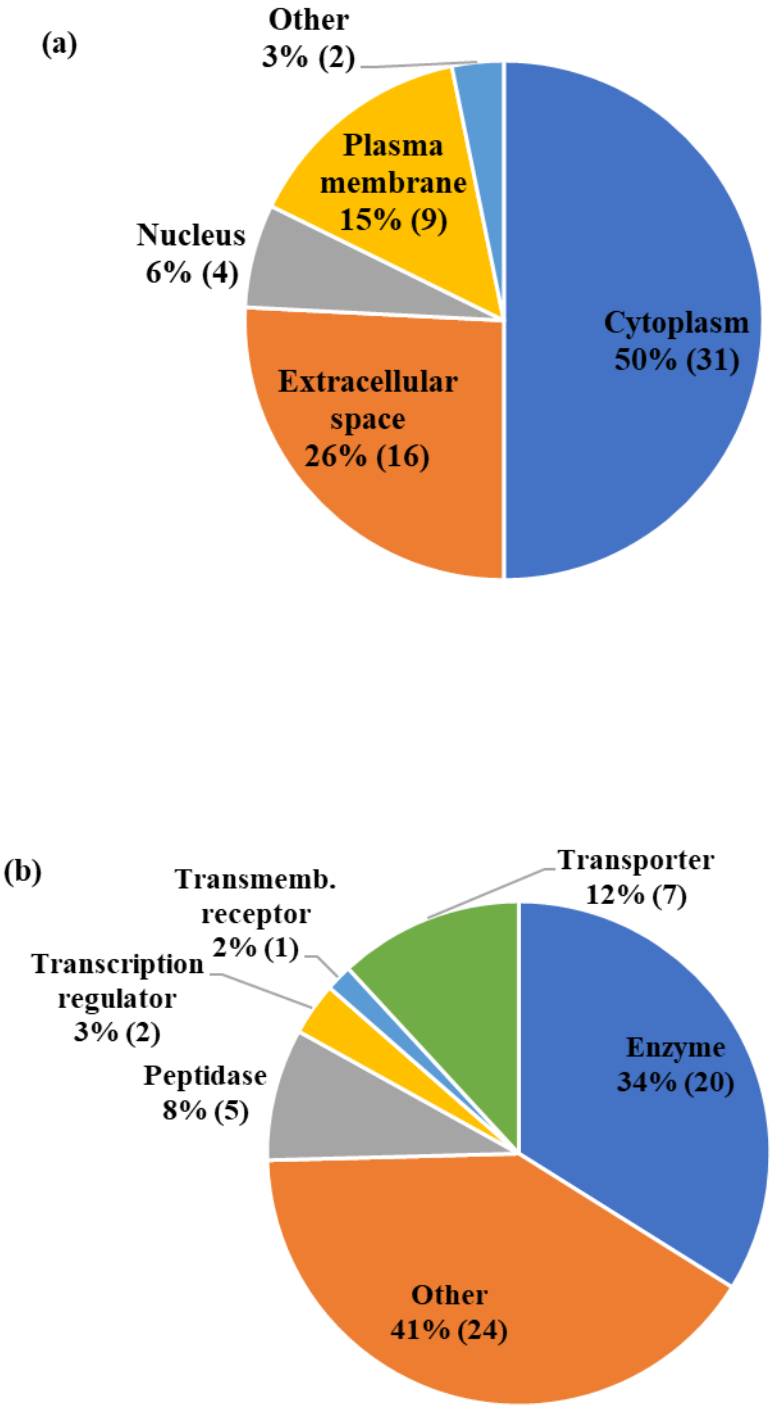


Figure 3-2. Molecular and cellular pathways altered by oral sodium salicylate in the subcutaneous adipose tissue of early lactating cows identified through Ingenuity Pathway Analysis (Qiagen). Proteins in parentheses are differentially abundant (P < 0.05 and fold change ± 1.5) between treatment groups.

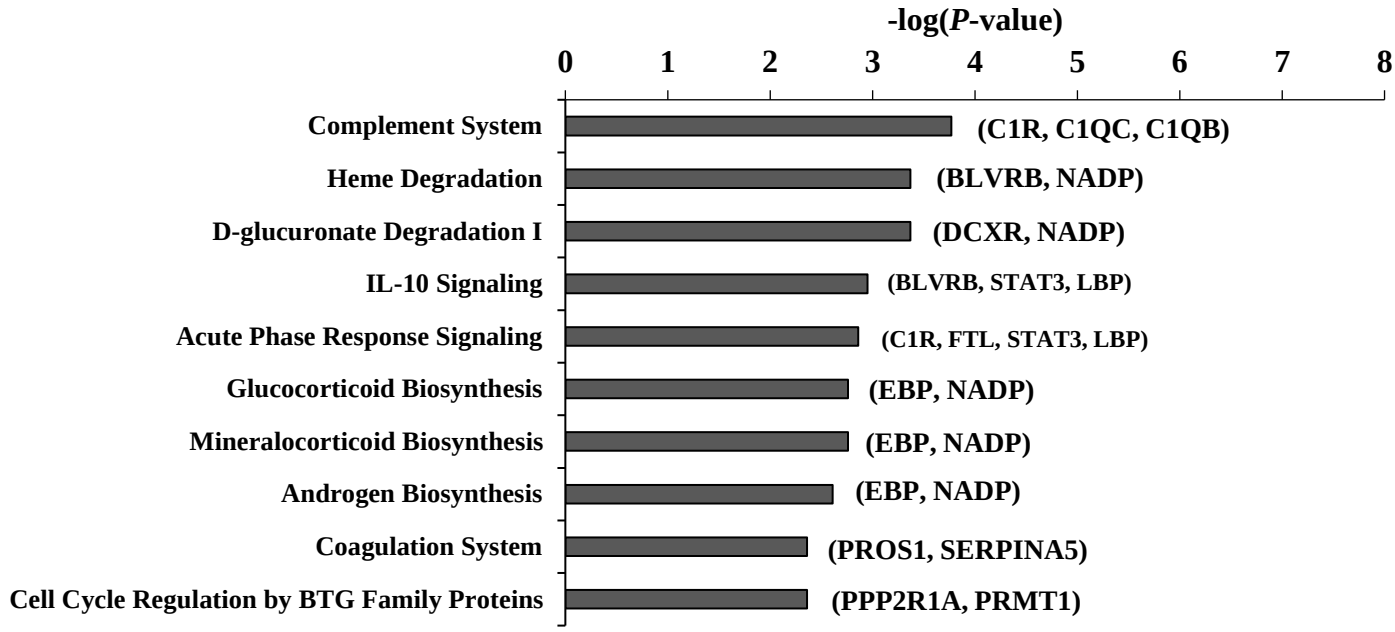


Figure 3-3. Abundance of proteins in the immune system pathways [complement system (panels A, B, C, and D), IL-10 signaling (panels E, F, and G), and heme degradation (panel H) that were differentially abundant between early lactating cows treated with sodium salicylate (SS) or not (control, CON), blue and red bars, respectively. (A) C1QB, complement C1q subcomponent subunit B [fold change (FC) = 360]; (B) C1R, complement C1r (FC = 1.65); (C) C1QC, C1qC protein fragment (FC = 1.59); (D) C6, complement 6 (FC = 1.5); (E) LBP, lipopolysaccharide binding protein (FC = 2.31); (F) STAT3, signal transducer and activator of transcription 3 (FC = 1.59); (G) BLVRB, flavin reductase (NADPH) (FC = 1.62); and (H) FTL, ferritin light chain (FC = -1.72). Data presented as mean abundances. Error bars represent SD. Cows treated with SS had greater abundance of most all described proteins in the adipose tissue compared with CON. P ≤ 0.01 (**) and P < 0.05 (*).

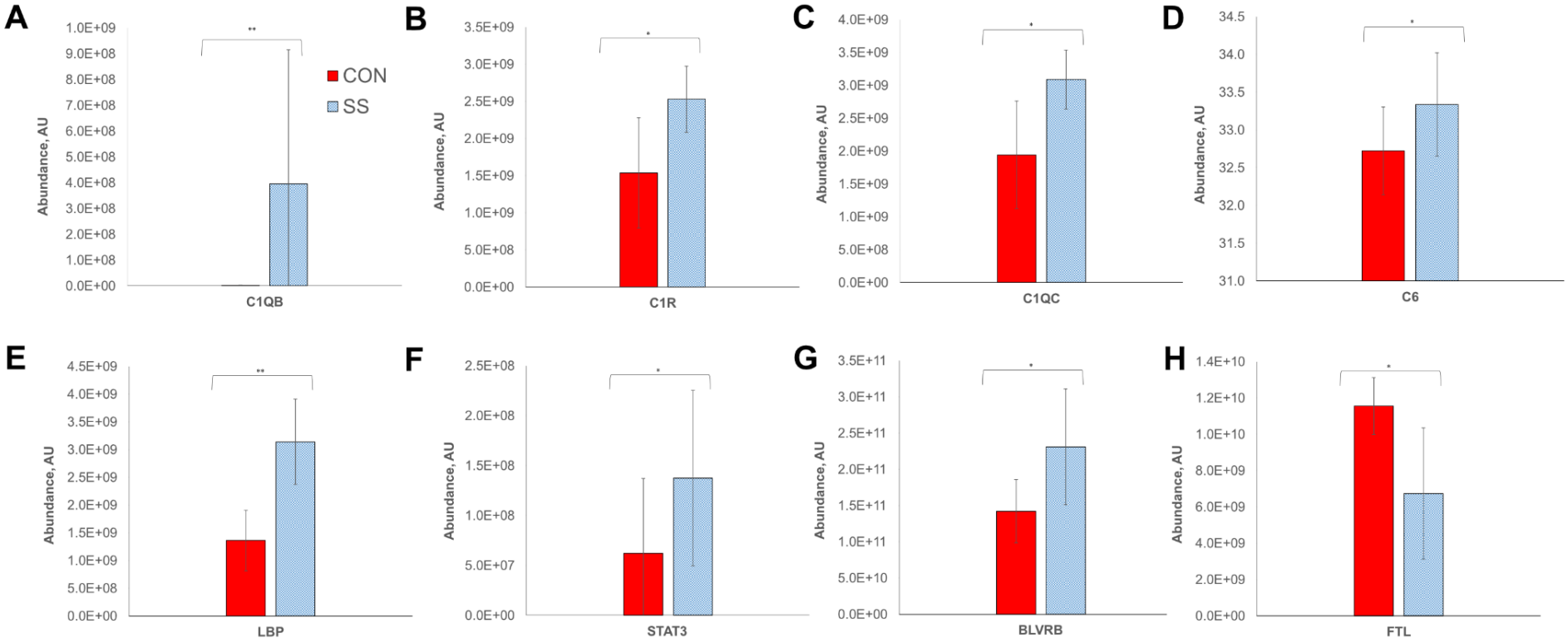


Figure 3-4. Mean intensity of protein bands determined by immunoblots as validation to proteomics data: (A) complement C1r, (B) complement C1q B chain (C1QB), (C) lipopolysaccharide binding protein (LBP). All abundances were corrected to tubulin as a loading reference. Error bars represent SD. Treatment *P*-values from *t*-test.

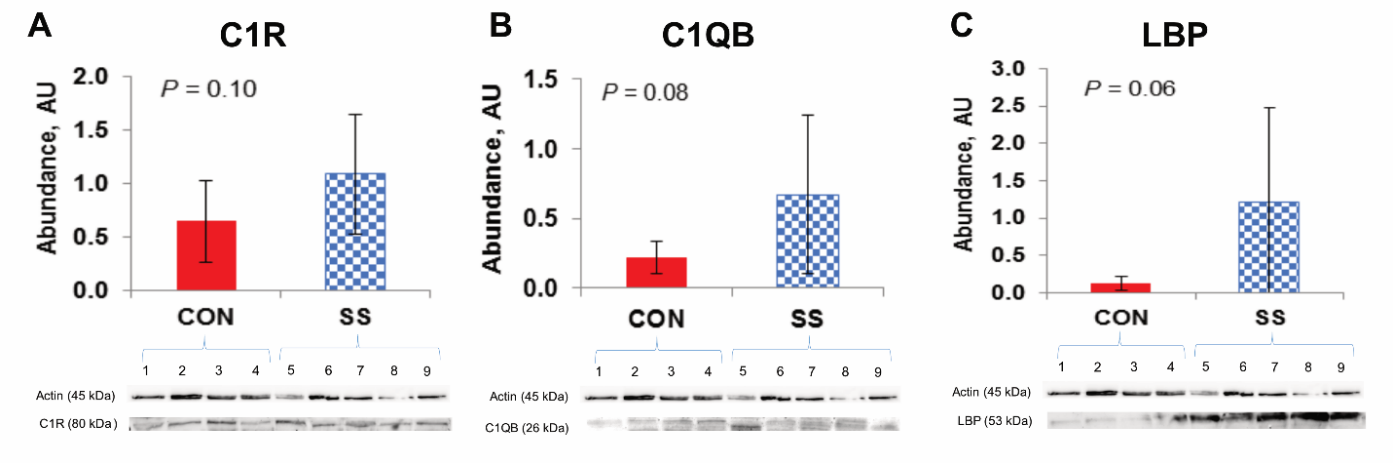
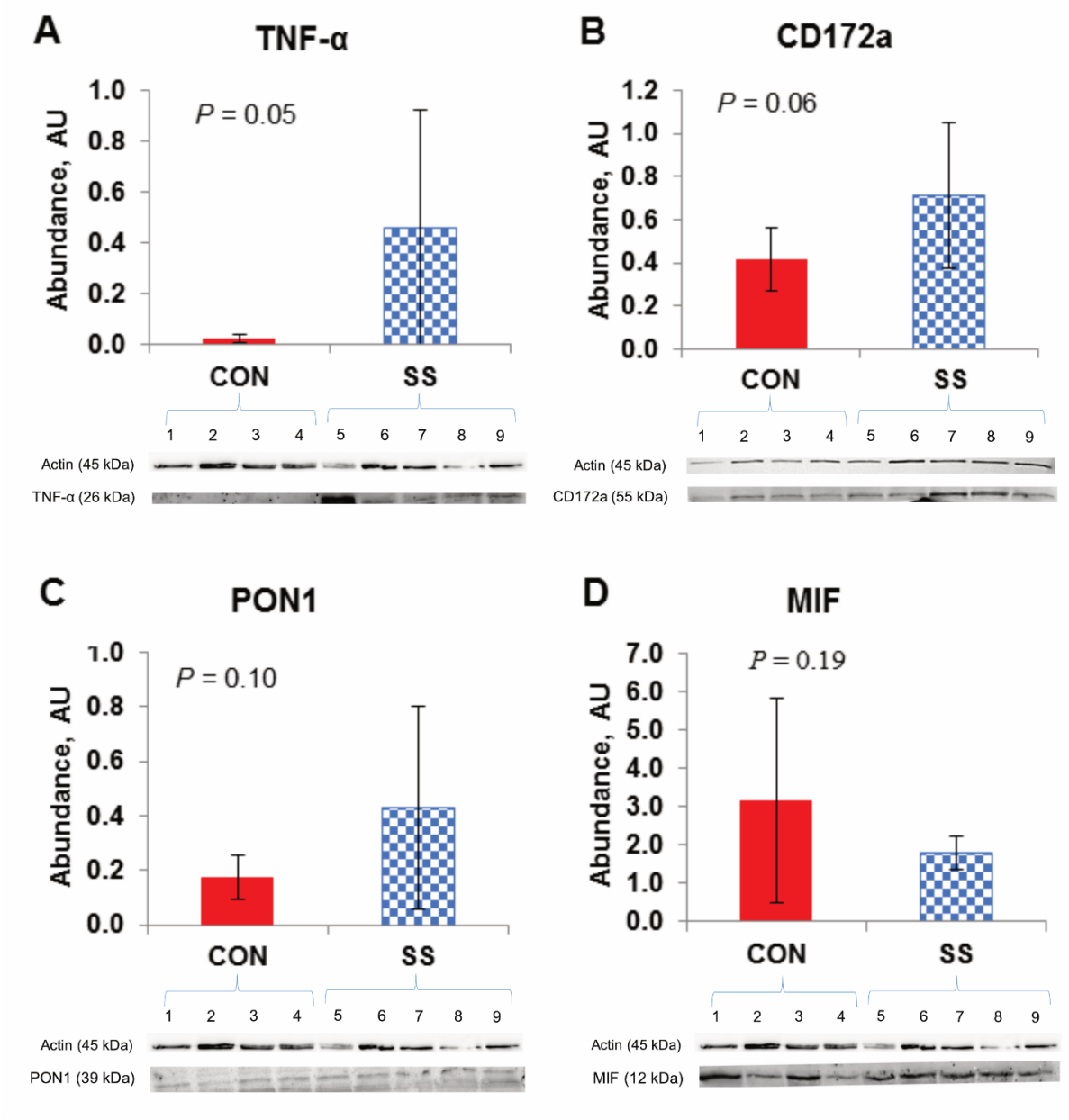


Figure 3-5. Mean intensity of protein bands in immunoblots: (a) tumor necrosis factor-alpha (TNF-α), (b) Cluster differentiation 172a (CD172a), (c) paraoxanase 1 (PON1), (d) macrophage inhibitor factor (MIF). All abundances were corrected to β-actin and tubulin as a loading reference. Error bars represent SD. $P < 0.05$ (*) for treatment effect using *t*-test.



Chapter 4 - Feeding Dairy Cows With "Leftovers" and the Variation in Recovery of Human-Edible Nutrients in Milk

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2019. Front. Sustain. Food Syst. 3: Article 114

ABSTRACT

Ruminants can convert feeds unsuitable and unpalatable for humans into milk and meat, and thereby play a key role in food security. Milk production efficiency is usually calculated as the ratio between nutrients secreted in milk and nutrient intake, but this metric does not address concerns about human/livestock feed competition. Our objective was to evaluate effects of diets composed of ecological leftovers (ECO; industrial by-products and feed produced on land unsuitable for human food production) on dairy cattle productivity compared with traditional diets used in the U.S. We also sought to estimate human-edible (HE) nutrient recovery rate (HE inputs vs. milk nutrients) in different scenarios: thrift (all potentially HE ingredients counted as such), choice (ingredients rarely consumed by humans considered not HE), and land use (land used for forage production could be used to grow corn and soybeans for direct human consumption). Experiment 1 evaluated effects of an ECO diet (ECO1), incorporating wheat straw and by-products, on performance of 12 mid-lactation cows in a crossover design with 20-d periods. Experiment 2 evaluated effects of a different ECO diet (ECO2), using winter crop forage and by-products with or without rumen-protected Lys and Met (ECO2-AA), on performance of 12 late-lactation cows in a 3 × 3 Latin square design with 21-d periods. Both ECO diets were compared

to lactation diets typical in North America (CON). Although ECO1 decreased feed efficiency (milk yield ÷ feed intake), both feed intake and milk yield were maintained for primiparous cows. ECO1 increased the HE recovery of metabolizable energy (ME) and protein relative to CON1 across all food system scenarios. In Experiment 2, ECO diets significantly decreased feed intake and milk yield, and in the thrift scenario, recovery of ME and protein were worsened by ECO2. All diets resulted in a positive net recovery of HE digestible essential amino acids, and ECO diets further improved their recovery. In conclusion, several factors affect recovery of HE nutrients fed to dairy cows, including dietary composition, land use, and human food system assumptions. Depending on these factors, ECO diets can either improve or reduce the efficiency of converting HE nutrients from feeds into milk.

INTRODUCTION

Growing world population, lack of arable land for agriculture expansion, energy demands (bio-fuels), and food waste may threaten global food security in coming decades. Food waste along the supply chain from the producer to the consumer it is estimated at 40% in the U.S. (Hall et al., 2009; Guners, 2017). The world's population is expected to grow from 7.6 to 10 billion between 2017 and 2067 (United Nations, 2017) while the global demand for milk is expected to increase by 48% between 2005 and 2050 (Alexandratos and Bruinsma, 2012). In addition, global life expectancy (Zijdeman and Ribeira da Silva, 2014), adult height (NCD-RisC, 2016), and body mass index (Finucane et al., 2011) have consistently increased over the years, thus affecting the overall maintenance nutrient requirements of population. A larger and wealthier global population will demand not only more food but also other goods and services that require land, water, energy, and minerals for their production. In the past 60 years, growth in demand for food has been met

primarily by steady increases in agricultural productivity; however, there are worrying signs that productivity gains are leveling off. For instance, the global rate of yield increase of cereal crops has steadily declined – dropping from 3.2%/year in 1960 to 1.5% in 2000 (FAO - Food and Agriculture Organization of the United Nations, 2009). Thus, new agricultural technologies that can reinvigorate productivity gains and approaches to enhance agricultural / food system efficiency are considered critical to meeting global food security goals.

Studies have suggested that net food production could be increased by shifting crop production away from bioenergy, livestock feed, and other non-food applications (Foley et al., 2011). These authors estimated that shifting major crops directly to human consumption would add over a billion tonnes to global food production or the equivalent of 3×10^{15} kcal annually (a 49% increase; Foley et al., 2011). Food security concerns, though, extend beyond caloric needs of the growing world population; in fact, amino acids and vitamins are more likely to be limiting for global health than energy (Wu et al., 2014; Chaudhary et al., 2018). As animal-source foods are high in protein, they should not be evaluated primarily as energy sources. In addition to the high protein content found in animal-source foods, protein quality of beef and milk is 1.40 to 1.87 times greater than human edible (HE) plant protein inputs in the dairy industry (Ertl et al., 2016a). Furthermore, some scenarios do not evaluate best practices in assessing the contribution of livestock to the agricultural system. In applied animal nutrition, feed processing methods (Giuberti et al., 2014), genetic modifications (Ferraretto and Shaver, 2015), and metabolic modifiers (McGuffey, 2017) have been consistently used to improve feed efficiency in livestock.

Ruminant rations are composed of a diversity of feed ingredients, of which some could be used as food for humans. Mottet et al. (2018) estimated that only 4 to 5% of feed consumed by domestic ruminants globally is human-edible (HE), a much smaller share compared with feed

consumed by monogastric livestock (83%). Both beef and dairy industries have a major impact on the food system because they can convert feeds unsuitable and unpalatable for humans into high-quality, protein-rich products (Tedeschi et al., 2015), with relatively high concentration and bioavailability of vitamin B_{12} (Matte et al., 2012). Feed efficiency of livestock is classically defined as the production of animal products (milk, meat, eggs, etc.) divided by the amount of feed ingested. An alternative measurement of feed efficiency in livestock is the calorie/protein retention proposed by Fry et al. (2018), which estimates the percentage from the total calorie and protein consumed by animals that is available for human consumption. These measurements, however, can be misleading because ruminants produce protein-rich meat and milk from fibrous feeds not suitable for many other species, including humans. Therefore, ruminants could be considered more efficient in terms of supporting the human food supply if they consume feeds unsuitable for human nutrition and do not displace human food production from arable land.

In contrast to ruminant systems that rely almost exclusively on grazing non-arable land, highly productive intensive ruminant systems such as those predominant in the U.S. utilize a greater proportion of HE grains and protein sources. Whereas intensive systems may decrease efficiency on a HE basis, they also enable the use of byproduct feeds in carefully balanced rations. North American dairy rations contain 20-30% by-product feedstuffs (Tricarico, 2016), and thus provide an important avenue for converting these materials into nutrient-dense foods for human consumption. Thus, use of by-product feeds in intensive systems can enhance efficiency in the overall food system. Despite the challenges of feeding by-products (i.e., risk of mycotoxin contamination, variation in nutrient composition between batches, etc.), studies have demonstrated that diets with relatively high proportions of by-products can maintain or even improve ruminant performance (Bradford and Mullins, 2012). Dairy cows fed by-products in place of cereals and

pulses had similar dry matter intake (DMI) and milk yield compared with cows fed conventional diets, and the by-product diet increased the conversion ratio of undesirable food for humans into milk (Ertl et al., 2015). More recently, dairy goats fed a diet wherein the concentrate portion was comprised entirely of by-products exhibited improvements in N utilization and feed efficiency and produced less methane in comparison with a traditional diet containing corn and sunflower meal (Romero-Huelva et al., 2017). These experiments support the concept that feeding by-products to ruminants can decrease feed costs, improve the environmental sustainability of milk production (VandeHaar and St-Pierre, 2006), and support high levels of productivity, if utilized carefully (Bradford and Mullins, 2012). Furthermore, Schader et al. (2015) modeled a food system in which animals were fed zero human-edible feeds (grassland and by-products) and predicted reductions in greenhouse emissions, pesticide use intensity, freshwater use, soil erosion potential, and N- and P-surplus while providing enough food for the 2050 reference scenario from Alexandratos and Bruinsma (2012). Thus, feeding animals with ingredients not edible for human consumption likely has a positive impact not only on global food security, but also on the environment.

Feeding animals with resources not suitable for human consumption, such as by-products and grass from marginal land unsuited for crop production, is referred to as producing livestock on "ecological leftovers" (ECO; Garnett, 2009). The ECO concept follows a set of normative principles including 1) arable land should primarily be designated to plant-based food for humans, 2) animals should be fed biomass unwanted by or unsuitable for humans, and 3) semi-natural grassland should be used for livestock, if grazing can be justified by reasons other than meat and milk production (e.g., biodiversity or soil conservation). Although there are many studies evaluating the replacement of cereals and pulses with by-products in ruminant diets, the literature lacks data evaluating the effects of full ECO diets on dairy cow performance and HE nutrient

recovery. The objective of this study was to evaluate impacts of different ECO diets on performance of mid- and late-lactation Holstein cows; we hypothesized that cows fed carefully-formulated ECO diets would maintain similar milk production while improving HE nutrient recovery (amount HE nutrients recovered per HE nutrients fed). Furthermore, we provide an alternative approach to estimating HE nutrient recovery rate for dairy cows.

MATERIALS AND METHODS

Two experiments were carried out between March and May 2015 (Experiment 1) and March and May 2017 (Experiment 2) at the Dairy Teaching and Research Center of Kansas State University (KSU), under the approval of the KSU Institutional Animal Care and Use Committee. In both experiments, cows were housed in a tie-stall barn containing rubber beds covered with wood shavings, and individual feed bunks and waterers.

Experiment 1

Twelve dairy cows (6 primiparous and 6 multiparous) after peak lactation (154 ± 20 days postpartum and 42.6 ± 5.4 kg/d milk yield at the start of experiment) were randomly assigned to treatment sequence (within parity) in a crossover design. Periods lasted 20 d; the first 17 d allowed for diet adaptation and d 18-20 were used for data collection and sampling. Treatments were a conventional diet containing 31% by-product feeds (CON1) or a diet with 95% by-product feeds (ECO1; Table 4-1). Cows were fed diets twice daily as total mixed rations (TMR) for *ad libitum* intake. Feedstuff and TMR samples were collected, composited, and analyzed for chemical composition (Table 4-2) using wet chemistry methods by the Dairy One Forage Laboratory (Ithaca, NY), including dry matter (method 930.15, AOAC), ash (method 942.05, AOAC), crude

fat (ether extraction method 2003.05, AOAC), acid detergent fiber (method 973.18, AOAC), neutral detergent fiber (NDF) adding sodium sulfite and alpha-amylase to the detergent (Van Soest et al., 1991), and crude protein [CP; methods 990.03 (animal feed) and 992.23 (cereal grain), AOAC]. Net energy of lactation was calculated according to the National Research Council (2001) and non-fiber carbohydrate (NFC) was calculated as: NFC = 100 – (ash-free NDF% + CP% + crude fat% + ash%). Total mixed ration samples were assessed for particle size distribution (Heinrichs and Kononoff, 2013).

Feed and refusals were weighed daily to calculate DMI over each sampling period. On d 20, blood samples were collected from coccygeal vessels into evacuated tubes (6 mL K_3EDTA Vacutainer tubes; Becton Dickinson, Franklin Lakes, NJ) before the morning feeding and analyzed for plasma glucose concentration by a colorimetric kit (kit #439–90901; Wako Chemicals USA Inc., Richmond, VA), and plasma insulin concentration by a bovine-specific sandwich ELISA (#10–1201–01; Mercodia AB, Uppsala, Sweden). Absorbance was measured on a spectrophotometer (PowerWave XS; BioTek Instruments Inc., Winooski, VT) and calculations were performed using Gen5 software (BioTek Instruments Inc.).

Cows were milked thrice daily, and milk samples were collected on the last three days of each experimental period at 9 consecutive milkings in plastic vials with preservative (Microtabs II, Advanced Instruments, Norwood, MA) and analyzed by DHIA Laboratories (Manhattan, KS) for milk fat, true protein, lactose, and urea N concentrations as well as somatic cell count (SCC) as described in Ylioja et al. (2018). Somatic cell linear score (SCLS) was calculated as: SCLS = $\log_2$ (SCC/100) + 3 (Shook, 1993). Body weight and body condition score were recorded at the beginning and end of each period (Wildman et al., 1982).

Data were submitted to analysis of variance using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC) to account for the fixed effects of diet, parity, and their interaction, as well as the random effects of cow and period. Residual normality was verified. When diet by parity interaction was significant, differences among least square means were analyzed using the Tukey option. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. One primiparous cow was removed from analysis due to refusal to consume the ECO1 diet.

Experiment 2

Twelve second lactation dairy cows (231 ± 40 days postpartum and 34.4 ± 7.2 kg/d milk yield at the start of the experiment) were assigned to a 3×3 Latin square design experiment balanced for carryover effects. Adaptation to diets was allowed for 17 d and 4 d were used for data collection and sampling. Cows were blocked (n = 4) according to fat-corrected milk yield and days in milk, and randomly assigned to treatment sequence within block. Treatments (Table 4-3) were: 1) a conventional TMR for lactating cows (CON2) containing 25.7% by-product feeds; 2) a TMR comprised entirely of ECO feedstuffs (ECO2); and 3) ECO2 with top-dressed rumen-protected lysine and methionine [ECO2-AA; 77 g/d AjiPro-L (Ajinomoto, Chicago, IL) and 45 g/d MetaSmart (Adisseo, Antony, France)]. Cows were milked and fed twice daily (0530 and 1600 h). The CON2 TMR was mixed before the morning feeding in a horizontal mixer wagon, whereas ECO2 TMR was prepared before the afternoon feeding in a stationary horizontal mixer. Spent coffee grounds were acquired from 3 coffee shops every other day and stored in plastic buckets at room temperature until mixed with other feeds. Amino acids were top-dressed and mixed into the top 2/3 of the TMR for each cow at both feedings for ECO2-AA. CON2 was formulated according to the National Research Council (2001). ECO2 and ECO2-AA were formulated using Formulate2

(Diet Formulation Systems LCC, Visalia, CA) based on the National Research Council (2001) model and requirements. Chemical composition of feeds is shown in Table 4-4.

Feed and refusals were weighed daily, and feeding rates targeted 10-15% refusals. During the last 4 d of each period, TMR samples were collected to assess particle size distribution and chemical composition as described earlier. Milk samples were collected during every milking of the last 4 d of each period, and analyzed by MQT Lab Services (Kansas City, MO) using the same methods described in Experiment 1. Body weight and body condition score were recorded at the start and end of each period.

Analysis of variance was performed using the Mixed procedure of SAS 9.4 (SAS Institute) in a model including the fixed effects of diet and block and the random effects of period and cow within block. Normality of residuals was verified. Differences among least square means were evaluated using the Tukey option. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$.

Human-edible nutrient recovery calculations

Maximum HE ME and protein contents of cattle diets were estimated based on sugar, starch, true protein, and fat concentrations in corn grain (including grain in silage), hominy feed, soybean meal products, wheat middlings, and molasses. Other feedstuffs were considered unsuitable for human consumption (such as spent coffee grounds). Metabolizable energy content of feeds and milk were calculated based on Atwater calorie factors (Atwater and Bryant, 1900): 4 kcal/g of starch or sugar, 9 kcal/g of fat, and 4 kcal/g of protein (Table 4-5). Corn silage was considered to contain 45.0% corn grain (DM basis), and the corn grain was assumed to contain 66% starch and 9.5% protein (DM basis), and 50% moisture. Soybean meal product (Soy Best,

Grain States Soya Inc., West Point, NE) was estimated to contain 6.6% fat (DM basis), 49% protein (DM basis), and 11% moisture. Hominy feed and wheat middlings nutrient values were retrieved from the National Research Council (2001). We calculated HE nutrient recovery in milk in three scenarios; the first considered hominy feed and wheat middlings suitable for human consumption (thrift scenario), the second considered hominy feed and wheat middlings as unlikely to be consumed by humans (choice scenario), and the third was similar to the thrift scenario but considered that the land used for alfalfa hay production could be used to grow soybeans and corn (land use scenario).

For the land use scenario, a blend of corn and dehulled soybeans (82% and 18% on DM basis, respectively) offering a ME:protein ration similar to milk (21.8 kcal/g protein; 3.5% fat, 3.2% protein, and 4.8% lactose) was evaluated in place of alfalfa. Thus, one hectare used for alfalfa hay production could be used to grow 0.57 ha of corn and 0.43 ha of soybeans. The annual yields (on DM basis) of alfalfa hay (7263 kg/ha), corn (7125 kg/ha), and soybeans (2240 kg/ha), retrieved from Kansas 2017 Annual Crop Production Summary (Bounds, 2018), were used to determine the potential HE ME and protein displaced by alfalfa hay production. Soybean yield was discounted 7.3% (Perkins, 1995) to account for inedibility of hulls. Employing these assumptions, the displaced HE nutrient potential of alfalfa hay was estimated at 2.24 Mcal/kg and 103 g protein/kg alfalfa.

To account for amino acid recovery, we estimated the HE digestible amino acid inputs and outputs using the feed total amino acid content and standardized ileal digestibility (SID) values from the National Research Council (2012) ingredient library, with the exception of milk values that we retrieved from INRA-CIRDA-AFZ tables (2002-2004). Standardized ileal digestibility of amino acids in pigs can be used when values in humans are not available (FAO, 2013). Recoveries

of HE nutrients in milk were calculated as the ratio between nutrients secreted in milk and the corresponding HE nutrient intake by cattle, where a value of 2 would indicate that for every HE unit (energy, protein, or amino acid) the cow consumes, she would secrete 2 units in milk.

RESULTS

Experiment 1

Cows fed ECO1 had decreased feed efficiency in terms of milk yield ($P = 0.025$) per kg of DMI compared with CON1 (Table 4-6). Feeding ECO1 instead of CON1 to cows also reduced yields of milk fat ($P = 0.001$) and milk protein ($P = 0.020$) and tended to decrease ($P = 0.084$) lactose yield. On the other hand, ECO1 reduced ($P < 0.001$) milk urea N concentration and increased ($P < 0.01$) blood glucose concentration compared with CON1. As expected, primiparous cows had lesser ($P \leq 0.001$) DMI, SCLS, and body weights compared to multiparous cows. The effect of diet on milk yield differed by parity, where the ECO1 diet decreased milk yield (interaction $P = 0.045$) only in multiparous cows.

In all 3 calculated scenarios, the ECO1 diet decreased ($P < 0.01$, Table 4-7) HE ME inputs by 27-119 MJ/d and HE protein inputs by 0.37-1.53 kg/d compared with CON1 diet. In the choice scenario, HE inputs differed by parity for the CON1 diet only, with multiparous cows consuming more than primiparous. However, the overall effects of diet were similar in magnitude; ECO1 had 90% lower ME inputs and 95% lower HE protein inputs than CON1. The land use scenario had outcomes similar to the thrift scenario, although CON1 HE input values for ME and protein were greater than in the thrift scenario so that reductions in HE inputs with ECO1 were even greater. In all food system scenarios, cows fed ECO1 had greater HE ME and protein recoveries than those fed CON1 ($P < 0.01$), although the magnitude of the difference varied greatly based on the scenario

(Figure 4-1). For thrift scenario and land use scenarios, recoveries of HE energy and protein were 0.6 to 0.94 for CON1 and increased by 5 to 50% for ECO1. However, for choice scenario ECO1 increased recoveries of energy and protein by 700 to 2000%. Human-edible digestible amino acid recoveries were analyzed in the choice scenario. Cows fed the ECO1 diet had greater ($P < 0.01$) recoveries of all amino acids evaluated in this study (Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val, Cys, and Tyr; Table 4-8).

Experiment 2

Cows fed ECO2 or ECO2-AA diets consumed less DM ($P < 0.01$) compared with those fed CON2 (Table 4-9). ECO2 and ECO2-AA likewise decreased ($P < 0.01$) milk yield of cows. Feed efficiency (milk yield ÷ DMI) was similar between cows fed CON2 and ECO2 but was reduced ($P = 0.02$) when cows were fed ECO2-AA. In general, ECO2 and ECO2-AA diets reduced the production of milk fat, protein, and lactose. Cows fed ECO2 diets also had greater ($P < 0.01$) SCLS relative to those fed the CON2 diet.

Feeding cows with ECO2 and ECO2-AA diets reduced ($P < 0.01$) outputs of HE ME and protein in milk in comparison with CON2 diet (Table 4-10). In the thrift scenario, ECO2 diets had no effect on HE ME inputs, and they increased ($P < 0.01$) HE protein input. In the choice, cows fed ECO2 diets had lesser ($P < 0.01$) HE ME and protein inputs compared with those fed CON2 diet. In the land use scenario cows fed ECO2 diets had lesser ($P < 0.01$) HE ME input but did not affect HE protein inputs. Recovery of HE ME and protein in milk were decreased (-30 and -40% on average, respectively; $P < 0.01$) by ECO2 diets under the thrift scenario (Figure 4-2 A and B). On the other hand, in choice scenario (Figure 4-2 C and D) ECO2 diets improved ($P < 0.01$) the recovery of HE ME and protein in comparison with CON2 diet. In the land use scenario (Figure

4-2 E and F), recovery of HE ME was not affected by diets and recovery of HE protein was worsened ($P < 0.01$) by ECO2 diets. In the choice scenario, HE ME and HE protein recoveries increased by 360% and 950%, respectively. Similar to observations in Experiment 1, ECO2 diets in a choice scenario improved ($P < 0.01$) the recovery of all amino acids evaluated (Table 4-11), with no detectable differences between ECO2 and ECO2-AA.

DISCUSSION

Dairy cattle productivity

The hypothesis of the current study was that cows fed ECO diets would maintain milk production while improving the HE nutrient conversion rate by dairy cows. Neither ECO1 nor ECO2 strategies were able to maintain equivalent milk yield in all cows. Furthermore, this study showed evidence that a dairy cow diet composed of by-products does not necessarily improve the HE nutrient conversion rate in dairy cows, as the outcome is dependent on parity, feed ingredients, and assumptions made in calculating the value of feed ingredients for human consumption.

In Experiment 1, cows had similar DMI across diets, but the ECO1 treatment decreased milk yield in multiparous cows and reduced overall feed efficiency in all cows, regardless of parity. Decreased milk yield might be attributed to decreased nutrient digestibility for ECO1 compared with CON1. Replacing alfalfa with wheat straw (on forage NDF basis) in diets of lactating cows decreased fiber digestibility, milk yield, and milk fat content (Poore et al., 1991). Anderson and Hoffman (2014) reported that wheat straw NDF digestibility (48 h *in vitro*) may range from 21 to 49% (39.0% average) whereas corn silage and alfalfa hay fiber digestibility ranges from 58-67% (Ivan et al., 2005) and 50-56% (Sulc et al., 2016), respectively. Oba and Allen (1999) summarized data from several experiments to determine the influence of fiber digestibility among forages with

similar fiber and crude protein content on dairy cow performance. These authors reported that a one-unit increase in forage fiber digestibility was associated with a 0.17 kg/day increase in DM intake and a 0.25 kg/day increase in fat-corrected milk yield. High-productivity dairy cattle systems are generally based on dedicated production of highly digestible forages, and this represents a significant challenge in attempting to avoid displacing arable land from human food crop production.

The responses to ECO1 differed by parity, and this interaction provides some insights into the likely cause of the decline in milk yield when feeding ECO diets. Multiparous cows produce more milk and thus have greater nutrient requirements than primiparous cows, and in this parity group, ECO1 apparently failed to provide adequate nutrients (either from poor digestibility or an imbalance in nutrient profile) to maintain the high level of milk production. On the other hand, primiparous cows, which produce more moderate yields, maintained productivity when fed ECO1. Not surprisingly, cows with greater levels of productivity are less flexible in terms of diet formulation.

As in the current study, Karlsson et al. (2018) reported similar DMI and a marginal decrease (32.1 vs. 30.8 kg/d, $P = 0.06$) in milk yield when replacing cereal grains and soybean meal with sugar beet pulp and distillers' grains. In contrast, Pang et al. (2018) fed cows a diet composed of grass silage with either a by-product or grain concentrate and found a trend for decreased DMI ($P = 0.06$) for the group fed the by-product concentrate with no differences in milk yield. Co-products often provide less rumen-degradable protein and more rumen undegradable protein in comparison with the original feed (Bradford and Carpenter, 2017). Moreover, feeds that are excessively heated may exhibit relatively high concentrations of Maillard products, thus decreasing protein digestibility. Although not explored in Experiment 1, the formulation software

highlighted a possible Met deficiency for dairy cows fed ECO1 (estimated supply of 55 g/d intestinally-available Met vs. estimated requirement of 59 g/d to maintain yield of control group; National Research Council, 2001), which is often considered to be the first-limiting amino acid for milk production in dairy cattle (Schwab and Broderick, 2017). It is important to highlight that it is difficult to meet nutrient requirements with ECO ingredients alone because by-products often lack valuable nutrients that have been removed for other purposes.

Based on the formulation software report and results from Experiment 1, the second experiment evaluated whether supplementing a by-product diet with rumen-protected amino acids would maintain milk production levels of cows. However, due to differences in feed availability, a modified formulation was used. Contrasting with experiment 1, cows fed ECO2 diets, regardless of amino acid supplements, had a substantial decrease in both DMI (-4.7 kg/d) and milk yield (-9.0 kg/d). This large negative impact on performance of cows can likely be attributed to the forage source used in this study. Supplementing ECO2 with amino acids tended to decrease milk urea N concentration, suggesting an improvement in N utilization. Furthermore, the triticale/clover hay had high crude protein content and high moisture content that favored spoilage and reductions in feed intake. These problems again highlight the challenges associated with avoiding the dedication of land towards growing high-quality forages production for dairy cattle.

Human-edible nutrient recovery

Bywater and Baldwin (1980) discussed the rationale of calculating animal efficiency based on total feed inputs versus inputs of HE energy and protein, since the ultimate concern is total human food production. For instance, returns of HE digestible energy and protein varied from 57 to 128% and 96 to 276%, respectively, depending on the feeding strategy adopted in some studies

(Baldwin et al., 1992; Oltjen and Beckett, 1996). More recently, the term edible feed conversion ratio was introduced by Wilkinson (2011) to relate HE nutrient inputs and outputs in animal products, and this approach has been used to estimate HE energy and protein recovery in milk in several studies (Ertl et al., 2015; Karlsson et al., 2018; Pang et al., 2018). However, this concept estimates the proportion of crops and crop by-products that are potentially suitable for human consumption in the UK, rather than considering the nutrient composition of each feedstuff as consumed by humans. For instance, cereal by-products, oilseed meals (excluding soybean meal), and other by-products (molasses, candy waste, spent coffee, and others) were assigned a HE nutrient coefficient of 0.2 (meaning the ingredients are considered 20% human edible), whereas soybean meal and cereal and pulse grains were assigned to a HE nutrient coefficient of 0.8. This concept would consider molasses and spent coffee grounds to contain the same nutritional value for humans. In addition, the cereal portion in silages is not considered as a HE nutrient by Wilkinson (2011). Corn silage is the main forage source for confined dairy cows in much of the world, and failure to consider the corn grain portion in plants as a HE food (before ensiling) would ultimately overestimate HE nutrient recovery of the diet. In addition, the above calculations ignored the fact that land used for growing forages could in some cases be used for growing crops to be consumed directly by humans. Vandehaar (1998) calculated the efficiency of land use by considering the HE value of corn and soybeans on a protein equivalent basis to milk to account for this possibility, postulating that a combination of corn and soybeans (similar to blends of other grains and legume seeds) would be the most effective way to feed people with limited land resources. Therefore, alternative methods to determine HE nutrient value of feeds should be considered to more precisely describe the efficiency of HE feed conversion. As an alternative approach, to describing efficiency of HE feed utilization, we calculated energy, protein, and

digestible amino acid content of feeds in cattle diets that could potentially be used for human consumption relative to secretion of the respective nutrients in milk.

Experiment 1 showed evidence that increased inclusion of by-product feeds in the diet improves the HE nutrient recovery in milk of cows, which has also been observed by others (Ertl et al., 2015; Karlsson et al., 2018; Pang et al., 2018). However, Experiment 1 demonstrated an average increase in HE recovery of just 13% in terms of energy and 22% in terms of protein (thrift scenario), whereas previous experiments have shown recoveries ranging from 367 to 495% in energy and 267 to 441% in protein (Ertl et al., 2015; Karlsson et al., 2018; Pang et al., 2018)– suggesting that calculations strategies (i.e. HE coefficients) heavily influence conclusions regarding HE nutrient recovery in milk. For instance, in the land use scenario of Experiment 1, the average increase in HE recovery was around 50 and 40% in terms of energy and protein, respectively. Ertl et al. (2016) calculated the HE conversion rate (based on minimum or maximum potential recoveries rates of HE energy and protein from the respective feedstuffs) from other studies that fed by-product-based concentrates to cows (Voelker and Allen, 2003; Dann et al., 2014). The authors reported no net HE energy or protein production when calculations used minimum HE nutrient coefficients; however, when calculating the conversion rate using maximum HE nutrient coefficients, authors reported net production of HE nutrients by lactating cows (Ertl et al., 2016b). It is important to note that the thrift scenario arguably represents an extreme, incongruous situation—one where human populations have become more open to consuming foods currently avoided, but where byproducts (frombiofuel production, for example) remain available for cattle diets. However, this scenario could arise through food technologies that enhance the incorporation of products like wheat middlings in human foods rather than out of desperation for food sources.

To demonstrate that HE nutrient conversion rate is highly dependent on model assumptions, we also estimated HE conversion values with the assumption that wheat middlings and hominy feed are unlikely to be consumed by humans (choice scenario). Although wheat middlings can be incorporated into certain human foods (i.e., pasta; Kaur et al., 2012), it is extensively marketed as a by-product feed for livestock, and its competitive price (Capehart et al., 2018) in relation to other energy and protein sources demonstrates its low perceived desirability in the human food supply. Hominy feed is a by-product from the manufacture of pearl hominy, hominy grits, or corn meal, and it is rarely used for human nutrition. Note that hominy feed is different from hominy food, where the former is a by-product of dry corn milling and the latter is a food made from kernels of corn soaked in alkali solution that removes hull and germ. The HE recovery outcome varied drastically depending on the HE values of these two ingredients. The proportional increase in HE nutrient conversion rate was much greater for ME (8.0-fold) and protein (24.8-fold) in the choice scenario compared with the thrift scenario (ME 1.13-fold and protein 1.25-fold) in Experiment 1 and for Experiment 2, the recovery of both ME and protein improved from a HE nutrient loss in the thrift scenario to a HE nutrient gain in the choice scenario.

Some approaches to calculating HE conversion rate of nutrients ignore forage displacement entirely, but it is more reasonable that only non-arable forage land (i.e., upland pastures) should be excluded in calculations. For instance, (Ertl et al., 2015) did not consider any "HE value" for alfalfa hay when estimating the HE conversion rate, potentially underestimating the HE inputs– since crops for human food production could be planted instead of alfalfa (acknowledging the complexity of this question in mountainous Austria). By incorporating the HE opportunity cost of land used for alfalfa in our calculations, the calculated HE energy recovery for CON diets dropped from ~ 96% to 60%, and from 84% to 62% for HE protein recovery. It is therefore quite meaningful

to consider land-use tradeoffs and the extent to which forage production could be shifted to human food crops in a given locale. Shortfalls with the land use approach are that fertilizer is needed to grow corn, crops usually increase erosion, and the protein quality from the blend of corn and soybeans designed is not as high as milk protein.

In areas that are seasonally unsuitable for grain production but have moderate climates, some forage crops can grow well (Wilke and Snapp, 2008) while improving soil physical, chemical, and biological properties (Fageria et al., 2005). We utilized winter cover crop forage (triticale and clover hay) produced off-season on the same land used for a food-producing crop in a dual-cropping system (Salmon et al., 2004) in Experiment 2. It is noteworthy that winter cover crops may have either negative or positive effects on subsequent corn yield, depending on agricultural practices adopted. A meta-analysis of winter cover crops under agricultural practices in the USA and Canada showed that a bi-culture of winter cover crops increased corn yield by 21%; legume cover crops had an overall positive effect on corn yield, whereas grass winter cover crops had a neutral effect (Miguez and Bollero, 2005). Although winter cover crops are usually planted by farmers due to potential forage harvest and economic benefits, cover crops also protect soil from erosion, protect water quality (reduce losses of pesticides, nutrients, or sediments from fields), and promote carbon sequestration and nitrogen fixation (Dabney et al., 2001). On the other hand, cover crops involve additional inputs and risks. For instance, winter forage conditions in many regions are not ideal for crop dry-down at harvest (hence the moisture content of triticale/hay used in this experiment). Cover crops also require additional costs compared with a single crop system, must be planted when time and labor are limited, may increase risk of diseases, and can reduce soil moisture (Dabney et al., 2001).

To account for protein quality, we calculated the recovery of HE feed digestible amino acids in milk. As mentioned earlier, ruminants can convert non-protein nitrogen or protein with poor amino acid profile into a high-quality protein that is better able to meet human amino acids requirements (Virtanen, 1966). Grains tend to have poorer amino acid profile and digestibility in comparison with animal-based products (Wu, 2016). Thus, cereal-based diets are able to meet total protein requirements but may fail to meet essential amino acid requirements (Bouis et al., 2011). Furthermore, removing animals from US agriculture would likely lead to deficiencies in supplies of several nutrients for the US population, including Ca, essential fatty acids, and vitamins A and B (White and Hall, 2017). Vandehaar (1998) estimated a 20% greater nutritional value in the protein from milk compared to protein from a mix of corn and soybeans, due to both protein availability and amino acid profile. Ritchie et al. (2018) estimated a 20.6% deficiency in average Lys requirement across the global food system if meat and dairy products were removed from supply chains. The contrast between Lys content in plant- and animal-based diets has been described elsewhere (FAO/WHO/UNU, 2007; Swaminathan et al., 2012). In Experiment 1, calculations from the choice scenario on amino acid recovery showed that feeding ECO1 greatly increased the milk recovery of all amino acids, including digestible Lys, which was secreted in milk at 471× the amount of HE Lys that cows consumed. Because molasses was considered the only HE ingredient in the choice scenario, relatively high values of digestible amino acids recovery in milk were expected. Interestingly, the CON1 diet also supported a net gain in HE supply of most amino acids, with the exception of Arg and Cys. For example, milk Lys secretion by cows fed CON1 was 291× greater than the amount of HE Lys consumed by those cows. In agreement with the current study, Karlsson et al. (2018) described improved net production of several human-digestible amino acids (His, Ile, Leu, Lys, Thr, Trp, Val, Phe+Tyr) when replacing corn and

soybean meal with by-products. Furthermore, the greatest improvement in net production of digestible amino acids was observed for Lys (Karlsson et al., 2018).

In Experiment 2, HE conversion rate was not necessarily improved by ECO diets. In the thrift scenario, ECO diets worsened HE ME and protein conversion, whereas in the choice scenario, ECO diets improved HE ME and protein conversion rates. The experiment 2 outcomes (at least the thrift scenario) oppose those reported in several studies (Ertl et al., 2015; Karlsson et al., 2018), mainly because of the negative impact of ECO diets on milk yield—which was not observed in the studies cited earlier. Although HE conversion rate was not necessarily improved by ECO diets, note that these diets theoretically do not use arable land and may present a higher efficiency of land use even with cows producing less milk during the lactation. For instance, Vandehaar (1998) estimated that milk protein produced by a dairy cow yielding 10,000 kg of milk per year when fed a diet without by-products requires more than twice the land base as a nutritionally equivalent supply of protein from corn and soybeans produced for direct human consumption. On the other hand, a cow producing half the amount of milk annually while consuming 33% by-products increases the efficiency of land use to 76% relative to the 35% on corn and soybean system (Vandehaar, 1998). Decreasing the utilization or increasing efficiency of land for crops is of great interest for sustainability of food systems (Peters et al., 2016).

It is important to note that our approach carefully accounts for HE inputs and outputs of specific nutrients in particular productive stages and food system scenarios but does not consider the feed required during non-productive stages of the cow's life nor the HE value of the cow carcass after culling. These are not trivial components of the overall dairy system; Thoma et al. (2013) attributed 13% of dairy farm greenhouse gas emissions to beef production, and Tichenor (2015) suggested that this may be an underestimate. Nevertheless, dietary strategies similar to

those evaluated herein can also contribute to net HE protein production in beef systems (Baber et al., 2018), and it is likely that our comparisons would hold for net beef + milk production, assuming ECO diets were employed across a farm.

Another caveat of this study is that we focused on a single aspect of sustainability—the utility of dairy cattle in an efficient food system, focusing primarily on arable land as a constraint. There are, of course, many other aspects of sustainability to be considered in implementing ECO diet strategies on dairy farms, not least the profitability of the farm. It is likely that the ECO diets we tested would decrease the gross cost of diet ingredients compared to CON diets, though the lack of commercial markets for some ingredients prevented us from evaluating this. However, the 28% decline in milk yield with ECO2 would almost certainly reduce revenue far more than feed costs, leaving little to cover fixed costs (i.e., infrastructure, labor, energy), and decreasing milk output per unit of these input. As is often the case, focusing on maximizing one narrow aspect of sustainability will likely harm sustainability in other ways.

In summary, HE nutrient conversion rate should be carefully analyzed, as values are highly dependent on whether individual feeds are considered HE and on the calculations performed (fixed coefficient for a group of ingredients vs. variable coefficients according to feed composition). Furthermore, ECO diets can be formulated using a wide range of ingredients depending on their availability and cost. As pointed out throughout this paper, diets with a relatively high inclusion of by-products can either improve, reduce, or maintain productive performance of animals. Under some scenarios, ECO diets do not necessarily improve HE feed conversion rate in dairy cows, and they will likely decrease feed efficiency of cows in terms of milk yield ÷ DM intake. Regardless of diet, parity, and productive stage, cows typically generate a net return of HE essential amino acids in milk. These findings contribute to our understanding of how dairy production contributes

to a sustainable global food system in alignment with competing environmental and development goals.

CONCLUSION

In this study, we showed that even with conventional diets on modern North American farms, the dairy industry is relatively efficient at using human-edible foods to produce milk and particularly in returning digestible essential amino acids. Moreover, we showed that inclusion of ecological leftovers can be used to effectively increase the use of human-edible foods, especially if foods that are not desirable by humans are not consumed by humans. This increase in human-edible recovery can occur even if milk production per cow declines.

ACKNOWLEDGMENTS

This work was a contribution (no. 20-053-J) from the Kansas Agricultural Experiment Station (Manhattan, KS), supported by USDA National Institute of Food and Agriculture (Washington, DC) Hatch project 1018048. The authors thank Ajinomoto (Chicago, IL) and Adisseo (Antony, France) for amino acid product donations and Ajinomoto for partially funding Experiment 2. Authors would also like to thank Mike Scheffel and employees of the Kansas State University Dairy Teaching and Research Center (Manhattan, KS) for their assistance in completing this study. CT appreciates the scholarship (88881.170046/2018-01) provided by the Coordination for the Improvement of Higher Education Personnel (CAPES, Brasília, Brazil).

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Table 4-1. Ingredients and chemical composition of diets provided in Experiment 1.

	Diet[1]	
Item	CON1	ECO1
Ingredient (% DM)		
Corn silage	17.2	
Alfalfa hay	21.6	
Corn gluten feed[2]	25.9	31.6
Whole cotton seed with lint	4.20	
Soybean meal[3]	5.70	
Ground corn	20.6	
Wheat straw		20.9
Wheat middlings		3.40
Hominy feed		26.8
Molasses		3.80
Porcine blood meal		0.70
Post-extraction algae residue		9.80
Limestone	1.60	1.80
Calcium salts of long-chain fatty acids[4]	0.80	
Sodium bicarbonate	1.10	
Vitamin and trace mineral mix[5]	1.30	1.30
Chemical (% DM)		
Dry matter (% as-fed)	50.9	50.3
Crude protein	17.4	17.0
Acid detergent fiber	18.6	18.2
Neutral detergent fiber (NDF)	30.5	32.9
Forage NDF	17.9	15.1
Non-fiber carbohydrate	37.1	35.6
Starch[6]	23.1	15.9
Ether extract	5.20	4.70
Ash	9.80	8.50
Net energy of lactation$_{3\times}$ (MJ/kg)	7.10	7.01
Particle size distribution[7] (g/kg as-fed)		
>19 mm	0.07	0.07
8-19 mm	0.33	0.24
4-8 mm	0.51	0.61
< 4 mm	0.09	0.07
Human-edible (HE) nutrient – Thrift scenario[8]		
HE metabolizable energy (MJ/kg)	4.51	3.65
HE protein (g/kg diet DM)	53.5	38.6
HE nutrient – Choice scenario[9]		

HE metabolizable energy (MJ/kg)	4.51	0.51
HE protein (g/kg diet DM)	53.5	2.05
HE nutrient – Land Use Scenario[10]		
HE metabolizable energy (MJ/kg)	6.49	3.65
HE protein (g/kg diet DM)	75.1	38.6

[1]Conventional lactation TMR (CON1), containing 31.0% co-product feeds, and TMR composed of ecological leftover feedstuff (ECO1) – water was added to achieve similar diet DM.

[2]Sweet Bran, Cargill, Blair, NE.

[3]Soy Best (Grain States Soya, Inc., West Point, NE).

[4]Megalac-R (Arm & Hammer Animal Nutrition -Church & Dwight Co., Inc. Trenton, NJ).

[5]CON1 mineral and vitamin mix (% DM): 13.2% vitamin E (44,000 IU/kg), 2.06% 4-plex C (Zinpro, Eden Prairie, MN), 1.49% Zinpro 100 (Zinpro), 2.25% Se, 1.32% vitamin A (30,000 IU/g), 0.39% Vitamin D (30,000 IU/g), 0.07 iodine (44,000 mg/kg), 0.56% Rumensin 90 (Elanco Animal Health, Greenfield, IN), 16.54% XP yeast (Diamond V. Cedar Rapids, IA), and 8.27% biotin. ECO1 mineral and vitamin mix (% DM): 52.9% vitamin E (44,000 IU/kg), 8.25% 4-plex C (Zinpro), 4.23% Zinpro 100 (Zinpro), 8.99% Se, 5.29% Vitamin A (30,000 IU/g), 1.59% vitamin D (30,000 IU/g), 0.26 iodine (44,000 mg/kg), 2.22% Rumensin 90 (Elanco Animal Health), and 16.3% XP yeast (Diamond V).

[6]Calculated.

[7]Particle size distribution (Heinrichs and Kononoff, 2013).

[8]Hominy feed and wheat middlings were considered to be foods for human consumption.

[8]Hominy feed and wheat middlings were not considered to be foods consumed by humans.

[9]Land used for alfalfa production could be used to grow corn and soybeans for direct human consumption

Table 4-2. Chemical composition of feeds used in Experiment 1 (% of DM, otherwise stated).

Ingredient[1]	DM (% as-fed)	CP	ADF	aNDF	NFC	EE	Ash
Corn silage	28.9	8.5	29.6	48.1	34.6	3.1	5.7
Alfalfa hay	89.6	20.7	35.2	43.1	24.6	2.2	9.36
Wheat straw	81.2	7.1	47.8	71.0	11.3	1.30	9.35
Whole cottonseed with lint	90.5	25.4	28.9	44.2	12.1	15.8	2.52
Corn gluten feed[2]	55.9	23.1	11.5	32.8	32.2	6.0	5.97
Molasses	28.6	5.6		0.4	78.05	3.7	12.3
Grain mix CON1[3]	88.4	15.3	4.2	8.6	53.7	6.5	15.9
Grain mix ECO1[4]	88.7	18.7	8.2	15.3	51.0	5.6	9.33

[1]Dry matter (DM), crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (aNDF) treated with sodium sulfite and alpha-amylase, non-fiber carbohydrate (NFC), ether extract (EE), human-edible metabolizable energy (HE ME), and human-edible crude protein (HE CP).

[2]Sweet Bran, Cargill, Blair, NE.

[3]Grain mix containing (% DM): 64.74% ground corn, 18.3% by-pass soybean meal (Soy Best, Grain States Soya, Inc., West Point, NE), 2.81% by-pass fat (Megalac-R, Arm & Hammer Animal Nutrition -Church & Dwight Co., Inc. Trenton, NJ), and 14.1% minerals and vitamins.

[4]Grain mix containing (% DM): 7.82% wheat middlings, 62.7% hominy feed, 22.9% post-extraction algae residue (*Chlorella spp.*), 1.69% porcine blood meal, and 4.83% minerals and vitamins.

Table 4-3. Ingredient, chemical composition, and particle size distribution of diets provided in Experiment 2.

	Diet[1]	
Item	CON2	ECO2
Ingredient (% DM)		
Corn silage	24.9	
Alfalfa hay	19.2	
Prairie hay	1.63	
Triticale/clover hay[2]		31.6
Corn gluten feed[3]	23.6	15.1
Whole cottonseed with lint	3.85	1.38
Ground corn	16.7	
Expeller soybean meal[4]	6.08	
Calcium salts of long-chain fatty acids[5]	0.76	
Wheat middlings		25.5
Hominy feed		12.8
Spent coffee grounds		4.36
Molasses		5.95
Limestone	1.22	1.28
Sodium bicarbonate	1.06	0.90
Potassium chloride	0.15	0.32
Minerals and vitamins[6]	0.88	0.60
Chemical (% DM)		
Dry matter (% as-fed)	58.5	54.1
Crude protein	17.1	18.0
Acid detergent fiber	19.5	20.4
Neutral detergent fiber (NDF)	34.4	37.3
Forage NDF	21.8	18.71
Non-fiber carbohydrate	34.4	25.9
Starch[7]	20.9	17.7
Ether extract	5.07	4.73
Ash	8.95	14.0
Total digestible nutrient (%)	69.0	62.7
Net energy of laction$_{3\times}$ (MJ/kg)	6.73	6.09
Particle size distribution[8] (g/kg as-fed)		
>19 mm	0.04	0.24
8-19 mm	0.22	0.09
4-8 mm	0.18	0.14
< 4 mm	0.56	0.53
Human-edible (HE) nutrient – Thrift Scenario[9]		
HE metabolizable energy (MJ/kg DM)	3.75	4.83

HE protein (g/kg DM)	46.9	73.9
HE nutrient – Choice Scenario[10]		
HE metabolizable energy (MJ/kg)	3.75	0.87
HE protein (g/kg diet DM)	46.9	3.49
HE nutrient – Land Use Scenario[11]		
HE metabolizable energy (MJ/kg)	5.23	4.83
HE protein (g/kg diet DM)	63.4	73.9

[1]Conventional lactation diet (CON2), containing 25.7% co-product feeds, and TMR composed of ecological leftover feedstuff (ECO2) – water was added to achieve similar diet DM.
[2]Hay from the winter intercropping of triticale and red clover.
[3]Sweet Bran, Cargill, Blair, NE.
[4]Soy Best (Grain States Soya, Inc., West Point, NE).
[5]Megalac-R (Arm & Hammer Animal Nutrition -Church & Dwight Co., Inc. Trenton, NJ).
[6]CON2 minerals and vitamin mix (% DM): 27.6% magnesium oxide, 34.5% salt, 17.2% vitamin E (44,000 IU/kg), 2.68% 4-plex C (Zinpro, Eden Prairie, MN), 1.37% Zinpro 120 (Zinpro), 2.91% Se, 1.72% vitamin A (30,000 IU/g), 0.51% vitamin D (30,000 IU/g), 0.08 iodine (44,000 mg/kg), 0.72% Rumensin 90 (Elanco Animal Health, Greenfield, IN), and 10.8% biotin. ECO2 minerals and vitamin mix (% DM): 27.1% magnesium oxide, 22.8% salt, 22.8% vitamin E (44,000 IU/kg),3.54% 4-plex C (Zinpro), 1.80% Zinpro 120 (Zinpro), 3.85% Se, 2.27% vitamin A (30,000 IU/g), 0.67% Vitamin D (30,000 IU/g), 0.10% iodine (44,000 mg/kg), 0.95% Rumensin 90 (Elanco Animal Health), and 14.2% biotin.
[7]Calculated.
[8]Particle size distribution (Heinrichs and Kononoff, 2013).
[9]Hominy feed and wheat middlings were considered to be foods for human consumption.
[10]Hominy feed and wheat middlings were not considered to be foods consumed by humans.
[11]Land used for alfalfa production could be used to grow corn and soybeans for direct human consumption.

Table 4-4. Chemical composition of feeds used in the Experiment 2 (% of DM unless otherwise labeled).

Ingredient[1]	DM, % as-fed	CP	ADF	aNDF	NFC	EE	Ash
Corn silage	35.6	9.2	21.5	39.2	41.9	3.70	6.05
Alfalfa hay	91.5	20.3	31.8	42.8	23.4	2.30	11.3
Prairie hay	93.3	5.70	43.1	68.9	14.0	2.30	9.14
Triticale/clover hay	72.3	19.1	40.9	59.2	1.55	2.23	17.6
Whole cottonseed with lint[2]	88.2	22.3	44.3	57.2	0.70	15.7	4.38
Corn gluten feed[3]	61.3	23.0	8.90	31.8	43.2	5.10	7.05
Spent coffee grounds	35.1	14.2	32.3	54.7	15.2	14.2	1.78
Molasses	70.7	5.80	-	-	-	5.70	15.4
Grain mix CON2[4]	86.4	16.1	6.80	19.8	45.3	5.35	13.6
Grain mix ECO2[5]	82.5	15.4	9.7	29.1	38.9	4.45	12.2

[1]Dry matter (DM), crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (aNDF) treated with sodium sulfite and alpha-amylase, non-fiber carbohydrate (NFC), and ether extract (EE).

[2]Hay was not harvested and stored properly which influenced in its moisture value.

[3]Sweet Bran (Cargill, Blair, NE).

[4]Grain mix containing (% DM): 63.9% ground corn, 23.5% by-pass soybean meal (Soy Best, Grain States Soya, Inc., West Point, NE), 3.3% by-pass fat (Megalac-R, Arm & Hammer Animal Nutrition -Church & Dwight Co., Inc. Trenton, NJ), and 9.18% minerals and vitamins.

[5]Grain mix containing (% DM): 61.4% wheat middlings, 30.4% hominy feed, and 8.22% minerals and vitamins.

Table 4-5. Human-edible (HE) nutrient values used for calculations (DM basis; g/kg unless otherwise labeled).*

Ingredient	Metabolizable energy (MJ/kg)	Protein	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val	Cys	Tyr
Alfalfa hay (land-use scenario)	9.81	108	-	-	-	-	-	-	-	-	-	-	-	-
Corn silage	2.40	16.0	0.543	0.336	0.388	1.41	0.312	0.252	0.560	0.364	0.081	0.526	0.257	0.347
Ground corn	14.2	95.0	3.22	1.99	2.30	8.35	1.85	1.49	3.32	2.16	0.480	3.12	1.52	2.05
Soybean meal[2]	10.7	490	32.4	11.5	19.0	31.9	26.3	5.94	21.1	15.8	6.01	19.4	5.88	13.9
Wheat middlings	8.13	185	-	-		-	-	-	-	-	-	-	-	-
Molasses	14.0	119	0.184	0.090	0.352	0.534	0.172	0.180	0.270	0.430	0.086	0.957	0.336	0.273
Hominy feed	11.3	56	-	-	-	-	-	-	-	-	-	-	-	-
Whole milk (as-fed)	0.697	32.0	0.938	0.845	1.481	2.67	2.09	0.674	1.39	1.27	0.391	1.64	0.173	1.06

*Values of HE metabolizable energy and protein were calculated based on chemical composition of feeds described in NRC (2001) and feed human-edible proportion of feeds. Values of HE amino acids were calculated based on HE protein value and standardized ileal digestibility of feeds described in NRC (2012).

[2]Soy Best (Grain States Soya, Inc., West Point, NE).

Table 4-6. Performance and blood metabolites of mid-lactation cows fed a conventional lactation diet or a by-product-based diet (Experiment 1).

	Primiparous		Multiparous			*P*-value		
Item	CON1	ECO1	CON1	ECO1	SEM	Diet	Parity	Diet × Parity
DMI (kg/d)	25.8	26.2	29.7	29.3	1.30	0.94	< 0.01	0.52
Milk yield (kg/d)	39.4^{ab}	39.3^{ab}	42.3^{a}	38.7^{b}	2.21	0.04	0.71	0.05
Milk yield ÷ DMI	1.53	1.50	1.43	1.32	0.084	0.03	0.21	0.17
Milk fat (kg/d)	1.37	1.27	1.58	1.33	0.079	< 0.01	0.23	0.06
Milk protein (kg/d)	1.14	1.12	1.30	1.18	0.062	0.02	0.22	0.08
Milk lactose (kg/d)	1.96^{ab}	1.97^{ab}	2.02^{a}	1.83^{b}	0.120	0.08	0.82	0.05
Milk urea nitrogen (mg/dL)	15.2	12.5	14.2	11.5	0.96	< 0.01	0.30	0.98
SCLS[1]	0.98	0.25	3.82	3.62	0.89	0.31	< 0.01	0.56
BW[2] change (kg/21 d)	5.37	1.16	-0.93	13.2	27.3	0.28	0.53	0.06
BCS[3] change (/21 d)	0.042	0.022	-0.013	0.017	0.076	0.92	0.63	0.72
Blood glucose (mg/dL)	57.4	66.2	58.0	61.2	2.23	< 0.01	0.48	0.11
Blood insulin (ng/dL)	0.42	0.34	0.36	0.51	0.084	0.55	0.62	0.11

[a-b]Least square means within rows with different superscripts differ significantly in Tukey's HSD ($P < 0.05$).

Treatments: conventional lactation TMR (CON1), containing 31% by-product feeds; and TMR composed of ecological leftover feedstuff (ECO1), containing 95% by-product feeds.

[1]Somatic cell linear score (SCLS) = log2 (somatic cell count /100) + 3 (Shook, 1993).

[2]Body weight.

[3]Body condition score change (1- 5, whereas 1 is thin and 5 is fat).

Table 4-7. Human-edible (HE) nutrient inputs and outputs of mid-lactation cows fed a conventional lactation diet or a by-product-based diet (Experiment 1).

	Primiparous		Multiparous			*P*-value		
Item	CON1	ECO1	CON1	ECO1	SEM	Diet	Parity	Diet × Parity
Thrift scenario[1]								
HE ME input (MJ/d)	116	96.2	134	107	3.51	< 0.01	< 0.01	0.21
HE protein input (kg/d)	1.38	1.01	1.59	1.13	0.04	< 0.01	< 0.01	0.12
Choice scenario[2]								
HE ME input (MJ/d)	116[b]	13.9[c]	134[a]	15.0[c]	3.02	< 0.01	< 0.01	< 0.01
HE protein input (kg/d)	1.38[b]	0.059[c]	1.59[a]	0.060[c]	0.03	< 0.01	< 0.01	< 0.01
Land use scenario[3]								
HE ME input (MJ/d)	167[b]	96.4[c]	193[a]	107[c]	4.51	< 0.01	< 0.01	0.04
HE protein input (kg/d)	1.94[b]	1.01[c]	2.23[a]	1.13[c]	0.05	< 0.01	< 0.01	0.03
Output								
Milk metabolizable energy (ME; MJ/d)	113[ab]	109[b]	126[a]	110[b]	5.98	< 0.01	0.38	0.03
Milk protein (kg/d)	1.14	1.12	1.30	1.18	0.06	0.02	0.22	0.07

[a-d]Least square means with different superscripts within rows differ significantly in Tukey's HSD ($P < 0.05$).

Treatments: conventional lactation TMR (CON1), containing 31% by-product feeds; and TMR composed of ecological leftover feedstuff (ECO1), containing 95% by-product feeds.

[1]Hominy feed and wheat middlings were considered to be foods for human consumption.

[2]Hominy feed and wheat middlings were considered not to be foods consumed by humans.

[3]Land used for alfalfa production could be used to grow corn and soybeans for direct human consumption.

Table 4-8. Human-edible (HE) digestible amino acid recovery (g output/ g input) in milk of mid-lactation cows fed a conventional lactation diet or a by-product-based diet (Experiment 1, Choice Scenario).

Amino acid	Primiparous		Multiparous			*P*-value		
	CON1	ECO1	CON1	ECO1	SEM	Diet	Parity	Diet × Parity
Arg	0.53	210	0.50	184	6.20	<0.01	0.061	0.061
His	1.08	387	1.01	339	11.4	<0.01	0.061	0.061
Ile	1.33	174	1.25	152	5.12	<0.01	0.062	0.060
Leu	1.00	207	0.94	181	6.09	<0.01	0.062	0.061
Lys	1.62	502	1.51	440	14.8	<0.01	0.061	0.061
Met	1.39	154	1.29	135	4.55	<0.01	0.062	0.060
Phe	1.01	212	0.95	186	6.26	<0.01	0.062	0.061
Thr	1.32	122	1.23	107	3.60	<0.01	0.062	0.060
Trp	1.28	188	1.19	164	5.53	<0.01	0.062	0.060
Val	1.29	70.6	1.20	61.9	2.08	<0.01	0.063	0.059
Cys	0.35	21.2	0.33	18.6	0.63	<0.01	0.063	0.059
Tyr	1.20	160	1.12	140	4.71	<0.01	0.062	0.060

Treatments: conventional lactation TMR (CON1), containing 31% by-product feeds; and TMR composed of ecological leftover feedstuff (ECO1), containing 95% by-product feeds.

Where a recovery value of 1 would indicate that for every gram of HE digestible amino acid the cow consumes, she would produce 1 g HE digestible amino acid in milk.

Table 4-9. Performance and sorting index of late-lactating cows fed a conventional lactation diet or by-product-based diets (Experiment 2).

	Diet[1]				
Item	CON2	ECO2	ECO2-AA	SEM	*P*-value[2]
Dry matter intake (kg/d)	26.2[a]	21.5[b]	22.5[b]	1.12	< 0.01
Milk yield (kg/d)	31.9[a]	22.9[b]	22.9[b]	3.03	< 0.01
Milk yield ÷ DMI	1.21[a]	1.07[ab]	1.02[b]	0.098	0.02
Fat (kg/d)	1.13[a]	0.87[b]	0.89[b]	0.049	< 0.01
Protein (kg/d)	1.02[a]	0.70[b]	0.75[b]	0.045	< 0.01
Lactose (kg/d)	1.53[a]	1.03[b]	1.05[b]	0.073	< 0.01
Milk urea nitrogen (mg/dL)	13.9	14.1	13.7	0.14	0.06
SCLS[3]	2.46[b]	3.69[a]	3.69[a]	0.25	< 0.01
Body weight change (kg/21 d)	31.7	21.9	21.7	4.96	0.28
Body condition score change (/21 d)	0.04	-0.04	-0.06	0.033	0.29

[a-b]Least square means with different superscripts within rows differ significantly in Tukey's HSD ($P < 0.05$).

[1]Conventional lactation TMR (CON2), containing 25.7% co-product feeds; TMR composed of ecological leftover feedstuff (ECO2); and ECO2 with top-dressed rumen-protected amino acids [ECO2-AA; 77 g/d AjiPro-L (Ajinomoto, Chicago, IL) and 45 g/d MetaSmart (Adisseo, Antony, France)].

[2]*P*-values associated with treatment effect.

[3]Somatic cell linear score (SCLS) = log2 (somatic cell count /100) + 3 (Shook, 1993).

Table 4-10. Human-edible (HE) nutrients inputs and outputs of late-lactation cows fed a conventional lactation diet or by-product-based diets (Experiment 2).

	Diet[1]				
Item	CON2	ECO2	ECO2-AA	SEM	*P*-value[2]
Milk metabolizable energy (ME) output (MJ/d)	93.8[a]	67.7[b]	67.7[b]	4.24	< 0.01
Milk protein output (kg/d)	1.02[a]	0.703[b]	0.747[b]	0.045	< 0.01
Thrift scenario[3]					
HE ME input (MJ/d)	98.4	104	109	4.30	0.11
HE protein input (kg/d)	1.23[b]	1.59[a]	1.67[a]	0.06	< 0.01
Choice scenario[4]					
HE ME input (MJ/d)	99.1[a]	18.8[b]	19.7[b]	2.08	< 0.01
HE protein input (kg/d)	1.23[a]	0.075[b]	0.079[b]	0.024	< 0.01
Land use scenario[5]					
HE ME input (MJ/d)	137[a]	104[b]	109[b]	4.72	< 0.01
HE protein input (kg/d)	1.66	1.59	1.67	0.068	0.54
Output					
Milk metabolizable energy (ME; MJ/d)	93.8[a]	67.7[b]	67.7[b]	4.24	< 0.01
Milk protein (kg/d)	1.02[a]	0.703[b]	0.747[b]	0.045	< 0.01

[a-b]Least square means within rows with different superscripts differ significantly in Tukey's HSD ($P < 0.05$).

[1]Conventional lactation TMR (CON2), co-product feeds; TMR composed of ecological leftover feedstuff (ECO2); and ECO2 with top-dressed rumen-protected amino acids [ECO2-AA; 77 g/d AjiPro-L (Ajinomoto, Chicago, IL) and 45 g/d MetaSmart (Adisseo, Antony, France)].
[2]*P*-values associated with treatment effect.
[3]Hominy feed and wheat middlings were considered to be foods for human consumption.

[4]Hominy feed and wheat middlings were not considered to be foods for human consumption.

[5]Land used for alfalfa production could be used to grow corn and soybeans for direct human consumption.

Table 4-11. Human-edible (HE) digestible essential amino acid recovery (g output/ g input) in milk of mid-lactation cows fed a conventional lactation diet or by-product-based diets (Experiment 2, Choice Scenario).

	Diet[1]			SEM	*P*-value
Amino acid	CON2	ECO2	ECO2-AA		
Arg	0.53[b]	87.7[a]	81.4[a]	7.47	< 0.01
His	1.16[b]	161[a]	150[a]	13.8	< 0.01
Ile	1.36[b]	72.3[a]	67.1[a]	6.17	< 0.01
Leu	1.14[b]	86.1[a]	79.9[a]	7.34	< 0.01
Lys	1.55[b]	209[a]	194[a]	17.8	< 0.01
Met	1.57[b]	64.4[a]	59.7[a]	5.50	< 0.01
Phe	1.07[b]	88.4[a]	82.1[a]	7.54	< 0.01
Thr	1.36[b]	50.8[a]	47.2[a]	4.34	< 0.01
Trp	1.24[b]	78.2[a]	72.5[a]	6.67	< 0.01
Val	1.37[b]	29.5[a]	27.3[a]	2.52	< 0.01
Cys	0.40[b]	8.84[a]	8.21[a]	0.76	< 0.01
Tyr	1.26[b]	66.6[a]	61.8[a]	5.68	< 0.01

[1]Conventional lactation TMR (CON2), containing 25.7% co-product feeds; TMR composed of ecological leftover feedstuff (ECO2); and ECO2 with top-dressed rumen-protected amino acids [ECO2-AA; 77 g/d AjiPro-L (Ajinomoto, Chicago, IL) and 45 g/d MetaSmart (Adisseo, Antony, France)].

Where a recovery value of 1 would indicate that for every gram of HE digestible amino acid the cow consumes, she would produce 1 g HE digestible amino acid in milk.

Figure 4-1. Human-edible (HE) metabolizable energy (left panels) and protein (right panels) conversion rate of mid-lactation cows fed a conventional lactation diet or a co-product-based diet (Experiment 1) in a thrift scenario (A-B), a choice scenario (C-D), or a a co-product-based diet (Experiment 1) in a thrift scenario (A-B), a choice scenario (C-D), or a land use scenario (E-F). A recovery value of 1 would indicate that for every HE unit (either MJ or protein) the cow consumes, she would produce 1 HE unit in milk. Thrift scenario considered that hominy feed and wheat middlings were edible by humans, whereas choice scenario considered that hominy feed and wheat middlings were not edible by humans. Land use scenario calculations were made based on thrift scenario with additional consideration that the land used for alfalfa production could have been used to grow corn and soybeans for direct human consumption. Treatments: conventional lactation TMR (CON1), containing 31% by-product feeds; and TMR composed of ecological leftover feedstuff (ECO1), containing 95% by-product feeds. Letters above columns describe treatment by parity differences ($P < 0.05$; Tukey's Honest Significant Difference test). Error bars are SE.

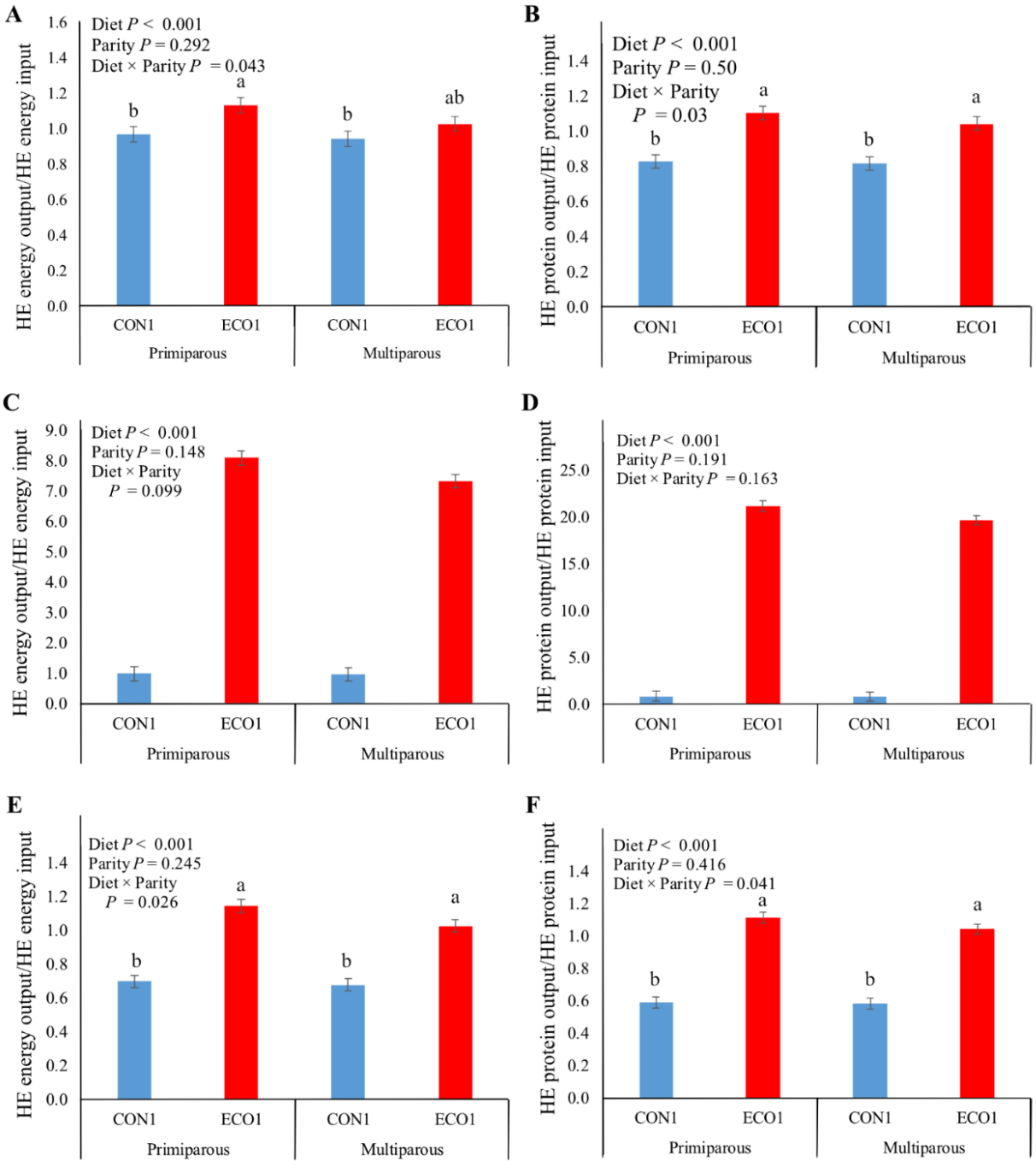
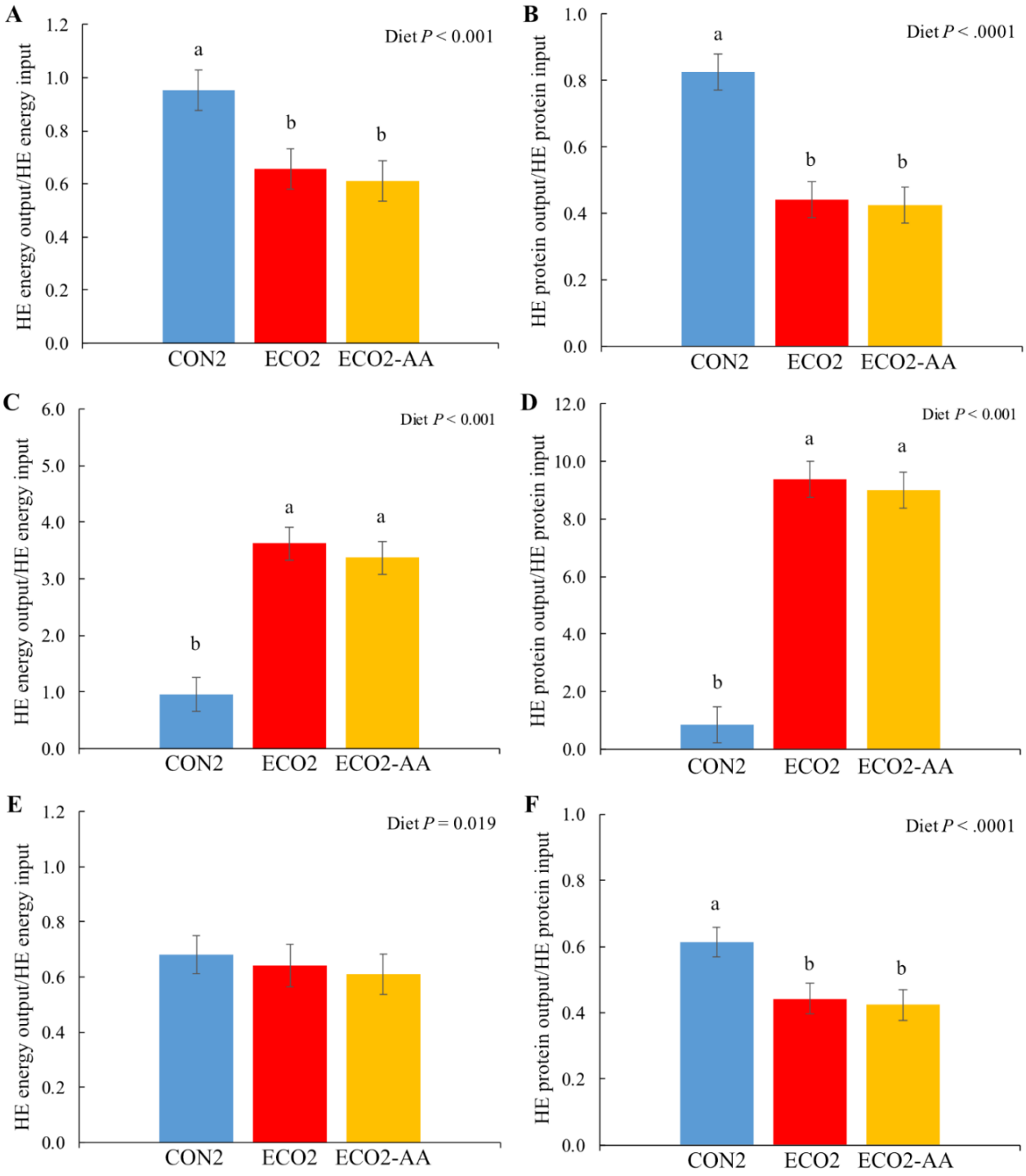
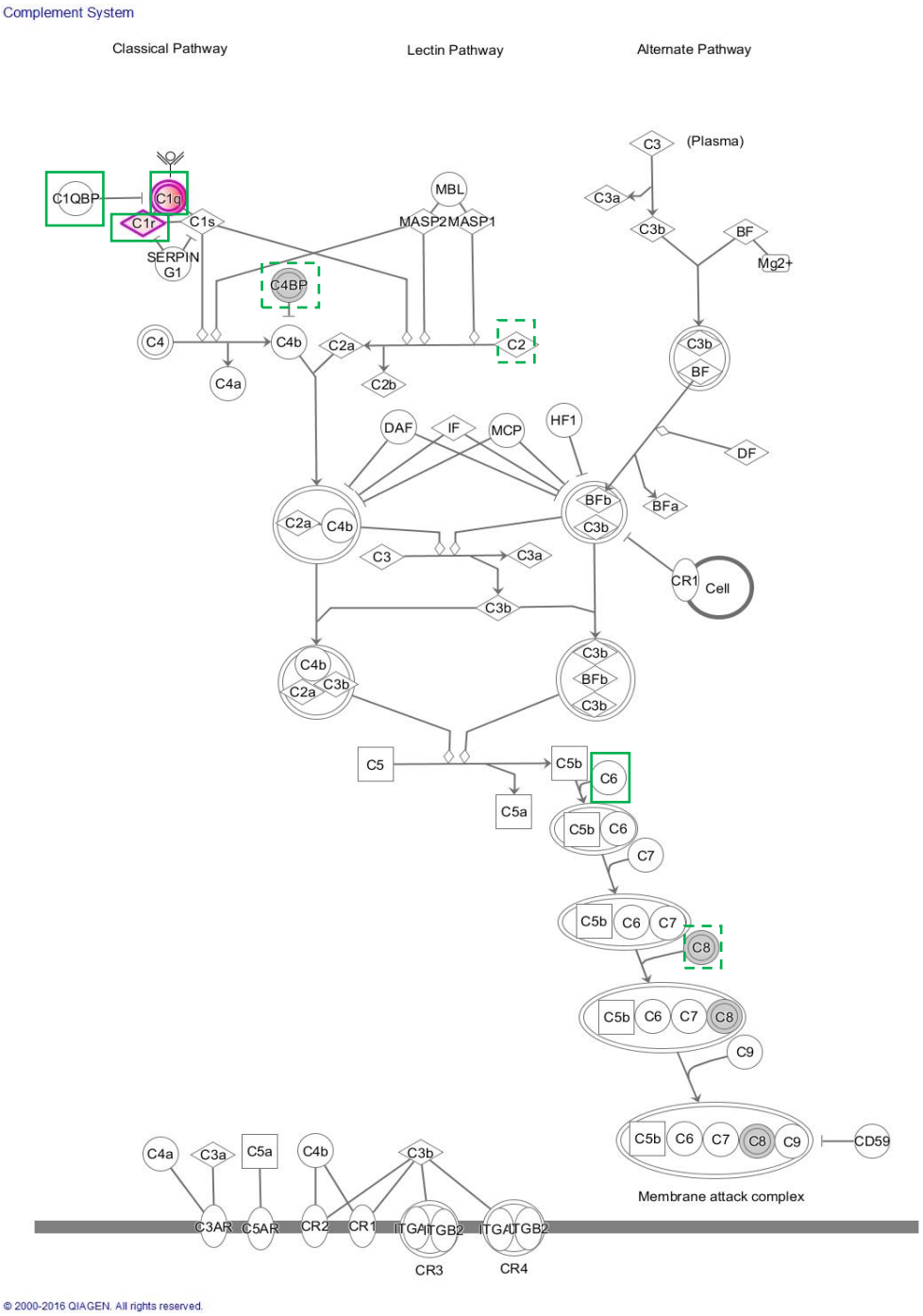

A
Diet P < 0.001
Parity P = 0.292
Diet × Parity P = 0.043
HE energy output/HE energy input
b
a
b
ab
B
Diet P < 0.001
Parity P = 0.50
Diet × Parity P = 0.03
HE protein output/HE protein input
b
a
b
a
C
Diet P < 0.001
Parity P = 0.148
Diet × Parity P = 0.099
HE energy output/HE energy input
D
Diet P < 0.001
Parity P = 0.191
Diet × Parity P = 0.163
HE protein output/HE protein input
E
Diet P < 0.001
Parity P = 0.245
Diet × Parity P = 0.026
HE energy output/HE energy input
b
a
b
a
F
Diet P < 0.001
Parity P = 0.416
Diet × Parity P = 0.041
HE protein output/HE protein input
b
a
b
a
CON1
ECO1
CON1
ECO1
Primiparous
Multiparous

Figure 4-2. Human-edible (HE) metabolizable energy (top panels) and protein (bottom panels) conversion rate of mid-lactation cows fed a conventional lactation diet or a co-product-based diet (Experiment 2) in a thrift scenario (A-B), in a choice scenario (C-D), or in a land use scenario (E-F), where a recovery value of 1 would indicate that for every HE unit (either MJ ME or protein) the cow consumes, she would produce 1 HE unit in milk. Thrift scenario considered that hominy feed and wheat middlings were edible by humans, whereas choice scenario considered that hominy feed and wheat middlings were not edible by humans. Land use scenario calculations were made based on thrift scenario with additional consideration that the land used for alfalfa production could be used to grow corn and soybeans for direct human consumption. Conventional lactation TMR (CON2), co-product feeds; TMR composed of ecological leftover feedstuff (ECO2); and ECO2 with top-dressed rumen-protected amino acids [ECO2-AA; 77 g/d AjiPro-L (Ajinomoto, Chicago, IL) and 45 g/d MetaSmart (Adisseo, Antony, France)]. ME stands for metabolizable energy. Letters above columns describe treatment differences ($P < 0.05$; Tukey's Honest Significant Difference test). Error bars are SE.


A
HE energy output/HE energy input
Diet P < 0.001
a
b
b
CON2
ECO2
ECO2-AA
B
HE protein output/HE protein input
Diet P < .0001
a
b
b
CON2
ECO2
ECO2-AA
C
HE energy output/HE energy input
Diet P < 0.001
b
a
a
CON2
ECO2
ECO2-AA
D
HE protein output/HE protein input
Diet P < 0.001
b
a
a
CON2
ECO2
ECO2-AA
E
HE energy output/HE energy input
Diet P = 0.019
CON2
ECO2
ECO2-AA
F
HE protein output/HE protein input
Diet P < .0001
a
b
b
CON2
ECO2
ECO2-AA

Appendix A - Complement system pathways



Complement system pathways and serine proteases involved in the membrane attack complex (Qiagen's Ingenuity® Pathway Analysis (IPA®, Qiagen Redwood City, CA). Proteins that were

differently abundant (FC > 1.5 and $P < 0.05$) in adipose tissue of cows treated with sodium salicylate compared to control are highlighted with green solid-line squares. Proteins that did not reach the 1.5 FC cut-off but had different abundance ($P < 0.05$) in adipose tissue of cows treated with sodium salicylate are highlighted with dashed-line green squares.