EFFECTS OF HOPS β-ACID EXTRACT (*HUMULUS LUPULUS* L.) ON CATTLE PERFORMANCE AND FERMENTATION BY RUMINAL MICROBES

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A.S., Pratt Community College, 2011
B.S., Emporia State University, 2013

A THESIS

Submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Animal Sciences and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2015

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Abstract

Hops β-acid extract was fed to 80 heifers (389 ± 23.6 kg initial BW) to assess impact on feedlot performance and ruminal fermentation. Heifers were randomly assigned to individual pens and fed once daily for 147 d. Treatments were a control (no additive); 33 mg monensin (Elanco Animal Health, Greenfield, IN)/kg diet DM; and 10, 25, 50 mg β-acid extract of hops (DSM Nutritional Products, France)/kg diet DM. Ruminal fluid was collected on d 44 and 86 by rumenocentesis for analyses of VFA, lactate, and NH_3 concentrations. Cattle were harvested at a commercial abattoir on d 147. Hops β-acids decreased propionate ($P = 0.01$) concentrations and increased caproate ($P = 0.05$), A:P ($P = 0.04$), and ammonia concentrations ($P = 0.03$) compared to monensin. Growth performance of heifers fed β-acid or monensin was not different than that of heifers fed the control diet. Additionally, two *in vitro* studies were conducted to evaluate effects of hops β-acid extract on starch fermentation by mixed microbial populations from the bovine rumen. In trial 1, 2 treatments were assigned in triplicate to fermentation bottle, fitted with Ankom^{RF1} Gas Production System modules (Ankom^{RF} Technology, Macedon, NY) using starch as substrate (Difco Soluble Starch; Dickinson and Company, Sparks, MD) and either 0 or 33 mg hops β-acid extract (10.99% active hops beadlet; DSM Nutritional Products, France)/kg substrate. Gas production was measured over 30 h. Terminal pH, IVDMD, and VFA and lactate were measured after 30 h of fermentation. Gas production increased in response to β-acid ($P \leq 0.05$). Terminal pH, IVDMD, VFA, and lactate were unaffected by addition of β-acid extract ($P \leq 0.05$). In trial 2, pH, VFA concentrations, and IVDMD were measured at 6-h intervals during a 30-h incubation period using 36 fermentation tubes. There was no effect of hops β-acid on in vitro fermentation ($P > 0.05$). In conclusion, under the conditions of these experiments, hops β-

acid extracts hops had little impact on feedlot performance, though there are indications of an impact on ruminal fermentation.

Key words: *β*-acid, hops, feedlot, *in vitro*

Table of Contents

List of Figures vii
List of Tables viii
Chapter 1 - Hops *β*-acid extract: A review 1
Abstract 1
Introduction 2
Literature review of ionophores 5
History of origin 5
Classification of ionophores 6
Neutral ionophores 7
Carboxylic ionophores 7
Channel-forming ionophores 8
Structure and function 8
Application in animal production systems 10
Effects on fermentation by ruminal microbes 11
Post-ruminal effects 13
Literature review of whole hops 15
Spent hops 17
Hops *β*-acid extract 19
History of origin 19
Formation and chemical composition 20
Preparation and extraction 20
Metabolism 22
Oxidation of hops 23
Function and antimicrobial effects 24
Effects on ruminal fermentation 28
Conclusion 29
References 31
Chapter 2 - Effects of hops *β*-acid extract (*Humulus lupulus* L.) on fermentation by ruminal microbes and performance of finishing heifers 48

Introduction 49
Materials and methods 50
In vivo 50
Animals and Diets 50
Sampling Procedures 51
Analyses of Ruminal VFA and Lactate 52
Analysis of Ruminal Ammonia 53
Statistical Analyses 54
In vitro 54
Experiment 1 54
Experiment 2 57
Results 58
In vivo 58
In vitro 59
Experiment 1 59
Experiment 2 59
Discussion 60
In vivo 60
In vitro 63
Experiment 1 63
Experiment 2 64
Acknowledgments 67
References 68

List of Figures

Figure 1.1 Examples of ionophores. 46
Figure 1.2 Chemical structures of α-acids and β-acids. 47
Figure 2.1 *In vitro* experiment 1: 30-h gas production (mL) during incubation with bovine ruminal fluid and starch with 0 or 33 hops β-acid extract (DSM Nutritional Products, France)/kg substrate. 78

List of Tables

Table 1.1 Examples of ionophore selectivity. 45
Table 2.1 Basal diet (DM basis) of heifers fed 0, 10, 25, or 50 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg diet DM or 33 mg monensin (Elanco Animal Health, Greenfield, IN)/kg diet DM. 72
Table 2.2 Average *β*-acid content (mg/kg) of diets consisting 0 (Control), 10 (Beta 10), 25 (Beta 25), 50 (Beta 50) mg *β*-acid extract (DSM Nutritional Products, France)/kg diet DM, or 33 mg monensin (Elanco Animal Health, Greenfield, IN)/kg diet DM (MON). 73
Table 2.3 Performance of heifers fed 0 (Control), 10 (Beta 10), 25 (Beta 25), 50 (Beta 50) mg *β*-acid extract (DSM Nutritional Products, France)/kg diet DM, or 33 mg monensin (Elanco Animal Health, Greenfield, IN)/kg diet DM (MON). 74
Table 2.4 Repeated measures analysis for feedlot performance of heifers fed 0 (Control), 10 (Beta 10), 25 (Beta 25), 50 (Beta 50) mg *β*-acid extract (DSM Nutritional Products, France)/kg diet DM, or 33 mg monensin (Elanco Animal Health, Greenfield, IN)/kg diet DM (MON). 75
Table 2.5 *In vivo* ruminal pH, VFA, lactate, and ammonia concentrations (mM) in heifers 24-h after feed delivery of diets containing 0 (Control), 10 (Beta 10), 25 (Beta 25), 50 (Beta 50) mg *β*-acid extract (DSM Nutritional Products, France)/kg diet DM, or 33 mg monensin (Elanco Animal Health, Greenfield, IN)/kg diet DM (MON). 76
Table 2.6 Composition of diet fed to cannulated steer used for *in vitro* cultures in experiment 1 and 2, DM basis. 77
Table 2.7 *In vitro* experiment 1: Terminal pH, IVDMD (%), VFA (mM), lactic acid (mM), and acetate:propionate following 30-h incubation of bovine ruminal fluid with starch and 0 or 33 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg substrate. 80
Table 2.8 *In vitro* experiment 2: pH at 0, 6, 12, 18, 24, and 30 h during incubation of bovine ruminal fluid with starch and 0 or 33 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg substrate. 81
Table 2.9 *In vitro* experiment 2: IVDMD (%) during 30-h incubation with bovine ruminal fluid with starch and 0 or 33 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg substrate. 82

Table 2.10 *In vitro* experiment 2: Acetic acid concentrations (mM) during 30-h incubation of bovine ruminal fluid with starch and 0 or 33 mg β-acid extract of hops (DSM Nutritional Products, France)/kg substrate. 83
Table 2.11 *In vitro* experiment 2: Propionic acid concentrations (mM) during 30-h incubation of bovine ruminal fluid with starch and 0 or 33 mg β-acid extract of hops (DSM Nutritional Products, France)/kg substrate. 84
Table 2.12 *In vitro* experiment 2: Butyric acid concentrations (mM) during 30-h incubation of bovine ruminal fluid with starch and 0 or 33 mg β-acid extract of hops (DSM Nutritional Products, France)/kg substrate. 85
Table 2.13 *In vitro* experiment 2: Total VFA concentrations (mM) during 30-h incubation of bovine ruminal fluid with starch and 0 or 33 mg β-acid extract of hops (DSM Nutritional Products, France)/kg substrate. 86
Table 2.14 *In vitro* experiment 2: Lactic acid concentrations (mM) during 30-h incubation of bovine ruminal fluid with starch and 0 or 33 mg β-acid extract of hops (DSM Nutritional Products, France)/kg substrate. 87
Table 2.15 *In vitro* experiment 2: Acetate:propionate during 30-h incubation of bovine ruminal fluid with starch and 0 or 33 mg β-acid extract of hops (DSM Nutritional Products, France)/kg substrate. 88

Chapter 1 - Hops *β*-acid extract: A review

Abstract

Hops (*Humulus lupulus* L.) have been utilized in beer manufacturing for centuries due to their antimicrobial and preservative properties. As our understanding of its many properties improves, the potential applications for hops and hops extracts is expanding. Hops have been observed to possess many useful characteristics, exhibiting anti-microbial, anti-cancer, anti-inflammatory, anti-metabolic disorder, and anti-oxidative properties. Hops are composed of numerous compounds that contribute to these effects. After harvest, compounds are extracted for use in a variety of manufacturing processes. Hop *β*-acids are one of the classes of compounds extracted from hops, and have chemical structure similar to ionophores. Ionophores are well known polyether antibiotics that primarily inhibit $Gram^+$ bacteria by altering the transmembrane ion gradient of the bacterial cell membrane. This similarity has sparked interest to elucidate whether these *β*-acid extracts have the potential to compete with ionophores as a natural-feed additive. Recently, a large amount of work has been done *in vitro* with mixed results. However, a very limited amount of work to confirm these observations has been performed with live animals. Researchers should be aware of multiple variables that can affect the outcomes to be observed from *β*-acid supplementation in animals such as age, species, diet, and type of extract. Hops *β*-acids have been shown to possess antimicrobial properties that have potential to alter ruminal fermentation and improve performance in production animals. These effects are reviewed herein and compared to similar effects observed for ionophores.

Keywords: hops, *β*-acid, ionophore, ruminant

Introduction

The female inflorescences of hops (*Humulus lupulus* L.*)* have played an integral role in beer manufacturing in many parts of the world due to their antimicrobial properties and enhancement of end-product stability. Seeded hops are grown in parts of America, Australia, and Great Britain, of which the seeds are responsible for between 10 and 30% of plant weight; remaining areas of the world cultivate seedless hops (Sharpe and Laws, 1981). Worldwide production of hops recently reached approximately 110,000 tons (Pavlovič, 2012) and use thereof has shown hops to possess antimicrobial properties against multiple species of bacteria (Batchvarov and Marinova, 2001; Bhattacharya et al., 2003; Siragusa et al., 2008).

Hops have been added to animal diets in the whole form, as spent hops, or as specific extracts from the hop plant. Utilization of hops as a feed additive has been limited to poultry, swine, and ruminants, with little work done in horses. Whole hops, deemed the fruiting body, contain yellow lupulin glands within cones that contain condensed tannins and secondary metabolites such as phenolic acids, flavonoid glycosides, resin, and essential oil compounds that are responsible for providing bitterness and aroma (Bhattacharya et. al., 2003; Cleemput et al., 2009). These compounds all contribute to the antimicrobial activity of hops (Schmidt et al., 2006; Siragusa et al., 2008), with the weak acids (α- and β-) from the resin contributing the most, but β-acids playing a larger role than α-acids (Hough et al., 1957; Michener and Anderson, 1949). Therefore, studies including whole hops should take into account that results represent a combined effect from all present compounds. For instance, when supplementing whole hops at a rate of 227 mg/kg, broilers witnessed improved rate of growth and feed utilization (Cornelison et al., 2006). The resin is further used as a preservative in beer solely for its antimicrobial attributes, especially against lactic-acid bacteria; which may provide efficacy when introduced in ruminant

diets (Bhattacharya et. al., 2003; Lavrenčič et al., 2014). The weak acids are also responsible for bitterness and aroma associated with beer (Bhattacharya et al., 2003). Alpha and β-acids of hops are also referred to as humulones and lupulones, respectively, and both consist of three predominant isomers: co-, n-, and ad-, of which their presence is dependent on the side chain chemical structure (Cattoor et. al., 2013). Hop extracts are widely known to be prone to oxidation which could potentially have an effect on the efficacy and activity of hops and hop derivatives (Taniguchi et al., 2013).

The most preferred method for modern extraction of hop acids is achieved via liquid or supercritical carbon dioxide (De Keukeleire et al., 2003). This extraction is typically performed to separate and collect the weak acids. Certain hop varieties are known to possess up to 19% (w/w) α-acids (De Keukeleire et al. 2003). Once extracted from the lupulin glands, α-acids are known to possess a variety of biological functions such as anti-cancer, anti-inflammatory, anti-metabolic disorder, and anti-oxidative properties (De Keukeleire et al., 2003; Taniguchi et al., 2014). Upon extraction, α-acids are used for flavoring in beer, leaving remnants abundant in β-acids which are similar in chemical structure to ionophores (Uwituze et. al., 2010).

Ionophores were deemed a new class of antibiotic in the 1970's when introduced by the Food and Drug Administration (Russell and Houlihan, 2003). These are small, lipid-soluble molecules that catalyze rapid transfer of ions down the transmembrane concentration gradient via antiporters, thus squandering ion-motive forces and causing loss of bacterial cell integrity that ultimately leads to cell death (Flythe, 2009). Ionophores are utilized to improve feed efficiency as much as 10 percent, in addition to decreasing the presence of digestive disturbances that often contribute to morbidity and mortality in ruminants (Russell and Houlihan, 2003; Uwituze et. al., 2010). Commonalities of ionophore-treated ruminants are decreased methane emissions,

decreased ammonia and lactate concentrations, decreased ruminal acetate to propionate ratio, modulation of feed intake, and mitigation of pasture legume bloat (Flythe, 2009; Russell and Houlihan, 2003). Routine use of ionophores provides improvements in animal production by targeting ruminal $Gram^+$ bacteria and ciliates, thus altering microbial fermentation to obtain more desirable end products (Flythe, 2009; Narvaez et al., 2013b). Although diet dependent, ionophores have been observed to inhibit the activity of hyper ammonia-producing bacteria (HAB), thereby mitigating the wasteful process of amino acid deamination that creates an accumulation of ammonia in the rumen (Flythe, 2009; Russell and Houlihan, 2003). Similar to ionophores, the mode of action of *β*-acid extract of hops concerns energy flow through living systems and cellular processes (Flythe, 2009). The growing trend to replace in-feed antibiotics in livestock production makes this plant derivative an attractive and potential alternative.

Furthermore, resistance to antibiotics by microbial populations is an increasing threat to food safety, animal health, and human health. This has been most notable in foodborne illnesses and is of outstanding concern with continued use of antibiotics in animal production. Specifically, due to their origins in food production this has been a particular problem with $Gram^-$ bacteria, such as *Salmonella* and *Escherichia coli* O157:H7 (Edrington et al., 2003). Additionally, strains of ruminal $Gram^-$ bacteria have shown characteristics of adaptation (Newbold et al., 1993), and in contrast, some strains of enterococci have not (Nisbet et al., 2008).

Beta-acids have been researched more so *in vitro* than *in vivo*, which warrants investigation to further our knowledge of their effects on live animal production. This paper reviews the literature, published up to 2014, pertaining to the efficacy of *β*-acid extracts of hops and ways they compare to ionophores.

Literature review of ionophores

The term ionophore was first adopted in 1834 by the English physicist and chemist, Michael Faraday. The term was later coined by Berton Pressman in 1964 to properly represent this ion transport mechanism. An ionophore, commonly referred to as a polyether antibiotic, can be defined as a lipophilic molecule, usually synthesized by microorganisms, that facilitates the transport of monovalent and divalent cations across biological membranes. To be classified as an antibiotic a substance must inhibit the growth of, or destroy, microorganisms. Ionophores achieve this by "shielding" the ion, allowing it to protrude through hydrophobic biological membranes. There are over 120 naturally occurring ionophores that have been discovered with the main commercial purpose to control coccidiosis and promote rate of gain in livestock (Rutkowski and Brzezinski, 2013). Ionophores typically are prepared by isolation and extraction from fermentation broths of their respective microbiological strains; however, they can also be produced synthetically, retaining properties similar to those of the natural products.

History of origin

The first discovery of polyether antibiotics was in 1951 and described as nigericin and lasalocid A; however, they were not known to be polyether antibiotics at the time (Berger et al., 1951; Harned et al., 1951). These compounds were observed to be effective against $Gram^+$ bacteria and mycobacteria; however, $Gram^-$ bacteria were resistant due to the presence of an outer membrane not pervaded by hydrophobic compounds (Chapman et al., 2010). Years later in 1967, the chemical structure of monensic acid provided the first detailed description of a polyether antibiotic. Monensin was synthesized by *Streptomyces cinnamonensis* (Agtarap et al., 1967), and the mode of action was provided by The Institute for Enzyme Research, University of Wisconsin-Madison (Estrada et al., 1968). During the same time period, Dutch researchers were

able to demonstrate that chloroform and carbon tetrachloride were effective in decreasing ruminal methane production *in vitro* (Russell, 1997). Furthermore, additional work proposed that methane production could be completely impeded in sheep with minute amounts of chloroform; and similar to ionophores, chlorinated hydrocarbons decreased ruminal acetate, increased propionate, and energy retention (Russell, 1997). Contrary to having profound effects on ruminal fermentation, alterations from these compounds led to increased hydrogen production, making their use less attractive (Russell, 1997).

The ionophore monensin was first introduced as a coccidiostat for poultry in 1971 (Chapman et al., 2010). In 1976 it became the first ionophore approved for use in ruminants to improve feed efficiency and in 1978 to increase rate of gain in pasture cattle (Chapman et al., 2010; Corah, 1991). Monensin was effective in reversing the poor performance and decreased productivity that coccidiosis created within poultry and other farm animals (Chapman et al., 2010). The pathology of monensin is uncommon when compared to other antibiotics for poultry in that it specifically targets the parasite, *Eimeria* (Chapman et al., 2010). Although monensin plays a large role as an ionophore, this category of antibiotics consists of numerous compounds that play many roles in animal production.

Classification of ionophores

Ionophores have been classified in thorough reviews published by Pressman (1976) and Westley (1982); therefore a brief discussion will be provided herein. Ionophores function in different ways and can be classified as one of two types: mobile-ion carriers and channel-forming ionophores, also known as quasi-ionophores (Pressman, 1976; Westley, 1982). Mobile-ion carrier dichotomy further includes neutral and carboxylic ionophores that function to physically shield ions to encourage their movement across plasma membranes (Pressman, 1976).

Mobile carrier turnover numbers are controlled by the rate at which they diffuse across membranes (Stein, 1968). Channel-forming ionophores function differently in that a favorable membrane potential must be present for pores to be formed by the ionophore to concede the flux of ions (Pressman, 1976). In contrast to mobile carriers, high rates of channel-forming carrier turnover is indicative of their function to open ion conduction channels (Pressman, 1976).

Neutral ionophores

Ionophores that fall under this category are free of ionizable or active functional groups within the compound. Functionality of this class of ionophores is attributed to a bend in the backbone of their structure, allowing them to conform six ester carbonyls into a sphere formation (Pressman, 1976). As with valinomycin and analogues thereof, selectivity is exhibited by the size of the ring structure and its specificity to fit cations of certain size during complex formation. With the aid of X-ray crystallography and NMR, it has been possible to observe that this "cage" formation is stable and opens only upon interaction with the membrane to release ions (Haynes and Kowalsky, 1969). Smaller ring sizes do not possess this capability and function in a more planar manner, as is true with enniatins (Dobler et al., 1969; Ovchinnikov et al., 1969). In addition to neutral ionophore classes listed above, further subcategories of this class include: macrotetralide nactins, synthetic polyethers, and antamanide, which is derived from the mushroom *Amanita phalloides* (Pressman, 1976).

Carboxylic ionophores

This class of ionophores is characterized by oxygenated heterocyclic rings with a terminal carboxyl group (Pressman, 1976). Ionophores in this group are special in that after forming an ion complex they are capable of carrying the ion as a zwitterion (Celis et al., 1974; Pressman, 1973). Nonetheless, carboxylic ionophores form ring conformations through

intramolecular interactions and the carboxyl group may or may not play a role in forming a cation ligand complex, which is dependent on compound structure (Pressman, 1976). In essence, carboxylic ionophores act as exchange diffusion carriers, which does not result in net transport of cation-for-proton exchange across the membrane (Pressman, 1976). Some members of this class exhibit selective transport of divalent rather than monovalent cations. Examples of ionophores in this group include: nigericin (Harned et al., 1951), grisoryxin (Alleaume and Hickel, 1970), monensin (Steinrauf et al., 1968), X-537A (Johnson et al., 1970), and A23187 (Pfeiffer et al., 1974; Reed et al., 1972)

Channel-forming ionophores

This class of ionophores is dependent on the magnitude of membrane potential difference to create surges of current across the membrane to form channels (Hladky and Haydon, 1970). Extensive research suggests that mobile carriers and channel-forming ionophores have similarities; however, the channel-forming mechanism does work in cooperation with excitable-membrane conductivity to produce ion-transport channels (Hille, 1971; Urry et al., 1971). Members of this class include but are not limited to: gramicidin (Neubert and Lehninger, 1962), monazomycin (McLaughlin and Eisenberg, 1975), alamethacin (McLaughlin and Eisenberg, 1975), and polyene antibiotics (Finkelstein and Holz, 1973).

These categories can be further broken down to the ion selectivity of ionophores, which is a function of two measures: energy for desolvation of the ion and ligand energy from complexation (Pressman, 1976). Examples are presented in Table 1.

Structure and function

The mode of action of ionophores has been extensively presented in multiple reviews (Bergen and Bates, 1984; Corah, 1991; Goodrich et al., 1984; Schelling, 1984; Sprott et al.,

1988). An abbreviated version will be provided herein as an overview of the pathology and effects of ionophores. The molecular structure of polyether ionophores greatly contributes to their biological activity. Polyether antibiotics are referred to as complex molecules that possess antimicrobial properties and are composed of multiple cyclic ether groups (Figure 1; Westley, 1982). Due to their chemical makeup, ionophores are capable of permeating lipid membranes and forming complexes with metal cations. As a result, ionophores are effective at penetrating the peptidoglycan layer of ruminal $Gram^+$ bacteria, however $Gram^-$ bacteria in the rumen may initially be sensitive but have been shown to adapt (Russell, 1997; Russell and Houlihan, 2003)). In conjunction, Newbold et al. (1993) observed that $Gram^-$ bacteria may adapt to ionophores through a mechanism that decreases the porosity of the outer cell membrane. Unlike antibiotic resistance, the theory that ionophore resistance is not rapidly transferred between bacteria is greatly supported by research, as was found by Aarestrup et al. (1998) in bacteria derived from Danish swine, cattle, and poultry administered ionophores. Additionally, one method utilized by bacteria to transfer antibiotic resistance is through plasmid-encrypted DNA. Mathers et al. (2004) observed different ionophores, antibiotics, and combinations thereof, to effectively inhibit the transfer of resistance to multiple antibiotics by the plasmid pBR325 *in vitro*. By incorporating typical concentrations used in animal production, the results from this study concur that the ionophores and antibiotics used have potential to alter cell envelope-associated DNA uptake channels or interfere with transformation and cell replication mechanisms necessary for transfer (Mathers et al. 2004).

Observed benefits from feeding ionophores are improved feed efficiency in finishing cattle, modulation of feed intake, decreased incidence of ruminal lactic acidosis and feedlot bloat, increased rate of gain in cattle on pasture, control of coccidiosis, decreased 3-methylindole

production in the rumen (Honeyfield et al., 1985; Nocerini et al., 1985), and increased efficiency of milk production (Corah, 1991; Lowe et al., 1991; Lynch et al., 1990). Transport of cations by polyether ionophores is achieved by exchanging protons and cations in an electro-neutral process. Furthermore, the ionophore in its anion state ($I\text{-}COO^-$) complexes with a metal cation or proton (H^+) to create a neutral salt ($I\text{-}COO^-M^+$) or an uncharged acidic form (I-COOH), and only these forms can permeate the hydrophobic cell membrane. Upon invading the cell membrane, ionophores create a flux of ions and stimulation of membrane associated ATP-ase pumps that function as ATP-coupled carrier systems to pump ions, such as (Na^+-K^+) (Chapman et al., 2010). This alters the ion concentration gradient and osmolality of the cell, ultimately leading to cell death. However, size plays a factor in characterizing the affinity for a cation to fit the cavity of an ionophore which contributes to the precise selectivity of ionophores (Lindoy, 1996; Pressman, 1985; Rutkowski and Brzezinski, 2013).

Application in animal production systems

Ionophores have been implemented in many facets of animal production. Monensin was first used as a coccidiostat in the poultry industry (Chapman et al., 2010). Shortly thereafter, a select number of ionophores were approved for use in ruminants and used to increase rate of gain and feed efficiency (Duffield and Bagg, 2000; Kennelly et al., 1998). In addition to the poultry industry, ionophores play a role in controlling coccidiosis in beef cattle and goats by targeting *Eimeria* spp. (Goodrich et al., 1984). Monensin and lasalocid are approved for use in ruminants and are among the most commonly used feed additives. Monensin is a sodium-proton anti-porter, but can also patrol potassium ions (Russell, 1997). Lasalocid, produced by *Streptomyces lasaliensis*, was approved for use in ruminant feedlot diets in 1982, and has effects similar to monensin, minus improvements in milk production (Erasmus et al., 1999). Laidlomycin was

introduced as a feed additive in 1994 and is derived from fermentation processes by *Streptomyces eurocidicus*. At high concentrations this antibiotic has shown efficacy against $Gram^+$ bacteria, and also possesses some activity against several *Mycoplasmas* (Rutkowski and Brzezinski, 2013). Laidlomycin generally serves to improve feed efficiency and rate of gain for cattle in confinement. Ionophores have also been implemented by the dairy industry with major benefits for lactating cows being improved efficiency of nitrogen and energy utilization (Duffield and Bagg, 2000; Ipharragerre and Clark, 2003; Kennelly et al., 1998).

Up until June 1996, monensin premix was not approved for use in Canada, but with introduction of a controlled-release capsule shortly thereafter, monensin was supplemented to prevent subclinical ketosis in lactating dairy cattle (Duffield and Bagg, 2000). With that being said, it has been confirmed that monensin is not present in milk or tissue of Holstein dairy cattle when fed up to 1274 mg/animal daily (Wilkinson et al., 1997). When comparing monensin and lasalocid administered to lactating dairy cows at 24 mg/kg of diet DM, Martineau et al. (2007) observed monensin to increase plasma urea-N, milk urea-N, excretions of urinary-N and total N, while decreasing N retention. These results suggest that different ionophores may function through different post-absorptive metabolic mechanisms in dairy cattle.

Although used to a lesser extent in animal production, salinomycin plays a role in controlling coccidiosis by affecting the merozoite stage of *Eimeria* spp. when used in domestic fowl (Mehlhorn et al., 1983). A few other polyether ionophores that are or have been used in beef cattle production are narasin and maduramycin (Rutkowsi and Brzezinski, 2013).

Effects on fermentation by ruminal microbes

Ionophores effectively inhibit certain ruminal bacteria and alter ruminal fermentation (Bergen and Bates, 1984; Nagaraja et al., 1982). As a result, more hydrogen is used to produce

propionate and succinate, thus favoring decreased production of acetate, butyrate, lactic acid, methane, hydrogen, and ammonia, which tends to be more characteristic of ruminal bacteria that are resistant to ionophores (Chen and Wolin, 1979; Kennelly et al., 1998). Increases in dry matter digestibility and hydrogen retention shifted towards propionic acid production result in improved metabolizable energy value of feedstuffs (Corah, 1991). When comparing effects of the most common ionophores used in beef production (lasalocid, laidlomycin propionate, and monensin) and corresponding carrier on *in vitro* fermentation of a high-concentrate substrate, Ponce et al. (2012) observed no differences in gas and methane production. However, *in vitro* dry matter disappearance was greater for ionophores at one time point.

Examination of the effects on methane production *in vivo* have revealed an adaptive mechanism by ciliate protozoa where populations are initially inhibited, but subsequently adapt within weeks (Guan et al., 2006). Additionally, molar proportions of acetate, acetate:propionate (A:P), and total volatile fatty acids decrease and propionate increases for ionophores, therefore supporting previous research (Ponce et al., 2012). In ruminal studies ionophores have been observed to exert effects that decrease ruminal ammonia, thus allowing for more protein available post-ruminally (Bergen and Bates, 1984; Corah, 1991). This could be demonstrated by an amino acid and protein-sparing effect caused by increased propionate production, effectively decreasing ruminal ammonia concentrations (Bergen and Bates, 1984). Essentially, this is due to a decrease in proteolytic, peptidolytic, and deaminase activities, which is characteristic of the remaining ionophore-resistant ruminal microflora (Corah, 1991). Newbold et al. (1990) speculated that this was due to be a synergistic effect resulting from inhibition of hyper-ammonia producing and $Gram^+$ bacteria, as well as adaptation by ionophore-resistant bacteria in the ecosystem. Spears and Harvey (1987) found ionophores to exert effects on mineral metabolism

such as apparent increases in sodium, magnesium, and phosphorus absorption; increases in magnesium and phosphorous retention; and alteration of soluble concentrations of specific minerals in ruminal fluid of steers fed finishing diets. These findings are in accordance with the definitive mode of action of ionophores and their effects on the flux of ions. Interesting observations by Harold and Knapp (1980) indicate that some proportion of monensin escapes metabolism in the gastrointestinal tracts of animals and can contribute to larvae mortality, and therefore a decrease in the fly population. Furthermore, monensin is not fed solely for this purpose but may possess the potential to be an added benefit.

Digestive disturbances, such as sub-acute or lactic acidosis, occur when cattle are abruptly transitioned from a high forage diet to a diet consisting of rapidly-fermentable carbohydrates (Elam, 1976). Subsequently, abnormal increases in L(+) and D(-) lactic acid are the primary culprit to these disturbances (Dunlop, 1972). Lasalocid and monensin are able to mitigate this process by inhibiting major lactate-producing ruminal bacteria (excluding *Selenomas*), although exhibiting no effect on lactate-fermenting ruminal bacteria (Nagaraja et al., 1982). This effectively decreases lactate production and improves utilization thereof. Supporting evidence by Nagaraja et al. (1982) demonstrated that in experimentally-induced acidosis, lasalocid and monensin effectively decreased populations of *Streptococcus bovis* and *Lactobacillus*, increased populations of lactate-utilizing bacteria, increased pH, and decreased ruminal lactate concentrations, thereby mitigating lactic acidemia.

Post-ruminal effects

Ionophores are useful for improving food animal production; however, at improper dosages they can have detrimental effects to animals, exhibiting greater toxicity than other antibiotics. Monensin metabolism occurs readily in aerobic environments via hydroxylation, O-

demethylation, and/or decarboxylation, which produces more than 50 compounds similar to the parent compound (Donoho et al., 1978; Donoho, 1984; Nebbia et al., 2001). Monogastrics are capable of metabolizing monensin more extensively than ruminants, and it is apparent that metabolic conversion is inversely proportional to the toxicity of the drug in broiler chicks, cattle, horses, pigs, and rats (Nebbia et al., 2001). Similarly, lasalocid is metabolized in cattle with 12% of liver residues containing the parent compound, compared to less than 10% of the monensin parent compound excreted in the feces of rats, chicken, and cattle (Donoho et al., 1978; Donoho et al, 1982; Weiss, 1990). Furthermore, the LD50 of lasalocid and monensin in adult rats (100 and 35 mg/kg, respectively) is substantially less than the LD50 for chlortetracycline or sulfamethazine (10,800 and 1,060 mg/kg, respectively; Union of Concerned Scientists, 2001). Additionally, toxicity is species dependent, with ionophores given at normal levels for chickens becoming highly toxic to horses and turkeys by expressing adverse effects on the heart, skeletal muscle, and liver (Dacasto et al., 1999; Matsuoka, 1976; Rollinson et al., 1987). The LD50 for monensin in horses is approximately 1.38 mg/kg of body weight (Matsuoka et al., 1996), whereas cattle and poultry can tolerate more than ten times this dose. This is believed to be due to the disruption of the sodium and potassium ion fluxes that control the contractility of the heart, leading to muscle degeneration and ultimately death in horses. However, it is still not understood why toxicity is much more aggressive in horses compared to other livestock. Monensin is excreted in the feces and is absent in the urine, suggesting it is metabolized by the liver (Blomme et al., 1999). In cattle, ionophore tolerance is mediated by two biological functions: liver enzymes that degrade parent compounds and recycling of ionophores back to the gut through bile (Russell and Houlihan, 2003). Perhaps these functions are not well mediated in horses, thus leading to more aggressive toxicosis at lower levels of monensin.

With an informative preview to ionophores, this review will now focus on the history of hops and the potential of its derivatives as feed additives in animal diets.

Literature review of whole hops

Hop or hops (*Humulus lupulus* L.) is a dioecious perennial that yields one crop per year, and has is a characteristically long-climbing vine with dark green leaves (Moreira et al, 2011). Hops are native to the Northern Hemisphere and belong to the *Cannabacea* family, which makes the plant related to hemp, marijuana, and hackberry (Koetter and Biendl, 2010). *Humulus,* originated from, *chemle,* a Slavic term later latinized and *lupulus* was inherited from the Latin word for wolf, *lupus* (Koetter and Biendl, 2010). According to Engels and John (2006), the common name for hops originated from the Anglo-Saxon word *hoppan* (to climb).

Hops typically grow from April to the beginning of July, and optimal growing conditions are a hot, dry temperate climate approximately 30 to 52 degrees North or South (Koetter and Biendl, 2010). These conditions can produce a daily growth rate of up to 30 cm and ultimately a height of 7 to 8 m (Koetter and Biendl, 2010). Within these degrees of latitude, areas that provide proper conditions and day length include Oregon, Washington, Germany, Great Britain, Poland, Czech Republic, China, Japan, Australia and New Zealand (Koetter and Biendl, 2010).

Prior to harvesting hops, cascade flowers, also called ‘cones’, begin to sprout at approximately three and a half months, with maturation occurring around four months. At harvest, hop plants are populated with an abundance of cones that house the resin and essential oils within lupulin glands. The most sought after ratio of α-acids:β-acids is 1:1; however, a majority of harvested hops contain a 2:1 ratio (Goldammer, 1999). Although both female and male inflorescences are active in biosynthesis of hop acids, male inflorescences produce them at a much lower level, therefore female inflorescences are commonly sought by industries utilizing

hops (De Keukeleire et al., 2003). Likens et al. (1978) state that hop male flowers do not develop into cones and are only valued in breeding programs for their pollen. Prime harvest time in the Northern Hemisphere occurs from August to September and during the month of February in the Southern Hemisphere (Koetter and Biendl, 2010). Post-harvest, inflorescences are immediately dried to approximately 10% water content and typically stored constantly with refrigeration due to poor stability of the bitter portions, which will be discussed later in this review (Koetter and Biendl, 2010). Without refrigeration, concentrations of the bitter portions of hops are degraded by 50 to 70% in six months, thereby decreasing usefulness of the crop (Hänsel and Schulz, 1986).

Ionophores are produced from bacteria, although plants also make antimicrobial compounds as is seen with hops (Nicholson and Haerschmidt, 1992). Whole hops contain condensed tannins and secondary metabolites such as phenolic acids, flavonoid glycosides, resin, and essential oil compounds that are responsible for a variety of different functions (Bhattacharya et. al., 2003; Cleemput et al., 2009). Condensed tannins are naturally occurring polyphenols. Limited work has been done to assess their role in whole hops, but research thereof in other areas has shown tannins to express anti-proteolytic properties (Tavendale et al., 2005). Essential oils have aromatic properties and the amount per plant is dependent on the extent of pollination and time of picking (Sharpe and Laws, 1981). The oils can be extracted from whole hops via steam distillation in approximately three hours; however, β-acids may be present with longer distillation times due to the volatility of β-acids (Sharpe and Laws, 1981). Investigations of various essential oils have shown decreases in ruminal ammonia production due to inhibition of bacterial activity rather than protozoa (McIntosh et al., 2003; Newbold et al., 2004; Benchaar et al., 2008). The phenolic acids are known to possess antioxidant properties and the resins,

which contain α- and β-acids, have effects on microbial populations (Bhattacharya et. al., 2003; Lavrenčič et al., 2014). Observations of hop flavonoid glycosides have shown promise as strategies for producing anti-allergenic and antioxidant medicines (Li et al., 2014; Segawa et al., 2006). Additionally, 8-prenylnaringenin is a flavonoid glycoside of hops known for its strong, phytoestrogenic properties (Kowaka and Kokubo, 1977) and xanthohumol possesses cancer chemopreventive properties (Bishop et al., 1974; Gerhäuser et al., 2001; Stevens and Page, 2004). Both of these compounds have the potential to be used in therapeutic medicine for their estrogenic effects, however more definitive evidence is needed along with a proper formulation of hops (Chadwick et al., 2006).

Spent hops

The use of whole hops and derivatization thereof by the brewing industry creates rather large amounts of by-products, one of which is spent hops. Hops are extracted by supercritical carbon dioxide extraction, thereby creating spent hops as a by-product. Following extraction, spent hops can be added to spent cereal grains and dried together into a mixture containing approximately 5% spent hops and 95% spent cereal grains, thus forming dried brewer's grain (Aniol and Zolnierczyk, 2008). Spent hops are rich in polyphenols, they are relatively inexpensive, and have been used mostly as a fertilizer, although other areas of use are currently being researched to extend the flexibility of this product (Aniol and Zolnierczyk, 2008; Fiesel et al., 2014).

Spent hops have been incorporated into animal diets such as dairy cows (Daenicke et al., 1991) and sheep (Wallen and Marshall, 1979). Due to the presence of compounds with bitter properties, spent hops is at a disadvantage for use as a feed supplement (Huszcza and Bartmańska, 2008). Additionally, animals may be dissuaded from consuming spent hops due to

sedative side effects of 2-methyl-3-buten-2-ol, a degradation product of bitter acids, and perhaps bitterness of the spent hops byproduct (Wolfhart et al., 1983). Despite its bitter properties, spent hops are of high nutritive value and can contain up to 29% crude protein, which provides justification for evaluation of spent hops as a feed ingredient in animal diets; although a limiting factor may be the ability to mask or remove the bitter acid portion (Huszcza and Bartmańska, 2008). In a recent study, Fiesel et al. (2014) observed only a small, non-significant decrease in feed intake when spent hops were fed at 1% of the diet. The authors fed spent hops to five-week old pigs and observed improvements in gain:feed, in spite of adverse effects of spent hops on apparent total tract digestibilities of crude protein, crude fiber, and organic matter (Fiesel et al., 2014). Spent hops decreased gene copy numbers of *Streptococcus* spp. and *Clostridium* Cluster XIVa, and tended to decrease total VFA in feces, thus suggesting alterations in microbial fermentation (Fiesel et al., 2014). In accordance, Lavrenčič et al. (2014) observed decreases in dry matter and crude protein degradability when increased doses of two varieties of milled hops were incubated *in vitro*. Feeding spent hops thus may provide means for improving ruminal bypass value of dietary protein; however, *in vivo* trials are needed to support this contention.

Spent hops consist of sugars and free amino acids, with small amounts of hop acids and essential oils (Aniol and Zolnierczyk, 2008). Interestingly enough, Huszcza and Bartmańska (2008) observed that yeast can aid in the degradation of hop bitter acids, potentially improving spent hops as a feed supplement. Similarly, Aniol and Zolnierczyk (2008) suggest it is feasible to remove hop bitter acids from spent hops by extraction with ethanol or methanol plus acetone. Upon fractionation of spent hops, Chadwick et al. (2004) observed spent hops to contain prenylated chalcones (most notably, xanthohumol), prenylflavanones, 4-hydroxybenzaldehyde, sitostero-3-*O*-*β*-glucopyranoside, humulinone, and cohumulinone. Rigby et al. (2009) suggests in

their patent claim (US 7,553,504 B2) that any warm or cold blooded animal, including but limited to mammals, poultry, fish, crustaceans, and various pets could benefit from supplementation of milled hops or hop acids.

Hops *β*-acid extract

History of origin

Hops are native to Europe, Asia, and North America, and historically have been used for medicinal purposes and as a constituent of beer (De Keukeleire et al., 2007). First documentation of hop inflorescences being used for beer brewing was in the 6th century B. C. (Sakamoto and Konings, 2003). In 1150-1160, Abbess Hildegard of St. Rupertsberg was first to report preservative qualities of hops (Hierynomous, 2012). German monks began to incorporate hops into beer in the 12th century and beer production began to increase in the 14th century, with hops becoming more widely noted for their flavor and preservative properties (Sakamoto and Konings, 2003). With that being said, hops did not become widely accepted until the 1500 and 1600's by Germans and British, and the bitter taste initially was not appreciated (Foster, 1999). Although hops was recognized to have preservative properties, the antibiotic and bacteriostatic effects did not become clear until about 70 years ago (Behr and Vogel, 2009). Furthermore, inhibition of bacteria from beer spoilage was found to occur through various avenues such as altering the permeability of the bacterial cell wall (Shimwell, 1937), proton-ionophore activity (Simpson, 1993b), and causing cytoplasmic leakage by consequence of inhibiting protein synthesis (Teuber and Schmalreck, 1973). Today there are approximately 100 varieties of hops commercially available, although the percentage of each variety used can change quickly. Noble hops are the most ancient variety of hops; albeit they produce low yields, are highly susceptible to diseases, and store poorly (Oliver, 2012).

Formation and chemical composition

Biosynthesis of hop acids within the cones can begin as early as the first stages of flowering, although relatively low for each variety measured (< 2%) (De Keukeleire et al., 2003). In a majority of the varieties investigated by De Keukeleire et al. (2003), lupulone and adlupulone reached maximum concentrations during stage 2, or small cone stage, whereas it is believed concentrations to increase throughout hop cone development. This formation occurs by acylation via apolar branched chain amino acids that ultimately lead to α- and β-acids (De Keukeleire et al., 2003).

The antimicrobial properties of hop β-acids are due to a pattern of congeners (adlupulone, colupulone, prelupulone, postlupulone, and adprelupulone) that consist of an allylic structure, 2, 4-cyclohexadiene-1-one (Figure 2; Siragusa et al., 2008) IUPAC for lupulone (3,5-dihydroxy-2-(3-methylbutanoyl)-4,6,6-tris(3-methylbut-2-enyl)cyclohexa-2,4-dien-1-one). The structural differences within congeners of α- and β-acids are due to variability in the acyl side chain at carbon two (Figure 2; Taniguchi et al., 2013). The lupulone parent compound consists of a benzene ring with one stereocenter characterized by two prenyl groups at carbon number six.

Preparation and extraction

It is of utmost importance for breweries to make hop extract preparations that are anactinic, or do not take part in photochemical reactions. This also holds true for use of hop extract preparations in the animal feeding industry to uphold the integrity of products. It was proposed in 1978 by Vitzthum et al. (U. S. Pat. No. 4,104,409) that hop extracts can be prepared by supercritical carbon dioxide addition to dried hops. Supercritical extraction, temperature and pressure in the processing vessel are maintained at levels in excess of the critical temperature (31.3º C) and pressure (approximately 73 atm) of carbon dioxide (Vitzthum et al., 1978).

Exceptions to this are in the precipitation tank where pressure is below critical pressure (Vitzthum et al., 1978). Separation also occurs in this tank and temperature can be above or below critical, which leads to a water-free extract, or a resin, essential oil, and water precipitate, respectively (Vitzthum et al. 1978). Modern methods of extraction implement similar preparation techniques, which allow for recovery of the resin and essential oil portions.

Goldstein et al. (1988; U.S. Pat. No. 4,767,640) describe a method for preparation of a light-stable product by pre-purification of a liquid carbon dioxide hop extract via liquid-liquid, which recovers the α-acid portion. Isomerization yields reduced isohumulones, and non-isohumulone light unstable products (NILUPS). The subsequent addition of alkali and water with heat and stirring separates the two portions and the reduced isohumulones can be extracted in the aqueous phase and used as a light stable constituent in beer. Due to the typical insolubility of β-acids or lupulones in beer, they contribute little to brewing processes and often are extracted for other uses or discarded (Goldstein et al., 1988). An earlier claim by Cowles et al. (1986; U.S. Pat. No. 4,590,296) suggests that β-acids present in extracts containing both α and β-acids can be separated by bubbling carbon dioxide through an aqueous hop extract with a pH range 9.6 to 13 until pH of the extract is lowered to between 8.5 and 9.5, which will precipitate the β-acids and leave the α-acids remaining, all without using organic solvents.

Čulík et al. (2009) state that use of supercritical fluid extraction with carbon dioxide is widely used, but they proposed that pressurized solvent extraction with the proper solvent mixture and inert matrix can provide similar recoveries while decreasing processing time. Both procedures can be implemented with sufficient efficacy and followed by HPLC for confirmation of the compounds extracted.

In an effort to characterize the oxidation products of hop acids, Taniguchi et al. (2013) made preparations consisting of humulones and lupulones. Similar to methods stated above, *β*-acids were prepared by supercritical carbon dioxide extraction and further dissolved in hexane and partitioned with 0.6 M sodium hydroxide, yielding an aqueous fraction rich with *β*-acids. The aqueous solution was further acidified with 6 N HCl and upon addition of hexane, free *β*-acids were extracted with a purity greater than 85%.

Metabolism

Research aimed at quantifying metabolism of *β*-acids by monogastrics or ruminants is relatively sparse. Research has been conducted to characterize *in vitro* metabolism of hop bitter acids quantification of oxidation products (Cattoor et al., 2013). Cattoor et al. (2013) evaluated *in vitro* metabolism of hop bitter acids when incubated with rabbit liver microsomes and further analysis via HPLC-MS/MS. The total amount of hop *β*-acids was metabolized and primarily identified as hulupones (known oxidation products), as well as further formation of tricyclic oxygenated products by oxidative biotransformation such as dehydrotricyclolupulones, hydroxytricyclolupulones, and hydroperoxytricyclolupulones (Cattoor et al., 2013). With these products formed, metabolism of *β*-acids may involve liver oxidation that is catalyzed by cytochrome P450 enzymes. This *in vitro* incubation including a *β*-acid mixture with microsomal enzymes also created congeners of co-lupulone with smaller amounts of n-lupulone and ad-lupulone. It should be noted that the native oxidized compounds are oxidized even further or undergo a transformation reaction, which often takes place in beer decomposition (Cattoor et al., 2013). Although physiological metabolism is different than oxidative processes in beer degradation, results regarding hop *β*-acid derivatives shed light on possible metabolites formed during biotransformation.

Oxidation of hops

It is well recognized that hops, specifically the hop acids, undergo rapid oxidation during storage in the presence of oxygen or light (Taniguchi et al., 2013). After harvesting, the inflorescences have a relatively low bulk density and approximately 60% are compressed into a pellet form (Koetter and Biendl, 2010). The flowers are cut, milled, and homogenized before being formed into granules, thus increasing density (Koetter and Biendl, 2010). They are then transported in a manner that protects them from air and light (Koetter and Biendl, 2010). Of the hop acid fractions, α-acids are most susceptible to post-harvest degradation (Koetter and Biendl, 2010). Temperature, environmental conditions, and variety all are factors that influence the extent of oxidative damage to the bitter acids. In terms of the β-acids, oxidation products formed from the oxidative reactions consist of hulupones (Figure 2.; Stevens and Wright, 1961). In this reaction, lupulones undergo a reduction in ring size during oxidation (6-member ring to 5-member ring), among other structural changes.

As mentioned earlier, Taniguchi et al. (2013) attempted to characterize the oxidation products of hop acids by grinding hop pellets and storing at different temperatures (20, 40, or 60º C). A sampling regimen was followed for at least seven weeks and samples were prepared for HPLC and NMR analyses. The HPLC analysis was performed to isolate hydrophilic, oxidized compounds together with α-, β-, and iso-α-acids. The authors observed that when hops were stored at 60º C for 48 h, concentrations of α- and β-acids clearly decreased, and it was apparent that humulinones and hulupones were the most prevalent oxidized compounds, along with numerous compounds present at far lower concentrations. Hulupones previously have been recovered from oxidized hops (Stevens and Wright, 1961); however, this was the first definitive observation that humulinones also are present as a main oxidation product (Taniguchi et al.,

2013). It should be noted that the authors observed decreases in α- and β-acid concentrations at lower temperatures as well, albeit at a slower rate over the sampling period.

Function and antimicrobial effects

The antibacterial compounds present in hops, mostly iso-α-acids, have been observed to possess proton ionophore activity in which H^+ ions are exchanged for monovalent or divalent cations to purge ion gradient across cell membranes (Behr and Vogel, 2009; Simpson, 1993a). Furthermore, iso-α-acids can be categorized as class I/II of proton ionophores or a proton/manganese exchanger, which function under a broad range of pH for proton transport with couplers across cell membranes (Behr and Vogel, 2009). Behr and Vogel (2009) observed this ionophore activity of a hop extract consisting of mostly iso-α-acids with trace amounts of α- and β-acids in the presence of bilayer lipid membranes with a pH or $MnCl_2$ gradient to compare to biological membranes found in nature. In some follow up work, Behr and Vogel (2010) used cyclic voltammetry analysis and found that a transmembrane redox reaction occurs at low pH and with manganese present, which is found at millimolar levels in lactic acid bacteria. Furthermore, hops alter the intracellular redox balance of cells, ultimately leading to oxidative damage (Behr and Vogel, 2010). With that being said, it is believed that hop-resistant bacteria must possess ionophore and oxidative-stress resistance mechanisms. Due to their prevalence in beer manufacturing processes, mechanisms concerning hop α-acids have been elucidated more so than those of β-acids. However, previous reports suggest that hop bitter acids (α- and β-acids) act as mobile-carrier ionophores that are pH dependent. The non-dissociated form at low pH encourages antibacterial inhibition and high pH decreases this activity (Simpson, 1993a). Additionally, hop acids can possess either bacteriostatic or bacteriocidal properties, depending on the environment (Simpson, 1993a). Therefore, the contention that iso-α-acids act as proton

ionophores also is believed to hold true for β-acids (Harlow et al., 2014; Teuber and Schalreck, 1973). These discoveries allow for comparisons to be made between hop acids and ionophores due to their similar effects on specific biological membranes.

It has been well established that ionophore (monensin) action in poultry diets functions very well as an antibiotic and regulator of coccidiosis. The antibacterial properties of hops have been implemented in poultry diets in a similar fashion. Cornelison et al. (2006) strived to elucidate the antimicrobial potential of hops and incorporated ground whole hops (0.6% α-acids and 9.3% β-acids; Teamaker variety) into male chick broiler diets. Feeding hops at 0.025% of the diet yielded improvements in feed efficiency compared to test birds receiving 50-g penicillin per ton of feed over a 42-d feeding period (Cornelison et al., 2006). Body weights of birds fed hops increased significantly by d 14 of the study and tended to be greater than those of birds in the negative control on d 42. It should be noted that no microbial challenge was administered and whole hops were incorporated into the broiler diets. Although the variety was rich in β-acids, it cannot be clear whether the improved performance or any antimicrobial effects are solely a result of the hop acids. Years later, Siragusa et al. (2008) applied strictly β-acids (62.5, 125, or 250 mg/kg) in water to a chick gastrointestinal colonization model, and observed that β-acids administered through water do have the potential to inhibit pathogenic clostridia that colonize the gastrointestinal tract of poultry. This is of relevance due to past, and future, removal of antibiotics as growth promotants which has had an impact on the poultry industry in the form of necrotic enteritis caused by *Clostridium perfringens*.

Furthermore, hops β-acid extract has been experimented with in horses, which is an interesting avenue of research given the known toxicity elicited by ionophores in equines and the similarities between ionophores and β-acid extracts of hops. Interestingly enough, Harlow et al.

(2014) prepared suspensions of uncultivated equine fecal microbiota with an inulin substrate and the inclusion of *β*-acid. The authors observed inhibition of lactate production and mitigation of pH, which they attributed to the ionophore-like activity of hop *β*-acid on *Streptococcus bovis* (Harlow et al., 2014).

In an experiment by Narvaez et al. (2013b) with the inclusion of three types of hops (Cascade, Millennium, and Teamaker) in an artificial rumen system, it was observed that alterations in fermentation were due to inhibition of methanogens and *S. bovis*. Increases in populations of R. *amylophilus* and *P. bryantii* were observed (Narvaez et al., 2013b); though it is recognized that *in vivo* studies are needed to confirm these findings.

The effects of hop acids have promulgated interest in their use as antiprotozoals, anticlostridials, antivirals, for food and feed applications, and most recently for their potential as antibiotics (Cornelison et al., 2006; Lewis and Ausubel, 2006; Mitsch et al., 2004). Bacterial species that have been observed to be sensitive to hop acids include *Lactobacillus* spp., *Pedicoccus* spp., *Bacillus* spp., *Clostridium botulinum*, *Clostridium difficile*, *Mycobacterium tuberculosis* (Chin et al., 1949), *Enterococcus* spp., *Staphylococcus aureus, Streptococcus bovis, Streptococcus mutans* (Bhattacharya et al., 2003), *Ruminococcus flavefaciens,* and a $Gram^-$ bacterium, *Helicobacter pylori* (Flythe and Aiken, 2010; Narvaez et al., 2013a; Siragusa et al., 2008; Srinivasan et al., 2004). In contrast, *β*-acids in hops have been observed to increase populations of *Selenomas ruminantium* (Narvaez et al., 2013a).

There are synergistic effects that exist between *β*-acids and antibiotics (Natarajan et al., 2008). Beta-acids combined with polymyxin inhibited $Gram^+$ bacteria, and also inhibited $Gram^-$ bacteria, which separately have minimal effects on $Gram^-$ organisms. Additionally, when bacitracin was replaced with *β*-acid, similar zones of inhibition to Neosporin® were produced

(Natarajan et al., 2008). Positive co-action also was witnessed with ciprofloxacin and tobramycin, which all vary in their respective modes of action. It should be realized that these results are preliminary and further research is warranted. The limited activity of *β*-acids on Gram$^-$ bacteria was elucidated when Shapouri and Rahnema (2011) investigated the effects of hop extracts when added to incubations containing *Brucella abortus* 544 and *Brucella melitnensis*. Although composition of the extract was not clear, addition of hop extracts in this study showed anti-*Brucella* activity, which is of relevance to animals and humans because *Brucella* spp. are intracellular bacteria that inhabit macrophages and are responsible for the widespread zoonotic disease, brucellosis (Shapouri and Rhnema, 2011).

Ionophores are known to inhibit hyper ammonia producing bacteria (HAB; *Peptostreptococcus anaerobis* C*, Clostridium sticklandii* SR and *Clostridium aminophilum* F), thereby protecting feed amino acids from degradation and allowing them to be available to the animal (Flythe, 2009). Flythe (2009) aimed to determine if these effects could be exploited in hops as well and when media were modified with *β*-acid extract ammonia production by mixed rumen bacteria was inhibited. Furthermore, pure cultures of HAB also were modified with 30 ppm *β*-acid, which inhibited growth and ammonia production, and bactericidal activity was witnessed at lower pH (Flythe, 2009).

Horses are known to consume diets rich in non-structural carbohydrates; however, excesses of carbohydrate can create hindgut acidosis and laminitis (Bailey et al., 2004). This is caused by a disruption of the normal hindgut microbiota, whereby non-structural carbohydrates fuel proliferation of lactic-acid bacteria (Milinovich et al., 2007). Therefore, a subsequent increase in lactic acid production ensues with a concomitant decline in hindgut pH. Ionophores are known to be highly toxic in the horse; nevertheless Harlow et al. (2014) evaluated effects of

hops *β*-acid on inhibition hindgut acidosis caused by fructan-fermenting fecal bacteria. In *in vitro* incubations, hops *β*-acid were able to inhibit lactate production and control decline in pH (Harlow et al., 2014). Hops *β*-acid successfully achieved this by dissipating the intracellular potassium of *Streptococcus bovis,* as observed in pure cultures.

Effects on ruminal fermentation

A plethora of experiments have been conducted *in vitro* to examine impact of hops *β*-acids; however, more insight is needed *in vivo* to confirm these findings. Work reported in 2011 by Narvaez et al. demonstrated that addition of ground whole hop pellets (Teamaker) to batch culture ruminal incubations was able to quadratically decrease methane production independent of substrate. However, in pure forage (0, 50, 100, 200, 400 μg/mL hops), hops linearly increased gas production at 6 h, linearly decreased at 24 and 48 h, increased true dry matter disappearance linearly at 48 h, decreased microbial N, increased A:P ratio with a decrease in the proportion of propionate, increased molar proportions of butyrate, and decreased branched chain VFA (Narvaez et al., 2011). In total mixed growing and finishing rations, hops was included at rates of 0, 200, 400, 800, or 1600 μg/mL which improved production of IVDMD and VFA with a finishing diet and had less than favorable effects in a growing diet (Narvaez et al., 2011). With that being said, hops decreased A:P ratio and methane in both diets, indicating that hops produce more favorable effects with increasing proportions of concentrate in the diet. This dosing range for hops can be supported by similar work done by Wang et al. (2010) in that lower doses may improve *in vitro* ruminal fermentation (IVDMD and VFA) but do not affect performance *in vivo*. Shortly after, Narvaez et al. (2013a) developed evidence supporting their previous findings. Although not pure hop extracts, varieties of hops that varied in *α*- and *β*-acid content were compared in an artificial rumen system (Rusitec). Results showed that hops may mitigate

methanogenesis by decreasing the population of methanogens, and with an unaffected NDF digestibility, this may be a promising avenue to target methane emissions in livestock (Narvaez et al., 2013a). These were not pure *β*-acid extracts, with Teamaker variety composed of greater than 90% *β*-acids and the other varieties contained less than 50% *β*-acids on a total hop bitter acid basis. In other work by Narvaez et al. (2013b), hops (Cascade variety) was applied alone (600 μg/mL) or with monensin (2.5 or 5 μg/mL) and incubated in a ground barley silage/barley grain growing cattle diet. Hops fed alone or in conjunction with monensin decreased methane production, microbial protein, NH_3-N accumulation, A:P ratio, and molar proportions of butyrate. From these *in vitro* studies it can be concluded that hops have the potential to alter rumen microbial populations and fermentation under these conditions. Additionally, when combined with monensin, hops exert even more pronounced effects.

Research conducted with the intent to characterize the effects hop *β*-acids have either used different varieties of hops that contain high proportions of *β*-acids among other antimicrobial constituents, as stated previously, or strictly used hops *β*-acid extract. With implementation of a *β*-acid extract of hops, Flythe and Aiken (2010) prepared incubations containing carbohydrates and witnessed increases in VFA production and decreased A:P ratio. This evidence is supportive of the contention that hops *β*-acids may alter fermentative processes to improve production of the more energetically favorable product of propionate.

Conclusion

Hops is an interesting plant due to its large number of components that have been chemically characterized and evaluated for a broad range of applications. The use of hops for purposes other than beer production is increasing due to an increased understanding of its many properties. It is understood that many components possess antimicrobial effects; though *β*-acids

may offer the greatest potential. As expressed in this review, there have been numerous *in vitro* experiments aimed at characterizing efficacy of hops *β*-acids as a feed additive in animal diets. Some of this evidence shows promise for use in animals, and other evidence provides conflicting results. Supporting evidence from live animal studies is more sparse and is needed to confirm findings from in vitro experiments and to justify its application in the feed industry.

If hops *β*-acids do elicit similar responses in live animals as they do *in vitro,* these findings would not only be exciting for animal and food production, but a logical next step would be to research the technology of a more-stable product. The stability of *β*-acids in the presence of light and/or oxygen poses an obstacle to create a foothold in the feed industry; however, with continued research and understanding of the chemical structure and biological properties, quite possibly a stable product can be formed to compete with synthetically made supplements.

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Table 1.1 Examples of ionophore selectivity[1]

Ionophore	Mol wt	Selectivity	K_i, Na^+
Neutral Ionophores			
Valinomycin	1110	Rb>K>Cs>Ag>Tl>>NH4>Na>Li	17,000
		Ba>Ca>Sr>Mg>	
Enniatin A	681	K>Rb≈Na>Cs>>Li	3.3
Nonactin	736	NH4>K≈Rb>Cs>Na	16
Monactin	750	NH4>K>Rb>Cs>Na>Ba	18
Tetranactin	792		
Carboxylic ionophores			
Monensin	670	Na>>K>Rb>Li>Cs	0.10
Nigericin	724	K>Rb>Na>Cs>>Li	45
X-S37A (lasalocid)	590	Cs>Rb≈K>Na>Li;Ba>Sr>Ca>Mg	3.0
A23187	523	Li>Na>K;Mn>Ca>Mg>Sr>Ba	
Salinomycin	706		
Channel-forming ionophores			
Gramicidin A[a]	≈1700	H^+>Cs≈Rb>NH4>K>Na>Li	1.8
Polyene antibiotics[a]		Low selectivity	
Alamethecin[b]		K>Rb>Cs>Na	

[1]Adapted from Pressman (1976).
[a]Conductivity is not voltage-dependent.
[b]Conductivity is voltage-dependent.

Figure 1.1 Examples of ionophores. Adapted from www.google.com.

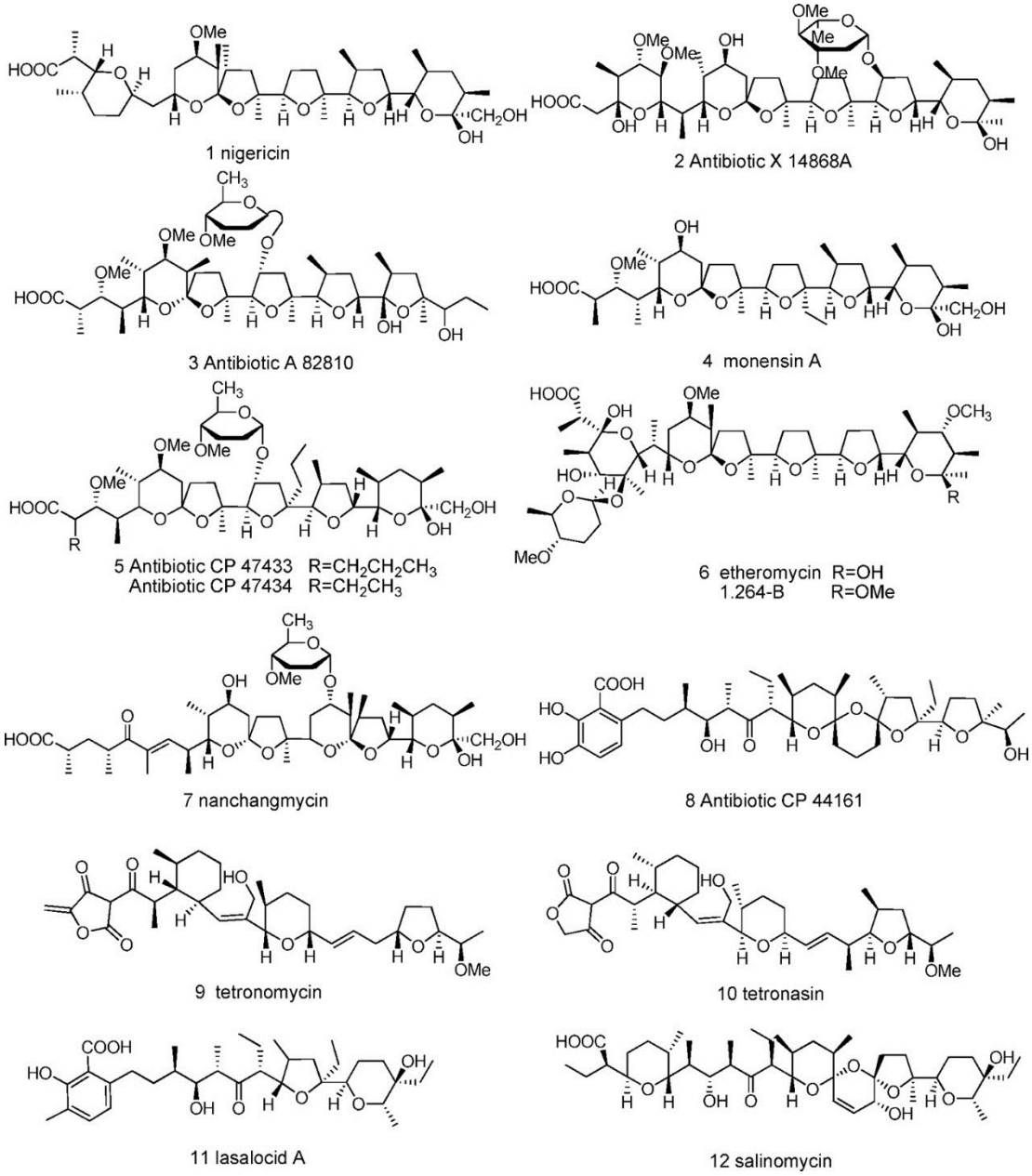
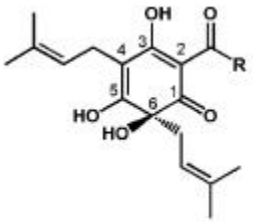
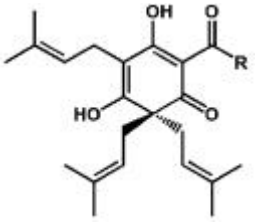


Figure 1.2 Chemical structures of *α*-acids and *β*-acids. Alpha-acids: (1a) cohumulone, (1b) n-humulone, (1c) adhumulone; and *β*-acids: (2a) colupulone, (2b) n-lupulone, (2c) adlupulone; and hulupulones, (3a) cohulupulone, (3b) n-hulupulone, (3c) adhulupulone. Adapted from Taniguchi et al. (2013).



1



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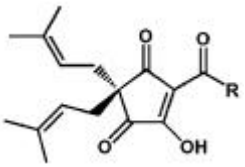

3

a: R = $CH(CH_3)_2$

b: R = $CH_2CH(CH_3)_2$

c: R = $CH(CH_3)CH_2CH_3$

Chapter 2 - Effects of hops *β*-acid extract (*Humulus lupulus* L.) on fermentation by ruminal microbes and performance of finishing heifers

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Introduction

Cattle are capable of consuming a variety of plant structural and non-structural carbohydrates (e.g. starch, fructan). Excess intake of non-structural carbohydrates is believed to be responsible for metabolic diseases such as acidosis and laminitis (Bailey et al., 2004). Acidosis is characterized by carbohydrate overload of fructan-rich pasture grasses or high-starch concentrates (Crawford et al., 2007), leading to lactate production, decreased pH, and allowing $Gram^+$ bacteria to predominate (Milinovich et al., 2008; Milinovich et al., 2010). Amines present in the rumen are suspect to decarboxylation by microbes and elicit a vasoconstrictive response in the hoof (Bailey et al., 2003), with these effects ultimately leading to laminitis. *Streptococcus bovis* produces lactate and is believed to produce amines and proteinases, thus playing a large role in laminitis in cloven-hoofed animals (Bailey et al., 2003; Milinovich et al., 2007; Thoefner et al., 2004).

Hops (*Humulus lupulus* L.*)* contain natural plant compounds that have played an integral role in beer manufacturing for centuries (Sakamoto and Konings, 2003). These compounds are widely known for their antimicrobial properties and preservative attributes. Hop resins consist of α-acids and β-acids; they are classified as humulones and lupulones, respectively, and differ structurally in their acyl side chains at C-2 (Taniguchi et. al., 2013). Alpha-acids are extracted and utilized to enhance beer flavoring, which leaves portions chiefly composed of β-acids (Cattoor et. al., 2013; Uwituze et. al. 2010). Beta-acids are structurally similar to ionophores and possess proton-ionophore activity on the $Gram^+$ cell membrane (Behr and Vogel, 2009; Simpson, 1993). Hop β-acids affect *Streptococcus* and *Lactobacillus* spp., so it is possible for this compound to be used as a strategy for mitigation of lactate production associated with acidosis and laminitis (Bhattacharya et al., 2003; Narvaez et al., 2013; Siragusa et al., 2008). Ionophores

are utilized to improve feed efficiency and have been shown to decrease the incidence of digestive disturbances that are important contributors to morbidity and mortality in ruminants (Russell and Houlihan, 2003). Feeding ionophores serves to disrupt the transmembrane potential of specific $Gram^+$ bacteria, thus leading to increased animal production and efficiency (Flythe, 2009). Extracts of hops effectively inhibit $Gram^+$ bacteria but are less effective against $Gram^-$ bacteria, thus exhibiting actions that are similar to the ionophore, monensin (Wang et. al., 2010). The majority of research performed on *β*-acid extracts of hops has been performed *in vitro.* These studies provide insight on its effects *in vivo* and *in vitro*. The objectives of these experiments were to assess the effects of *β*-acid extract of hops on heifer feedlot performance when fed high-concentrate diets, to determine responses to varied doses of *β*-acid extract of hops, and to determine the effect of *β*-acid extract on starch fermentation in mixed microbial populations from the bovine rumen. Our hypothesis was that hops *β*-acid extract would mitigate the decrease in pH associated with excess lactate production from the fermentation of rapidly fermented carbohydrates.

Materials and methods

In vivo

Procedures in this study were approved by the Kansas State University Institutional Animal Care and Use Committee protocol number 3333.

Animals and diets

The study was a randomized complete block design consisting of five treatments with 16 replicates. Initial weight served as the blocking criterion and feedlot pen constituted the experimental unit. Eighty crossbred heifers (BW = 389 ± 23.6 kg) purchased from multiple sources were shipped to the Kansas State University Beef Cattle Research Center. Cattle were

implanted (Component TE-200; Zoetis Inc., Florham Park, NJ), dewormed (Dectomax; Zoetis Inc.), and vaccinated against common viral and clostridial diseases (Bovi-Shield Gold and Ultra-bac-7; Zoetis Inc.) prior to onset of the trial. Heifers were randomly allotted to 5 treatments consisting of a no additive (negative control); 33 mg monensin (Elanco Animal Health, Greenfield, IN)/kg diet DM; and 10, 25, or 50 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg diet DM. The *β*-acid extract consisted of 10.99% active hops beadlet (lupulone, colupulone, adlupulone) and was stored in a -20ºC freezer for duration of trial. Heifers were housed individually in partially covered pens equipped with individual feed bunks and water fountains that allowed access to feed and water *ad libitum*. The basal diet (DM basis; Table 1) contained a combination of steam-flaked corn, wet corn gluten feed, and roughage, supplemented with vitamins A and E, macro minerals (calcium, potassium), and inorganic trace minerals (Na, Cl, Co, Cu, I, Mn, Se, and Zn). Feed additives (monensin and *β*-acid) were pre-blended into a carrier consisting of finely ground corn. The monensin premix contained 1,320 mg/kg active ingredient, and the *β*-acid premixes contained 400, 1,000, and 2,000 mg *β*-acid extract of hops/kg premix (DM basis). The negative control consisted of ground corn only. Diets were mixed daily using a stationary mixer (H.D. Davis and Sons, Model HD-5) and the mixer was cleaned between batches to avoid carryover. Order of mixing was: negative control, 10 mg/kg *β*-acid, 25 mg/kg *β*-acid, and 50 mg/kg *β*-acid, followed by monensin. Each diet was mixed, weighed, and delivered to respective pens once daily at approximately 1100 h. Samples of each diet were collected daily and stored in a -20ºC freezer. All samples were composited weekly, packaged, labeled, and delivered to a commercial laboratory (John I. Haas, Inc., Yakima, WA) for analysis of *β*-acid content (data presented in Table 2).

Sampling procedures

Seven experimental periods were used over the 147-d feeding period to assess feedlot performance (ADG, DMI, Gain:feed; Table 3 and 4) with each period lasting approximately three wk in duration. On animal weighing days, and additionally when excess fed accumulated within bunks, unconsumed feed was weighed from each pen and DM content was determined by drying in a 55ºC oven for 48 h. Dry matter intakes were estimated using the as-fed feed deliveries and actual feedstuff DM values minus the amount of unconsumed DM. Ruminal digesta samples (4 to 8 mL) were collected via rumenocentesis on days 44 and 86 and used to determine ruminal pH and concentrations of VFA, lactate, and ammonia (Table 5). Rumenocentesis was performed by trimming and disinfecting (chlorhexidine; Aspen Veterinary Supply, Liberty, MO) a 10 cm × 10 cm area on the left side of the animal between the last rib and stifle. A 60-mL syringe fitted with a 14 gauge × 11 cm needle was inserted through the peritoneum and abdominal wall, into the ventral sac of rumen. The plunger was then drawn back to collect ruminal fluid in the syringe and then placed into a scintillation vial where each sample was immediately tested for pH using a Thermo Orion benchtop pH meter (model 230 A, Thermo Fisher Scientific Inc., Waltham, MA). Ruminal contents (4 mL) then were combined with 1 mL of 25% w/v *m*-phosphoric acid solution and stored in a freezer until further analysis. These samples were each used for VFA, lactate, and ammonia analyses.

Analyses of ruminal VFA and lactate

After thawing, 2 mL of each mixture were placed into micro-centrifuge tubes and centrifuged at 17,000 × *g* for 15 min. Supernatant was removed, placed into gas chromatography vials, and stored in a freezer at -20ºC. Ruminal fluid contents were analyzed using an Agilent gas chromatograph (model 7890A, Santa Clara, CA) equipped with a Nukol capillary column (15 m × 0.52 i.d.). The injection temperature was 225°C, the split ratio was 1:30, flame-ionization

detector was set at 250°C and used hydrogen (35 mL/min), air (400 mL/min), makeup helium (25 mL/min), and helium carrier gas at constant flow (0.65 mL/min). The oven temperature program was set as follows: held at 105°C for 1 min, increased 8°C/min to 135°C, then increased 30°C/min and held at 240°C for 1.75 min. Supelco Volatile Standard Mix (46975-U Supelco, Sigma-Aldrich, St. Louis, MO) was used as the standard.

Lactate content was analyzed using a colorimetric determination of lactic acid in biological material procedure (Barker and Summerson, 1941). Ruminal fluid samples containing 25% w/v *m*-phosphoric acid solution were thawed and centrifuged for 18 min at 1,400 × *g* to remove all protein. Next, 0.5 mL of sample was transferred to a 15-mL disposable tube with 0.5 mL of 20% copper sulfate, 4 mL water, and 0.5 g calcium hydroxide and shaken vigorously until a bright blue color appeared. Samples were allowed to rest at room temperature for 30 min and then centrifuged at 1,000 × *g* for 10 min. A 0.5-mL sample of supernatant was transferred into a new 13 × 100 mm culture tube. Next, 25 µL of 4% $CuSO_4$, and 3.0 mL of sulfuric acid were added and homogenized using a vortex mixer. Tubes were placed in a boiling water bath for 5 min and then cooled in cold water until temperature reached ≥ 20°C. After cooling, 50 µL of *p*-hydroxydiphenyl were added and quickly mixed by vortexing each tube. Next, the tubes were placed in a 30°C water bath for 30 min and mixed once during this time period. The tubes were transferred to a boiling bath for 90 s to destroy any alkali, allowing a purple color to develop. After cooling the tubes to room temperature, absorbance was recorded on a spectrophotometer at 560 nm using reagent blank as zero.

Analysis of ruminal ammonia

After thawing of ruminal fluid containing 25% w/v *m*-phosphoric acid solution, 2 mL of each mixture were placed into micro-centrifuge tubes and centrifuged at 17,000 × *g* for 15 min.

Supernatant was removed and placed into gas chromatography vials. Ruminal ammonia concentrations were measured using a colorimetric procedure on an Autoanalyzer III (SEAL Analytical Inc.; Mequon, WI) as described by Broderick and Kang (1980).

Statistical analyses

Feedlot performance data were analyzed using the MIXED procedure of the Statistical Analysis System (version 9.0 of SAS; Cary, NC) with a model that included diet as a fixed effect. Feedlot performance were also analyzed as repeated measures using the MIXED procedure. Weight block constituted the random effect. Least-squares means (LSMeans) were calculated for each group and treatment was tested against the residual error at 5% level of significance. Ruminal fermentation parameters were analyzed using the MIXED procedure with a model that included diet as a fixed effect and weight block as the random effect. Treatment tested against the residual error at 5% level of significance. Pre-planned orthogonal contrasts of least-squares means of performance and fermentation parameters were made consisting of control vs. monensin; control vs. average of β-acids; average of β-acids vs. monensin; linear effect of β-acids; quadratic effect of β-acids. Trends are discussed at $P > 0.06$ and ≤ 0.10.

In vitro

Experiment 1

The experiment was a randomized complete block design with two treatments and three replicates. Treatments consisted of hops β-acid extract added to cultures at a rate of 0 or 33 mg β-acid (DSM Nutritional Products, France)/kg substrate. Ruminal digesta was obtained from a single ruminally-fistulated Holstein steer fed a diet (DM basis; Table 6) that consisted of 30% wet corn gluten feed, 25% ground alfalfa hay, 25% ground brome hay, 16.50% steam-flaked corn, 2.16% feed additive premix, and 1.34% vitamin/mineral premix. Ruminal contents were

obtained at approximately 1100 h, prior to feeding. Ruminal fluid was placed directly into pre-warmed insulated containers to maintain temperature after being strained through four layers of cheese cloth, and transported 2 km to the Pre-Harvest Food Safety Laboratory.

In the laboratory, ruminal fluid was decanted into separatory funnels, purged with nitrogen gas for several minutes, and incubated at 39ºC for approximately 1 h, allowing the fluid to stratify into three layers. The bottom sediment and mat layers were discarded, and the bacteria-rich middle layer was retained for use as inoculum. Batch cultures were prepared by combining 3.0 g soluble starch (Difco Soluble Starch; Dickinson and Company, Sparks, MD) with 50 mL strained ruminal fluid and 100 mL buffer solution in 250 mL screw-top bottles. The buffer contained (per liter) 9.80 g $NaHCO_3$, 3.71 g Na_2HPO_4, 0.57 g KCl, 0.47 g NaCl, 0.12 $MgSO_4$-$7H_2O$, 0.80 g NH_2CONH_2, and 0.04 g $CaCl_2$. The *β*-acid product consisted of a stabilized beadlet with 10.99% active ingredient (lupulone, colupulone, adlupulone; DSM Nutritional Products, France) and was stored in a -20ºC freezer. Hops *β*-acid extract was solubilized in methanol:water (1:1) prior to adding to cultures. Treatments without *β*-acid were given the same dose of methanol:water (1:1) without added *β*-acid. Methanol:water solutions were added to respective treatments at a rate of 0.10 mL per culture to provide 0 or 33 mg *β*-acid/kg substrate. Three bottles were used as blanks, containing only buffer, inoculum, and methanol:water solution. Culture bottles were gassed with nitrogen, capped with Ankom^{RF1} modules (Ankom^{RF} Gas Production System; Ankom Technology, Macedon, NY), and placed into a shaking incubator (New Brunswick Scientific Co., Inc., New Brunswick, NJ) maintained at 39ºC with gentle agitation for 30 h. Gas pressure was recorded at 15-min intervals. After 30 h, cultures were removed from the shaker and final pH was determined using a benchtop pH meter (Thermo Orion model 230 A; Thermo Fisher Scientific Inc., Waltham, MA). Subsequently, 4 mL

of the particle-free fluid layer were mixed with 1 mL of a 25% w/v *m*-phosphoric acid solution and frozen overnight (-20ºC). After thawing, 2 mL of each mixture were placed into micro-centrifuge tubes and centrifuged at 17,000 × *g* for 15 min. Supernatant was removed, placed into gas chromatography vials, and stored in a freezer at -20ºC until analyses of VFA and lactate. The remaining contents of each fermentation flask were transferred into a 19.0 × 12.7-cm aluminum pan and placed into a 105ºC oven until dry to determine IVDMD. Calculation of IVDMD was as follows: (substrate/fluid final DM – final blank wt.)/(initial substrate weight)*100.

For VFA and lactate analyses, after thawing, 2 mL of ruminal fluid with 25% *m*-phosphoric acid solution was inserted into micro-centrifuge tubes and centrifuged at 17,000 × *g* for 15 min. Supernatant was pipetted into 12 × 75 tubes, and tubes were then vortexed. Pivalic internal standard was added at a rate of 120 µL/tube and mixed (final pivalic acid internal standard concentration was 8 mM). One mL of the mix was transferred to a gas chromatography vial and refrigerated at 4ºC until analysis. Analyses were carried out using an Agilent gas chromatograph (model 7890A, Santa Clara, CA) equipped with a Nukol capillary column (15 m × 0.52 i.d.). The injection temperature was 225°C, the split ratio was 1:30, flame-ionization detector was set at 250°C and using hydrogen (35 mL/min), air (400 mL/min), makeup helium (25 mL/min), and helium carrier gas at constant flow (0.65 mL/min). The oven temperature program was set as follows: held at 105°C for 1 min, increased 8°C/min to 135°C, then increased 30°C/min and held at 240°C for 1.75 min. Supelco Volatile Standard Mix (46975-U Supelco, Sigma-Aldrich, St. Louis, MO) was used as the standard.

Statistical Analyses. Gas production, terminal pH, IVDMD, VFA, and lactate were analyzed using the MIXED procedure of SAS version 9.3 (SAS Inst. Inc., Cary, NC) with a model that included *β*-acid as the fixed effect and block as the random effect. Ankom module

was the experimental unit. Time and β-acid x time also were included in the model statement as a fixed effects for statistical analysis of gas production. Means were separated using the PDIFF of the LSMeans statement. Significance was determined at $P \leq 0.05$ and trends are mentioned between $P > 0.06$ and ≤ 0.10.

Experiment 2

Procedures for collection of bovine ruminal digesta and β-acid product were similar to those described for in vitro experiment 1, with the exception digesta was collected at approximately 0900 h, prior to feeding.

In vitro experiment 2 was a randomized complete block design with incubations containing bovine ruminal fluid and starch with either 0 or 33 mg hops β-acid extract/kg substrate, and incubation times of 0, 6, 12, 18, 24, and 30 h. Batch cultures were carried out in triplicate using 36, 50-mL conical centrifuge tubes (Thermo Scientific, Rochester, NY) equipped with vented rubber stoppers. Soluble starch was again used as the substrate, and was included at 0.5 g/culture tube. To each culture tube 20 mL buffer and 10 mL strained ruminal fluid were added, along with the appropriate methanol:water mixture (i.e., with and without β-acid) added at the rate of 0.1 mL/tube. Nine blanks were used and contained buffer, ruminal fluid, and methanol:water solution without β-acid. After all components were added, tubes were gassed with nitrogen, capped, and placed into the shaking incubator at 39ºC with gentle agitation. A portion of the tubes were removed at 6-h intervals, pH was immediately measured, and 4 mL of the fluid portion was mixed with 1 mL of 25% w/v *m*-phosphoric acid solution and frozen overnight (-20ºC). After thawing, 2 mL were transferred to micro-centrifuge tubes and centrifuged at 17,000 × *g* for 15 min. Supernatant was removed, placed into gas chromatography vials, and stored in a freezer at -20ºC until VFA analyses. Tubes containing the remaining

digesta were centrifuged at 2,200 × *g* for 15-min in a free spin centrifuge (Beckman, Model J-6B) for IVDMD analysis. The supernatant was decanted and tubes containing the remaining pellet were placed into a 105ºC oven until dry to determine IVDMD. Calculations of IVDMD were as follows: [1 – (dry residue of tube – dry residue of blank)/(dry weight of initial substrate)]*100. Analyses of VFA and lactate were performed as described for in vitro experiment 1.

Statistical analyses. For pH, IVDMD, VFA, and lactate were performed using the MIXED procedure of SAS version 9.3 (SAS Inst. Inc., Cary, NC) with a model that included *β*-acid, hour, and *β*-acid × hour as fixed effects and block as the random effect. Culture tube was the experimental unit. Treatment means were separated using the PDIFF option of the LSMeans statement. Significance was determined at $P \leq 0.05$ and trends are mentioned between $P > 0.06$ and ≤ 0.10.

Results

In vivo

One heifer from the Beta 10 treatment was diagnosed with digital dermatitis, causing severe lameness and depressed feed consumption. All data from this animal were removed from the study.

No interaction was observed between diet and day, or an effect of day for all ruminal fermentation parameters assessed, ($P > 0.05$; Table 5). As a result, means are expressed as the average of means for both sampling days for ruminal fermentation parameters. Heifer performance data are presented in Table 3. Additionally, these data are separated by period and analyzed as repeated measures ($P > 0.05$; Table 4). Average daily gain, DMI, and feed efficiency throughout the 147-d experiment were unaffected by treatment ($P > 0.05$; Table 3). Ruminal

VFA concentrations and A:P are reported in Table 5. Hop β-acids decreased propionate concentrations ($P = 0.01$; Table 5) and increased A:P ($P = 0.04$; Table 5), caproate ($P = 0.05$; Table 5), and ruminal ammonia concentrations ($P = 0.03$; Table 5) compared to monensin. Additionally, there was a tendency for a quadratic effect of β-acids on ruminal butyrate concentrations ($P = 0.08$; Table 5) where increasing butyrate concentrations were observed with greater amount of β-acid in the diet. No differences were noted for ruminal pH ($P > 0.05$; Table 5), however we detected a linear effect of β-acids for lactate ($P = 0.06$; Table 5) where lactate increased with increasing β-acid in the diet.

In vitro

Experiment 1

In terms of gas production, we observed no interaction between β-acid and time ($P > 0.05$; Fig. 1). However, we detected an effect of β-acid ($P \leq 0.05$; Figure 1) and an effect of time ($P \leq 0.05$; Figure 1), where the addition of β-acid increased gas production compared to no addition of β-acid and gas production increased over the incubation period. Terminal pH, IVDMD, VFA, and lactate were unaffected by hops β-acid extract ($P > 0.05$; Table 7) compared to absence of β-acid. However, we observed an increase in A:P with the addition of hops β-acid extract compared to absence of β-acid ($P \leq 0.05$; Table 7).

Experiment 2

We observed no interaction between β-acid and hour, or effect of β-acid for any parameter assessed ($P > 0.05$; Tables 8 to 15). However, time of incubation affected pH ($P \leq 0.05$; Table 8), with pH declining from h 0 to 18. As expected, IVDMD increased with longer incubation times ($P \leq 0.05$; Table 9). However, compared to experiment 1, gas production, which is an indicator of microbial activity *in vitro*, proceeded more slowly than IVDMD.

Similarly, incubation time impacted production of acetate ($P \leq 0.05$; Table 10), with concentrations increasing over the range of 0 to 18 h. Additionally, an hour effect was observed for propionate ($P \leq 0.05$; Table 11) where concentrations increased from h 0, 6, 12, 18, and 24. We detected an hour effect for butyrate ($P \leq 0.05$; Table 12) where concentrations increased from h 12, 18, 24, and 30. Total VFA concentrations were unaffected by β-acid ($P > 0.05$); however, we observed an hour effect ($P \leq 0.05$; Table 13) where concentrations increased from h 0, 6, 12, and 24. We detected an hour effect for lactate ($P \leq 0.05$; Table 14) where concentrations increased drastically from h 6 to 12. Lastly, we observed an hour effect for A:P ($P \leq 0.05$; Table 15) where ratios decreased from h 0, 6, 12, 18, and 24.

Discussion

In vivo

Chemical structures of β-acids are similar to those of ionophores, and incorporation of these additives into cattle diets could potentially impact fermentation in a manner similar to that achieved with ionophores such as monensin. Ionophores effectively inhibit certain classes of ruminal microorganisms, thus altering ruminal fermentation. Feeding ionophores improves feed efficiency (Bergen and Bates, 1984; Nagaraja et al., 1982), an effect that presumably occurs as a result of increased production of propionate and succinate at the expense of acetate, butyrate, lactic acid, methane, and ammonia (Chen and Wolin, 1979; Kennelly et al., 1998). Although differences in performance for cattle fed monensin or β-acid, were not significant ($P > 0.05$; Table 3), the magnitude of response to monensin (approximately 4.5%) was more or less typical. Response to the Beta 25 treatment was of similar magnitude to monensin, but again was not significant ($P > 0.05$; Table 3). When comparing the average of β-acid treatments to monensin, ruminal contents of cattle fed monensin contained more propionate and less ammonia ($P = 0.01$,

$P = 0.03$, respectively; Table 5). The lack of difference between animals fed the control diet and animals fed the different feed additives seemingly refutes the notion that either additive has a protein-sparing effect. Wang et. al. (2010) reported no differences in intake, ADG, or feed efficiency when steers were given increased levels of whole hops on either a growing or finishing diet, which is consistent with observations in the current study when heifers were fed a hops β-acid extract. Al-Mamun et al. (2009) researched effects of hops on metabolism parameters in sheep and found that glucose, NEFA, VFA and lactic acid concentrations were similar between treatments. The authors did report a tendency for increased ruminal NH_3-N concentration in animals fed the diet containing hops ($P = 0.08$) compared to those fed a mixed-hay diet. Feeding β-acids in our study elicited no change in ruminal ammonia compared to cattle fed the control diet, but ruminal ammonia concentrations were greater for β-acid treatments compared to monensin ($P = 0.03$; Table 5), suggesting if there is a protein sparing effect of monensin this property is not shared by β-acid extract. Furthermore, speculation that β-acid extract of hops may have an effect on hyper-ammonia producing bacteria (HAB) is not supported by these data, which is a departure from that observed for monensin (Chen and Russell, 1989). Lactic acid bacteria that contaminate beer fermentation possess $Gram^+$ cell envelopes, which also are found in *Clostridium perfringens*. Siragusa et al. (2008) reported that addition of hop extracts decreased survival rate of this pathogen. This can infer that HAB strains may be sensitive to hop extracts due to lack of an outer membrane. Using an *in vitro* model, Flythe (2009) observed that three HAB strains were sensitive to antimicrobial properties of hops added to mixed ruminal bacteria. In follow up work, Flythe and Aiken (2010) reported increased propionate to acetate ratio and rates of VFA production over 24 h *in vitro* when mixed ruminal microbes were introduced to hops extract. Flythe and Aiken (2010) also stated that *Megasphaera elsdenii,* a

Gram$^-$ organism, was less sensitive to β-acid concentrations used in their study (30 mg/kg). This is important because this bacterium plays a large role in propionate production from lactate (Russell 2002). Ruminal lactic acid bacteria (LAB) ferment carbohydrates to lactate, and *M. elsdenii* converts lactate to propionate (Gottschalk, 1986). When co-cultured with *Selonomas ruminantium*, a Gram$^-$ organism, propionate production was not affected, thereby supporting the contention that outer membranes are not susceptible to the activity of β-acid extract of hops (Flythe and Aiken, 2010). With that said, in this study it appeared that addition of β-acid extract did not have an effect on lactate concentrations observed compared to the control ($P > 0.05$; Table 5). However, there was a tendency for a linear effect of β-acids on lactate concentrations, with lactate increasing as amounts of β-acid in the diet increased ($P = 0.06$; Table 5).

Hops are highly susceptible to oxidation and Taniguchi et al. (2013) observed that α- and β-acids are susceptible to rapid autoxidation. Additionally, the authors observed that storing hops at 60°C for 48 h resulted in formation of oxidation products and decreased concentrations of α-acids and β-acids. Thus, improper storage of hops, and even exposure to air, can quickly diminish the potency of β-acids as was discovered in the current study (Table 2). Under the conditions of the current study, β-acid extract was stored in a -20ºC freezer and mixed daily in an effort to minimize degradation and autoxidation. Diet analysis of β-acid content showed that less than 50% of the expected concentration was present in each of the total diets which could explain the lack of an effect of hops β-acid extract in this study (Table 2). To support this contention, Taniguchi et al. (2013) found that less than 5% of initial concentration was observed for α- and β-acids when stored at 40ºC. Additionally, the authors observed at 60ºC the decrease in concentration occurred at a more rapidly (approximately four weeks vs three days). Although the authors elucidated the effects of elevated temperature on the stability of β-acid extracts of hops,

our storage temperature conditions were much lower, however conditions while in transit could have been equivalent (Table 2). Nevertheless, feeding hops β-acid extract resulted in no significant improvements in performance or ruminal fermentation of feedlot heifers, as was the case with monensin.

In vitro

Experiment 1

Beta-acid extract of hops have been observed to exhibit antibacterial activity and may affect fermentation products (Simpson, 1993; Harlow et al., 2014). Incubations in this study consisted of starch with strained bovine ruminal fluid and either β-acid extract of hops at 0 or 33 mg/kg of substrate. In terms of gas production expressed in mL, we detected a β-acid effect ($P \leq 0.05$; Fig. 1) and time effect ($P \leq 0.05$; Fig. 1). This showed that addition of β-acid increased gas production compared to the absence of β-acid. This supports previous research by Narvaez et al. (2011) where gas production linearly increased ($P < 0.05$) in batch cultures including ground whole hop pellets in bovine cultures with barley grain. However, gas production linearly decreased ($P < 0.001$) with substrates including whole hop pellets with pure forage or a total mixed ration. In terms of A:P in the current study, ($P \leq 0.05$; Table 7) greater A:P were detected with β-acid addition compared to the absence of β-acid. The absence of a β-acid effect for lactate suggests that lactic acid bacteria were not affected, resulting in low pH and increased lactate concentration when provided rapidly fermentable substrate. There was a tendency for lower lactate concentrations ($P = 0.06$; Table 7) in this experiment, which is consistent with observations found with ionophores (Flythe, 2009; Russell and Houlihan, 2003).

It is understood that consuming excesses of readily fermented carbohydrates can predispose ruminants to acidosis related maladies. Our goal was to add β-acid extract of hops to

incubations containing rapidly fermentable carbohydrate to see if this could mitigate the rapid decline in pH often associated with bovine ruminal acidosis. Availability of rapidly fermentable carbohydrate potentially allowed for rapid microbial growth and fermentation, which created an acidic environment and could explain the high production of lactate.

Bailey et al. (2003) performed an *in vitro* study similar using equine cecal contents and observed that *Streptococci* and *Lactobacilli* are the major proliferative bacteria in the presence of readily fermentable carbohydrates. Hop β-acids are capable of inhibiting $Gram^+$ bacteria such as *Lactobacillus* spp., *S. aureus*, *S. bovis*, and *S. mutans* (Bhattacharya et al., 2003; Narvaez et al., 2013; Siragusa et al., 2008). We did not enumerate micro-organisms, but under the conditions of this experiment it is possible that lactate-producing bacteria were not affected by β-acid and were allowed to flourish, potentially due to a decrease in competing bacteria or a rapid decline in pH at the beginning of the fermentation. On the contrary, *S. bovis* is primarily responsible for ruminal acidosis (Owens et al., 1998), and concentrations of lactate tended to decrease in bovine cultures including β-acid compared to the absence of β-acid ($P = 0.06$; Table 7). Albeit, this decrease could be due to less competition at lower pH and more efficient conversion of lactate to acetate or propionate. These results may indicate that lactate-producing bacteria were unaffected under the conditions of this experiment. *In vitro* cell cultures are an important tool for obtaining insights into cellular processes in an isolated system and a supplement to *in vivo* animal experiments. However, a direct correlation cannot be made between the conditions of our experiment and *in vivo* processes.

Experiment 2

There were no interactions between β-acid and hour ($P > 0.05$) or effect of β-acid ($P > 0.05$). In this experiment, pH, IVDMD, VFA, and lactate were measured every 6 h for 30 h to

correlate with exp. 1. When monitoring pH every 6 h we observed an hour effect that showed pH to decrease at h 0, 6, 12, and 18, with a subsequent increase from h 24 to 30 ($P \leq 0.05$; Table 8). The readily available carbohydrate possibly allowed for proliferation of $Gram^+$ bacteria such as *Streptococci* and *Lactobacilli* that produce acidic products from metabolism (Bailey et al., 2003). Perhaps the addition of *β*-acid was not able to combat this rapid proliferation under the conditions of this study.

In terms of IVDMD, there was an effect of hour ($P \leq 0.05$; Table 9) where IVDMD increased at h 0 to 6, increased further from h 12 to 18, and h 30 was greater than h 18. Results appear to be difficult to explain and abnormally high, however it is evident that a majority of the dry matter disappearance occurred between 0 and 6 h ($P \leq 0.05$; Table 9). This supports the contention by Thoefner et al. (2004) that the abrupt availability of excess and rapidly fermentable carbohydrate leads to rapid proliferation of $Gram^+$ bacteria, which could explain the dramatic increase in IVDMD within the first 6 h.

W we observed an effect of hour ($P \leq 0.05$; Table 10) for acetate, propionate ($P \leq 0.05$; Table 11), butyrate ($P \leq 0.05$; Table 12), total VFA ($P \leq 0.05$; Table 13), lactate ($P \leq 0.05$; Table 14), and A:P ($P \leq 0.05$; Table 15). With the exception of butyrate that did not plateau, these possibly plateaued due to end product inhibition of the microbial environment. In terms of *in vitro* concentrations of lactate, we observed a main effect of hour over 30 h and spiked at h 12 and was different compared to h 6 ($P \leq 0.05$; Table 14). Lactate concentrations remained high at h 18 ($P > 0.05$; Table 14) and decreased at h 24 and 30 compared to h 18 ($P \leq 0.05$; Table 14). Flythe and Aiken (2010) performed a similar study, however when grown in pure culture and exposed to *β*-acid, *S. bovis* was inhibited, as well as lactate production. In the current study, *β*-acid could have affected *S. bovis* initially, however at h 12 there were spikes in lactate

production, perhaps suggesting some form of adaptation by this organism and other lactate producing bacteria that stain Gram^{+} (Table 14). We could speculate that these increased concentrations of lactate had a deleterious effect on the microbial environment, and therefore possibly explaining the lack of an effect of *β*-acid. In summary, under the conditions of this experiment, the addition of *β*-acid to *in vitro* incubations had no effect on microbial fermentation.

Acknowledgments

The authors would like to thank DSM Nutritional Products for financial support and provision of hops β-acid extract used in this experiment, and Jake Thieszen for assistance and expertise with laboratory procedures.

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Table 2.1 Basal diet (DM basis) of heifers fed 0, 10, 25, or 50 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg diet DM or 33 mg monensin (Elanco Animal Health, Greenfield, IN)/kg diet DM.

Ingredient, %	Step 1	Step 2	Step 3	Final
Wet corn gluten feed	30.47	30.47	30.46	30.45
Ground alfalfa hay	30.37	22.85	15.33	7.81
Steam-flaked corn	24.89	35.85	46.81	57.76
Wheat straw	10.30	6.87	3.43	-
Feed additive premix[1]	2.01	2.01	2.01	2.01
Supplement[2]	1.96	1.96	1.96	1.96
Calculated composition, %				
CP	14.77	14.57	14.37	14.18
Ca	1.00	0.90	0.80	0.71
P	0.45	0.46	0.47	0.49
K	1.07	0.95	0.82	0.70
NDF	36.55	30.85	25.16	19.47
NE_m, Mcal/lb	0.76	0.83	0.90	0.96
NE_g, Mcal/lb	0.48	0.54	0.60	0.66

[1]Feed additives (monensin and *β*-acid beadlet) were blended into ground corn and are included in the percentage. Premixes contained 1,320 mg/kg monensin, 400, 1,000, or 2,000 mg/kg *β*-acid daily (DM). The negative control consisted of ground corn only.

[2]Provided 2,205 IU/kg vitamin A, 22 IU/kg vitamin E, 0.1% added sodium, 0.15% added chlorine, 0.7% calcium, 0.7% potassium, 10 mg/kg copper, 60 mg/kg zinc, 60 mg/kg manganese, 0.5 mg/kg iodine, 0.15 mg/kg cobalt, and 0.3 mg/kg selenium in the total diet DM.

Table 2.2 Average *β*-acid content (mg/kg) of diets consisting 0 (Control), 10 (Beta 10), 25 (Beta 25), 50 (Beta 50) mg *β*-acid extract (DSM Nutritional Products, France)/kg diet DM, or 33 mg monensin (Elanco Animal Health, Greenfield, IN)/kg diet DM (MON).

	Treatment					
Shipment	Control	Beta 10	Beta 25	Beta 50	MON	SEM[1]
1	0	0 (0)**	0 (0)	2.9 (.05)	0	1.98
2	0	0 (0)	3.0 (11)	11.7 (21)	0	
3	0	0 (0)	5.0 (18)	9.5 (17)	0	
4	0	1.3 (12)	6.2 (23)	18.7 (34)	0	
5	0	4.6 (42)	14.0 (51)	20.8 (38)	0	
6	0	15.3 (139)	12.3 (45)	22.4 (41)	0	
7	0	13.4 (121)	13.3 (48)	24.4 (45)	0	
Mean	0	4.9 (45)	7.7 (28)	15.8 (29)	0	
STDEV[2]	0	6.7 (60)	5.5 (20)	7.9 (14)	0	

*The *β*-acid beadlet additive was expected to provide 10% of active product and after analysis was observed to contain 10.99% active product on average.

[1]SEM = Standard error of the mean.

**Values in parentheses represent the actual percentage of expected value.

[2]STDEV = Standard deviation of the mean.

Table 2.3 Performance of heifers fed 0 (Control), 10 (Beta 10), 25 (Beta 25), 50 (Beta 50) mg *β*-acid extract (DSM Nutritional Products, France)/kg diet DM, or 33 mg monensin (Elanco Animal Health, Greenfield, IN)/kg diet DM (MON).

	Treatments						Contrast, *P*-value[3]				
Item	Control	Beta 10[1]	Beta 25	Beta 50	MON	SEM[2]	1	2	3	4	5
Number	16	15	16	16	16						
Days on feed	147	147	147	147	147						
Initial BW, kg	391.2	389.0	387.5	386.4	388.7	2.30	0.40	0.15	0.68	0.12	0.57
Final BW, kg	595.7	598.3	602.0	593.3	601.7	7.34	0.55	0.80	0.64	0.78	0.40
ADG, kg	1.37	1.38	1.44	1.40	1.44	0.14	0.33	0.57	0.53	0.67	0.41
DMI, kg/d	9.88	9.88	9.87	9.96	9.92	0.282	0.76	0.83	0.88	0.55	0.70
G:F	0.1394	0.1405	0.1465	0.1394	0.1457	0.0132	0.35	0.62	0.52	0.94	0.28

[1]One heifer from the Beta 10 treatment group was diagnosed with severe lameness caused by digital dermatitis, and was removed from the study.

[2]SEM = standard error of the mean.

[3]No interaction between diet and day, $P > 0.05$. 1 = Control vs. MON; 2 = Control vs average of *β*-acids; 3 = Average of *β*-acids vs. MON; 4 = Linear effect of *β*-acids; 5 = Quadratic effect of *β*-acids.

Table 2.4 Repeated measures analysis for feedlot performance of heifers fed 0 (Control), 10 (Beta 10), 25 (Beta 25), 50 (Beta 50) mg *β*-acid extract (DSM Nutritional Products, France)/kg diet DM, or 33 mg monensin (Elanco Animal Health, Greenfield, IN)/kg diet DM (MON).

	Treatments						
Item	Control	Beta 10[1]	Beta 25	Beta 50	MON	SEM[2]	Pr>F[3]
Period 1							
ADG, kg	2.03	1.71	2.11	1.78	1.86	0.30	0.31
DMI, kg/d	9.49	9.09	9.21	9.61	9.35	0.603	0.85
G:F	0.2131	0.1927	0.2298	0.1848	0.2000	0.0061	0.26
Period 2							
ADG, kg	1.30	1.69	1.37	1.62	1.69		
DMI, kg/d	9.84	10.01	9.88	9.80	9.75		
G:F	0.1775	0.1711	0.1825	0.1718	0.1752		
Period 3							
ADG, kg	1.69	1.74	1.86	1.67	1.74		
DMI, kg/d	9.57	10.17	10.17	9.84	9.89		
G:F	0.1775	0.1752	0.1711	0.1825	0.1718		
Period 4							
ADG, kg	1.20	1.17	1.21	1.15	1.15		
DMI, kg/d	10.17	10.30	10.12	10.28	9.95		
G:F	0.1180	0.1121	0.1191	0.1092	0.1153		
Period 5							
ADG, kg	1.23	1.19	1.26	1.15	1.48		
DMI, kg/d	10.05	10.09	10.04	10.04	10.24		
G:F	0.1235	0.1156	0.1258	0.1131	0.1439		
Period 6							
ADG, kg	1.42	1.21	1.22	1.36	1.41		
DMI, kg/d	10.45	10.18	10.09	10.36	10.45		
G:F	0.1364	0.1172	0.1199	0.126	0.1338		
Period 7							
ADG, kg	0.76	0.98	1.08	1.06	0.79		
DMI, kg/d	9.74	9.55	9.71	9.94	9.96		
G:F	0.0745	0.1053	0.1101	0.1079	0.0787		

[1]One heifer from the Beta 10 treatment group was diagnosed with severe lameness caused by digital dermatitis, and was removed from the study.

[2]SEM = standard error of the mean.

[3]No interaction of treatment and period, $P > 0.05$. No effect of diet, $P > 0.05$. No effect of period, $P > 0.05$. Pr>F = Probability values for overall F-test.

Table 2.5 *In vivo* ruminal pH, VFA, lactate, and ammonia concentrations (mM) in heifers 24-h after feed delivery of diets containing 0 (Control), 10 (Beta 10), 25 (Beta 25), 50 (Beta 50) mg *β*-acid extract (DSM Nutritional Products, France)/kg diet DM, or 33 mg monensin (Elanco Animal Health, Greenfield, IN)/kg diet DM (MON).

	Treatments						Contrast, *P*-value[3]				
Item	Control	Beta 10[1]	Beta 25	Beta 50	MON	SEM[2]	1	2	3	4	5
pH	6.35	6.34	6.35	6.27	6.28	0.19	0.69	0.84	0.78	0.62	0.79
Acetic acid	47.3	47.8	48.4	45.7	50.3	2.89	0.35	0.75	0.13	0.68	0.52
Propionic acid	39.0	41.2	37.6	39.6	45.0	3.44	0.23	0.34	0.01	0.73	0.60
Isobutyric acid	0.7	0.8	0.8	0.7	0.8	0.05	0.80	0.55	0.76	0.61	0.37
Butyric acid	10.3	10.0	9.9	12.0	11.0	1.15	0.32	0.38	0.73	0.51	0.08
Isovaleric acid	2.8	2.6	2.8	2.6	2.6	0.3	0.92	0.57	0.48	0.56	0.78
Valeric acid	3.8	4.1	3.4	4.2	3.4	0.6	0.19	0.49	0.35	0.99	0.87
Isocaproic acid	0.05	0.05	0.05	0.06	0.05	0.01	0.94	0.54	0.59	0.40	0.57
Caproic acid	0.57	0.56	0.63	0.67	0.53	0.11	0.31	0.48	0.05	0.95	0.23
Heptanoic acid	0.13	0.11	0.12	0.12	0.10	0.03	0.82	0.57	0.38	0.59	0.89
Acetate:Propionate	1.3	1.2	1.5	1.3	1.2	0.09	0.56	0.19	0.04	0.70	0.47
Total VFA	105	107	104	106	114	7.3	0.31	0.66	0.08	0.85	0.84
Lactate	6.6	6.5	7.0	7.9	7.6	0.89	0.21	0.45	0.44	0.06	0.58
Ammonia	0.32	0.37	0.46	0.36	0.21	0.11	0.28	0.37	0.03	0.63	0.22

[1]One heifer from the Beta 10 treatment group was diagnosed with severe lameness caused by digital dermatitis, and was removed from the study.

[2]SEM = standard error of the mean.

[3]No interaction between diet and day, $P > 0.05$. No effect of day, $P > 0.05$. 1 = Control vs. MON; 2 = Control vs average of *β*-acids; 3 = Average of *β*-acids vs. MON; 4 = Linear effect of *β*-acids; 5 = Quadratic effect of *β*-acids.

Table 2.6 Composition of diet fed to cannulated steer used for *in vitro* cultures in experiment 1 and 2, DM basis.

Ingredients, %	
Wet corn gluten feed	30.00
Ground brome hay	25.00
Ground alfalfa hay	25.00
Steam-flaked corn	16.50
Feed additive premix[1]	2.16
Vitamin/mineral premix[2]	1.34
Calculated composition, %	
CP	16.67
Ca	0.77
P	0.49
K	1.36
NDF	40.46
NE_M, Mcal/lb	0.79
NE_G, Mcal/lb	0.50

[1]Formulated to provide 300 mg/d monensin and 90 mg/d tylosin (Elanco Animal Health, Greenfield, IN) in a ground-corn carrier.

[2]Formulated to provide 2,200 IU/kg vitamin A; 22 IU/kg vitamin E; 10 mg/kg added Cu; 60 mg/kg added Zn; 60 mg/kg added Mn; 0.5 mg/kg added I; 0.25 mg/kg added Se; and 0.15 mg/kg added Co.

Figure 2.1 *In vitro* experiment 1: 30-h gas production (mL) during incubation with bovine ruminal fluid and starch with 0 or 33 hops *β*-acid extract (DSM Nutritional Products, France)/kg substrate. [1]SEM = Standard error of the mean of the combination of *β*-acid and time. No interaction between *β*-acid and time, $P > 0.05$. Effect of *β*-acid, $P \leq 0.05$. Effect of time, $P \leq 0.05$. 0 mg *β*-acid/kg = Cultures containing bovine ruminal fluid and starch with 0 mg *β*-acid extract of hops/kg substrate; 33 mg *β*-acid/kg = Cultures containing bovine ruminal fluid and starch with 33 mg *β*-acid extract of hops/kg substrate.

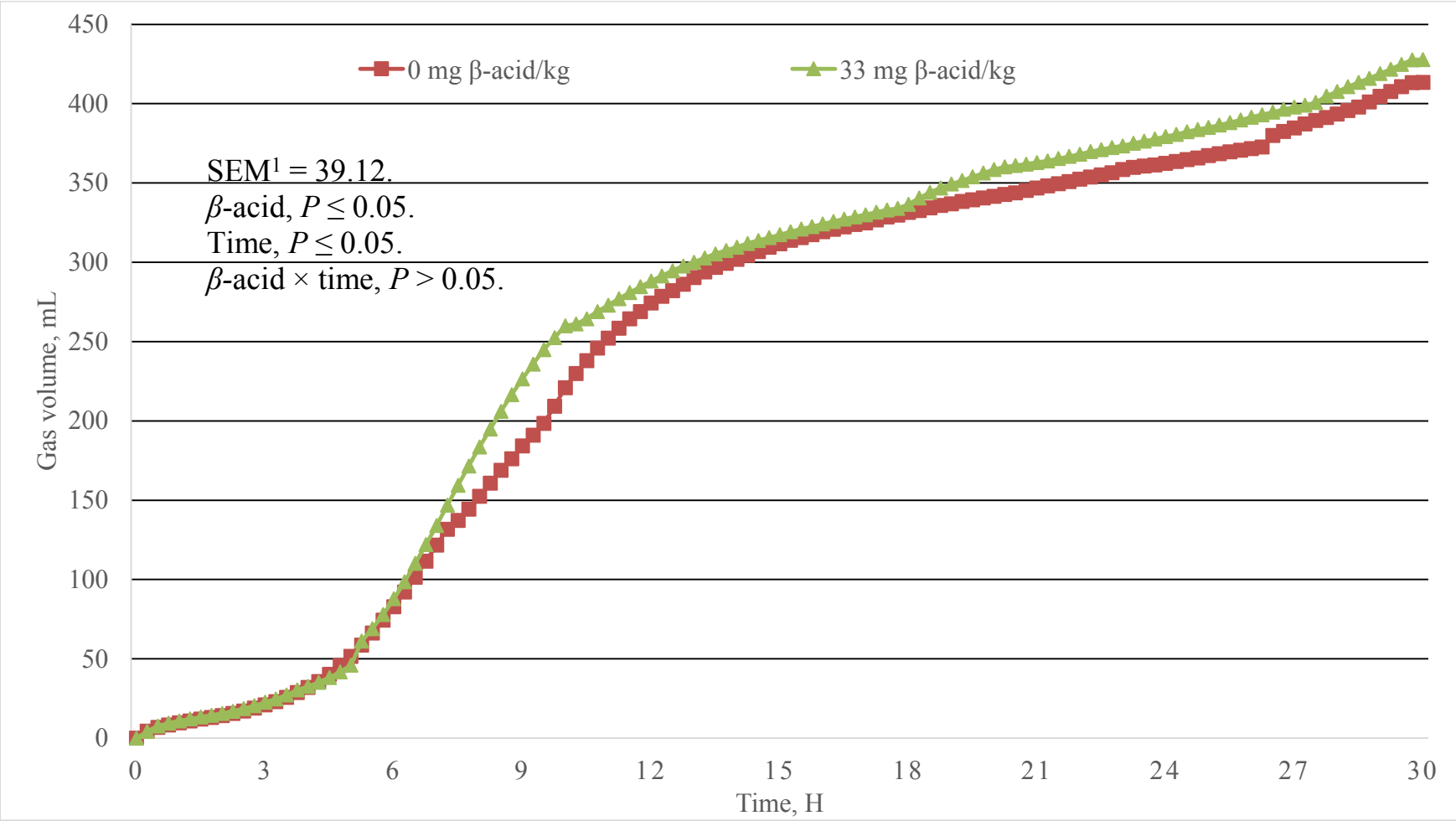
450
400
350
300
250
200
150
100
50
0
Gas volume, mL
0 mg β-acid/kg
33 mg β-acid/kg
SEM[1] = 39.12.
β-acid, P ≤ 0.05.
Time, P ≤ 0.05.
β-acid × time, P > 0.05.
0
3
6
9
12
15
18
21
24
27
30
Time, H

Table 2.7 *In vitro* experiment 1: Terminal pH, IVDMD (%), VFA (mM), lactic acid (mM), and acetate:propionate following 30-h incubation of bovine ruminal fluid with starch and 0 or 33 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg substrate.

Item	0 Beta	33 Beta	SEM[1]	P-value[2]
pH	5.25	5.32	0.04	0.23
IVDMD, %	45.79	44.20	3.99	0.79
Acetic acid	56.4	54.6	1.92	0.39
Propionic acid	50.4	48.5	2.04	0.29
Butyric acid	13.8	13.2	1.55	0.47
Total VFA	124.6	120.2	5.16	0.17
Lactic acid	48.4	42.0	6.52	0.06
Acetate:Propionate	1.12	1.13	0.014	0.003

[1]SEM = Standard error of the mean.

[2]No effect of *β*-acid, $P > 0.05$.

Table 2.8 *In vitro* experiment 2: pH at 0, 6, 12, 18, 24, and 30 h during incubation of bovine ruminal fluid with starch and 0 or 33 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg substrate.

Hour*	0 Beta	33 Beta	SEM[1]
0[a]	7.43	7.35	0.05
6[b]	6.59	6.55	
12[c]	5.71	5.66	
18[de]	5.54	5.44	
24[d]	5.43	5.45	
30[e]	5.51	5.59	

[1]SEM = Standard error of the mean of the combination of *β*-acid and hour.

*No interaction between *β*-acid and time, $P > 0.05$. No effect of *β*-acid, $P > 0.05$. Effect of hour, $P \leq 0.05$.

[a-e] Hours without common superscripts represent an effect of hour, $P \leq 0.05$.

Table 2.9 *In vitro* experiment 2: IVDMD (%) during 30-h incubation with bovine ruminal fluid with starch and 0 or 33 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg substrate.

Hour*	0 Beta	33 Beta	SEM[1]
0[a]	40.54	40.28	3.23
6[bd]	69.37	77.92	
12[b]	71.34	72.92	
18[de]	80.95	77.32	
24[ce]	79.57	81.05	
30[c]	83.83	87.56	

[1]SEM = Standard error of the mean of the combination of *β*-acid and hour.

*No interaction between *β*-acid and time, $P > 0.05$. No effect of *β*-acid, $P > 0.05$. Effect of hour, $P \leq 0.05$.

[a-e] Hours without common superscripts represent an effect of hour, $P \leq 0.05$.

Table 2.10 *In vitro* experiment 2: Acetic acid concentrations (mM) during 30-h incubation of bovine ruminal fluid with starch and 0 or 33 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg substrate.

Hour*	0 Beta	33 Beta	SEM[1]
0[a]	13.4	13.2	1.40
6[b]	16.9	17.7	
12[c]	24.0	25.5	
18[d]	27.7	30.6	
24[d]	30.1	31.4	
30[d]	31.6	30.2	

[1]SEM = Standard error of the mean of the combination of *β*-acid and hour.

*No interaction between *β*-acid and time, $P > 0.05$. No effect of *β*-acid, $P > 0.05$. Effect of hour, $P \leq 0.05$.

[a-d] Hours without common superscripts represent an effect of hour, $P \leq 0.05$.

Table 2.11 *In vitro* experiment 2: Propionic acid concentrations (mM) during 30-h incubation of bovine ruminal fluid with starch and 0 or 33 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg substrate.

Hour*	0 Beta	33 Beta	SEM[1]
0[a]	4.1	4.2	1.06
6[b]	8.6	9.4	
12[c]	15.6	16.1	
18[d]	19.8	22.7	
24[e]	25.7	26.7	
30[e]	26.0	26.4	

[1]SEM = Standard error of the mean of the combination of *β*-acid and hour.

*No interaction between *β*-acid and time, $P > 0.05$. No effect of *β*-acid, $P > 0.05$. Effect of hour, $P \leq 0.05$.

[a-e] Hours without common superscripts represent an effect of hour, $P \leq 0.05$.

Table 2.12 *In vitro* experiment 2: Butyric acid concentrations (mM) during 30-h incubation of bovine ruminal fluid with starch and 0 or 33 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg substrate.

Hour*	0 Beta	33 Beta	SEM[1]
0[a]	2.5	2.5	0.32
6[a]	2.8	3.1	
12[b]	4.1	4.1	
18[c]	4.6	5.2	
24[d]	6.3	6.5	
30[e]	8.4	8.1	

[1]SEM = Standard error of the mean of the combination of *β*-acid and hour.

*No interaction between *β*-acid and time, $P > 0.05$. No effect of *β*-acid, $P > 0.05$. Effect of hour, $P \leq 0.05$.

[a-e] Hours without common superscripts represent an effect of hour, $P \leq 0.05$.

Table 2.13 *In vitro* experiment 2: Total VFA concentrations (mM) during 30-h incubation of bovine ruminal fluid with starch and 0 or 33 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg substrate.

Hour*	0 Beta	33 Beta	SEM[1]
0[a]	20.6	20.9	2.69
6[b]	28.7	31.0	
12[c]	45.3	46.7	
18[d]	51.7	59.6	
24[e]	63.6	66.0	
30[e]	66.8	67.0	

[1]SEM = Standard error of the mean of the combination of *β*-acid and hour.

*No interaction between *β*-acid and time, $P > 0.05$. No effect of *β*-acid, $P > 0.05$. Effect of hour, $P \leq 0.05$.

[a-e] Hours without common superscripts represent an effect of hour, $P \leq 0.05$.

Table 2.14 *In vitro* experiment 2: Lactic acid concentrations (mM) during 30-h incubation of bovine ruminal fluid with starch and 0 or 33 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg substrate.

Hour*	0 Beta	33 Beta	SEM[1]
0[a]	4.3	0.0	5.16
6[a]	0.4	2.5	
12[b]	18.6	21.5	
18[b]	23.8	23.6	
24[b]	17.2	12.0	
30[b]	15.4	18.4	

[1]SEM = Standard error of the mean of the combination of *β*-acid and hour.

*No interaction between *β*-acid and time, $P > 0.05$. No effect of *β*-acid, $P > 0.05$. Effect of hour, $P \leq 0.05$.

[a-b] Hours without common superscripts represent an effect of hour, $P \leq 0.05$.

Table 2.15 *In vitro* experiment 2: Acetate:propionate during 30-h incubation of bovine ruminal fluid with starch and 0 or 33 mg *β*-acid extract of hops (DSM Nutritional Products, France)/kg substrate.

Hour*	0 Beta	33 Beta	SEM[1]
0[a]	3.2	3.1	0.05
6[b]	1.9	1.9	
12[c]	1.6	1.6	
18[d]	1.3	1.4	
24[e]	1.2	1.2	
30[e]	1.1	1.1	

[1]SEM = Standard error of the mean of the combination of *β*-acid and hour.

*No interaction between *β*-acid and time, $P > 0.05$. No effect of *β*-acid, $P > 0.05$. Effect of hour, $P \leq 0.05$.

[a-e] Hours without common superscripts represent an effect of hour, $P \leq 0.05$.