EXAMINING THE EFFECTS OF ADDING FAT, IONOPHORES, ESSENTIAL OILS, AND MEGASPHAERA ELSDENII ON RUMINAL FERMENTATION WITH METHODS IN VITRO AND IN VIVO

DISSERTATION

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ABSTRACT

Much attention has been drawn to the fatty acids (FA) present in the meat and milk from ruminant animals, which are often blamed for their high content of saturated FA, the intake of which is correlated with an increased risk of cardiovascular diseases in humans, and for the relatively low content of health promoting polyunsaturated FA (PUFA). Ruminal biohydrogenation (BH) can be manipulated to alter the amount of BH intermediates, such as PUFA, and decrease the flow of trans-10, cis-12 conjugated linoleic acid (CLA), which is an inhibitor for milk fat synthesis in dairy cattle. However, the BH of different FA in different feeds for ruminants is not fully understood in terms of microbes and metabolic processes related to BH. Methane mitigation and reducing the excretion of urinary N with dietary strategies are the major foci for ruminant nutritionists in terms of reducing the environmental impact of dairy operations. The combination of monensin and essential oil has been reported to suppress protozoa and methane production, while maintaining normal rumen function and minimizing the risk of metabolic diseases for cows in early lactation. A higher level of concentrate in the ration postpartum than prepartum places cows at high risk for rumen acidosis. Administration of the lactate utilizer, Megasphaera elsdenii, to ruminants has been suggested for reducing the incidence of acidosis.
In the first experiment, the objective was to determine the effects of raw and roasted soybeans, corn oil, soybean oil, and dried distillers grains with solubles (DDGS) and different particle sizes on the BH pattern of FA in vitro. Fat sources combined with grass hay were formulated to achieve 10% FA (dry matter basis; DMB) in each treatment. Incubation was maintained for 24 h in tubes, and fermentations of each treatment were stopped at 0, 1, 2, 4, 8, 12, 16 and 24 h. The fractional rates of disappearance of 18:2 and 18:3 were estimated by a single pool, first-order kinetic model. The BH differed with fat sources. Overall, DDGS had the highest BH among the treatments. Roasted soybeans, corn oil, and soybean oil had similar BH, which were lower compared with DDGS. Roasting and particle size did not affect overall BH. However, the roasting process and particle size affected the rate of disappearance of 18:2 in soybeans. Particle size exerted minimum effect on BH, but the particle sizes differed at most by 1 mm in this study.

The second experiment was conducted in a modified dual flow continuous culture system. The objective was to determine the effects of feeding Rumensin® (ionophore with active component of monensin sodium) and Cinnagar® (essential oil of cinnamon and garlic) in diets on ruminal fermentation characteristics. Four continuous culture fermenters were maintained in 4 periods in a 4 X 4 Latin square design with 4 dietary treatments arranged in a 2 x 2 factorial: (1) control diet, 40 g of a 50:50 concentrate:forage diet containing no additive; (2) Rumensin at 11g/909 kg of DM; (3) Cinnagar at 0.0043% (DM basis); and (4) a combination of Rumensin and Cinnagar at the levels used in (2) and (3). Protozoa counts were used to calculate their generation.
times. A by-difference procedure involving boiling and sonication was developed to determine the protozoal N per cell to minimize feed contamination. There were no effects of treatment on protozoal generic distribution, concentration of NH$_3$-N, total N flow of effluent, production of total VFA, and flows of CLA and total C18. Rumensin decreased acetate:propionate ratio and BH of total C18 and cis-9 18:1; Rumensin increased concentration of peptide, flow of trans-11 18:1, and protozoal generation time from 22 to 27 h. Rumensin tended to decrease protozoal counts in effluent flow and flow of 18:0, and increase propionate production. Cinnagar decreased true OM digestibility and protozoal N flow of effluent. Cinnagar increased non-ammonia non-microbial N flow of effluent. Cinnagar tended to decrease protozoal counts, microbial N flow of effluent, NDF digestibility, and protozoal N per cell. Cinnagar tended to increase BH of total C18, 18:2, and 18:3. Cinnagar and Rumensin tended to interact for increased methane production and bacterial N flow. Under the conditions of our study, we did not detect an additive response for Rumensin® and Cinnagar® to decrease protozoal counts or methane production.

The final experiment was designed to (1) determine if _Megasphaera elsdenii_ (Lactipro; MSBiotec, Littleton, CO) orally administered to transition dairy cows would improve milk yield and reduce the risk for metabolic disease, and (2) to monitor the establishment and persistence of _M. elsdenii_ in the rumen using real-time PCR. Thirty primi- and multiparous Jersey cows were used in a randomized complete block design until 90 DIM. Within each block, the cows were assigned to 1 of 2 treatments: 1) control (no dose), or 2) 200 mL Lactipro by oral drenching at 1 to 2 d postpartum (_M. elsdenii_, 1
x $10^8$ cfu/mL). Close-up cows were fed 60% forage, and after calving, cows were fed 2 different diets (30 DIM and 31 to 90 DIM) containing 50% forage. The DMI, BCS, and BW change were similar between treatments, and no treatment by time interactions were detected. There was no difference in milk yield between the treatments (29.1 and $28.9 \pm 0.89$ kg/d). A treatment by parity interaction was found for milk yield ($P < 0.01$), and a trend ($P = 0.06$) for a treatment by parity interaction was found for milk fat yield. For cows with $\geq 3$ lactations, those dosed with *M. elsdenii* had higher milk yield and tended to have higher milk fat yield than the control cows. No treatment effect or treatment by time interactions were detected for milk fat and protein percentages. Based on the urine ketones, 6.7% of the cows experienced clinical ketosis. The cows with $\geq 15$ mg/dL urine ketones were 13.3% for the control cows and 6.7% for the dosed cows. The *M. elsdenii* population was numerically greater in the 2 dosed cows than the 2 control cows, and the higher population may have persisted for about 2 d after dosing. Dosing Jersey cows with *M. elsdenii* may enhance the milk yield in early lactation cows with $\geq 3$ lactations.

In summary, the one-stage incubation *in vitro* allowed us to assess the BH of certain FA sources in a closed system. BH *in vitro* can provide information to relate BH of FA in different fat sources *in vivo*. The continuous culture system, as a two-stage *in vitro* incubation, with retained diverse protozoal population allowed the study on ruminal fermentation characteristics under different dietary conditions in a controlled environment. Further research is needed on cell size change of ruminal protozoa upon the Cinnagar addition and how to inhibit protozoa enough to decrease their negative effects but not so much to disrupt normal microbial ecology of the rumen. The study of dosing
dosing *M. elsdenii* to dairy cows shed light on the potential benefit of this probiotic on the ruminal conditions and performance of dairy cows. The molecular techniques allowed us to monitor the establishment and persistence of *M. elsdenii* in the rumen. A greater number of cannulated cows are needed to better understand the change of population of *M. elsdenii*. 
Dedicated to my family and friends who have always been there for me.
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x
TABLE OF CONTENTS

ABSTRACT .......................................................................................................................................................... ii
DEDICATION ......................................................................................................................................................... vi
ACKNOWLEDGMENTS ........................................................................................................................................ viii
VITA .................................................................................................................................................................... x
TABLE OF CONTENTS ......................................................................................................................................... xi
LIST OF TABLES ...................................................................................................................................................... xiii
LIST OF FIGURES .................................................................................................................................................. xv

CHAPTERS                                      PAGES

1. INTRODUCTION .....................................................................................................................................1
2. REVIEW OF LITERATURE ...................................................................................................................5
   MANIPULATION OF RUMINAL BIOHYDROGENATION ..........................................................5
   METHODS TO STUDY RUMINAL FERMENTATION AND METHANE PRODUCTION..........................11
   DIETARY ADDITIVES ALTER RUMINAL FERMENTATION AND NITROGEN METABOLISM.....................14
   METHODOLOGIES FOR DETERMINATION OF CHANGES IN MICROBIAL POPULATION .........................17
   TRANSITION COWS AND MEGASPHAERA ELSDENII ..................................................................21
   SUMMARY .................................................................................................................................................27
3. EFFECTS OF DIFFERENT SOURCES OF FAT ON THE BIOHYDROGENATION OF FATTY ACIDS IN VITRO .................................................................29
   ABSTRACT ..................................................................................................................................................29
INTRODUCTION ...........................................................................................................30
MATERIALS AND METHODS ..................................................................................32
RESULTS AND DISCUSSION ..................................................................................36
CONCLUSIONS .........................................................................................................42

4. ESSENTIAL OIL AND RUMENSIN AFFECT RUMINAL FERMENTATION IN CONTINUOUS CULTURE ..........................................................51
   ABSTRACT ..............................................................................................................51
   INTRODUCTION ....................................................................................................52
   MATERIALS AND METHODS .............................................................................55
   RESULTS AND DISCUSSION .............................................................................59
   CONCLUSIONS ....................................................................................................73

5. EXAMINING THE EFFECT OF ORAL ADMINISTRATION OF MEGASPHAERA ELSDENII ON PERFORMANCE OF JERSEY COWS DURING EARLY LACTATION ..........................................................82
   ABSTRACT ..............................................................................................................82
   INTRODUCTION ....................................................................................................83
   MATERIALS AND METHODS .............................................................................86
   RESULTS ...............................................................................................................91
   DISCUSSION .......................................................................................................95
   CONCLUSIONS ...................................................................................................103

6. CONCLUSIONS .....................................................................................................114

LIST OF REFERENCES ..............................................................................................117

Appendix A: Protocol of Repeated Bead Beating Plus Column (RBB+C) Method ..........................................................141
Appendix B: Protocol of Real-Time PCR ................................................................144
LIST OF TABLES

Tables

3.1 Fatty acid profiles of the treatments with grass hay and different sources of fat ..... 43
3.2 Fractional rates (k) of disappearance of 18:2 and 18:3 and biohydrogenation (BH) of C18 after incubation for 24 hours \textit{in vitro} ................................................................. 44
3.3 Areas under the curve (mg x h) of C18 during incubation \textit{in vitro} with various treatments ........................................................................................................................................... 45
4.1 Ingredient and nutrient composition of diets without (-) or with (+) Rumensin\textsuperscript{®} and Cinnagar\textsuperscript{®} ............................................................................................................................... 74
4.2 Fatty acid (FA) composition of the dietary treatments without (-) or with (+) Rumensin\textsuperscript{®} and Cinnagar\textsuperscript{®} .................................................................................................................................................. 75
4.3 Protozoal counts, generation time, generic distribution, and protozoa cell volume in continuous cultures fed diets without (-) or with (+) Rumensin\textsuperscript{®} and Cinnagar\textsuperscript{®} ................................................................................................................................................. 76
4.4 The neutral detergent fiber (NDF) and organic matter (OM) digestibilities, ammonia nitrogen, peptide nitrogen and N fractions and bacterial efficiency in continuous cultures fed diets without (-) or with (+) Rumensin\textsuperscript{®} and Cinnagar\textsuperscript{®} .... 77
4.5 Fermentation characteristics in continuous cultures fed diets without (-) or with (+) Rumensin\textsuperscript{®} and Cinnagar\textsuperscript{®} .................................................................................................................................................. 79
4.6 Flow and biohydrogenation of long-chain fatty acids (FA) in effluent from continuous cultures fed diets without (-) or with (+) Rumensin® and Cinnagar® .... 80

5.1 Ingredients and chemical compositions of the close-up, fresh, and lactating cow TMR (% of DM)........................................................................................................................................ 104

5.2 Least square means of dry matter intake, body weight, body condition score, urine ketones, serum NEFA and BHBA, milk production, and milk composition in cows not dosed and dosed with Megasphaera elsdenii................. 105
LIST OF FIGURES

Figures

2.1 Pathways of the ruminal biohydrogenation of 18:2 and 18:3. ........................................... 28

3.1 Biohydrogenation of FA averaged by replicate during incubation of treatments in vitro with grass hay (GH) and different sources of fat. .................................................. 46

3.2 Presence of 18:0, 18:1, 18:2, and 18:3 during incubation in vitro for the 7 treatments ........................................................................................................................................ 47

4.1 Procedure of boiling and sonication on protozoa ................................................................. 81

5.1 Dry matter intake from week 1 to 4 of lactation for control cows and cows dosed with \textit{Megasphaera elsdenii} ........................................................................................................ 106

5.2 Body weight change by week of lactation ........................................................................... 107

5.3 Body condition score from 2 weeks prepartum to 90 DIM for control cows and cows dosed with \textit{Megasphaera elsdenii}. ............................................................................................................ 108

5.4 Milk production from week 1 to 13 of lactation for control cows and cows dosed with \textit{Megasphaera elsdenii} ........................................................................................................... 109

5.5 Treatment by parity interaction ($P < 0.01$) for milk yield from week 1 to 13 of lactation for control cows and cows dosed with \textit{Megasphaera elsdenii}. ......................... 110

5.6 Treatment by parity interaction ($P = 0.06$) for milk fat yield from week 1 to 4 for control cows and cows dosed with \textit{Megasphaera elsdenii}. ........................................... 111
5.7 Treatment by parity interaction (P < 0.05) for milk urine nitrogen (MUN) from week 1 to 4 for control cows and cows dosed with *Megasphaera elsdenii*. ........ 112

5.8 Populations of *Megasphaera elsdenii* in cannulated control cows and cows dosed with *Megasphaera elsdenii* ............................................................................................................ 113
CHAPTER 1
INTRODUCTION

Agriculture is the second largest methane source in the United States, accounting for 6 to 8% of the total US greenhouse gas emissions with methane emission estimated to be 428.4 Tg CO$_2$E (EPA, 2011). The growing concern over the contributions of animal agriculture to global warming has drawn attention to the research on reducing the methane production from ruminants. Dairy farms are the major agricultural source for that because of the number of cows and the magnitude of feed intake and associated fermentation. Worldwide, ruminants excrete 70 billion kg of N each year (Sheldrick et al., 2003), including 1 billion kg N for U.S. dairy cattle (St-Pierre and Thraen, 1999). Understanding the relationships among ruminal bacteria, protozoa, and methanogens, and the production and utilization of hydrogen in the rumen ecosystem is important for methane mitigation.

Ruminants have a lower efficiency of N utilization compared with non-ruminants. The overall average efficiency of N utilization (g N in product/g N intake) for ruminants is 25%, with a wide range from 15 to 40% among experiments (Kohn et al., 2005; Huhtanen and Hristov, 2009). Excessive ammonia produced by protein degradation in the rumen is released into the blood stream and converted to urea by the liver. The urea is excreted in urine or reenters the digestive tract by diffusion into saliva or directly across
the gut wall. The existence of epithelial transport proteins facilitating the transfer of urea-N from the blood to the gut provides the possibility that the urea-N transfer could be manipulated by dietary management or genetic means (Calsamiglia et al., 2010). The urea in the urine is converted to ammonia, nitrates, or nitrous oxide by the microorganisms in soil and feces. These compounds can contribute N to water or air and cause a health hazard to humans, as well as aquatic species. Generally, in terms of environmental pollution, urinary N has a greater impact than fecal N. Reducing methane emission and excretion of urinary N with whole farm nutrient balancing should be the major focus in terms of reducing the environmental impact of dairy operations.

Another health related topic is the fatty acids (FA) in the meat and milk from ruminant animals, which are often blamed for their high content of saturated fatty acids (SFA), the intake of which is correlated with an increased risk of chronic diseases such as cardiovascular diseases in humans (Burlingame et al., 1999), and for the relatively low content of health promoting polyunsaturated fatty acids (PUFA). Attempts have been made to increase the amount of cis-9, trans-11 conjugated linoleic acid (CLA) in ruminant fat because of its reported health benefits including anticarcinogenic and antiatherogenic effects (Kritchevsky et al., 2004; Lock et al., 2004). Inhibition of methane production in the rumen might also affect biohydrogenation (BH) of unsaturated fatty acids (UFA). Two groups of bacteria are involved in the BH of FA. The first is group “A”, including some fiber digesting bacteria (Polan et al., 1964; Kepler et al., 1966; Kemp et al., 1975), which isomerize and reduce UFA to trans-11 C 18:1. The other is group “B” bacteria which catalyze the rate-limiting step for BH, reduction of trans-11
18:1 to 18:0. In co-cultures, if methane production is inhibited, the growth of group “A”
bacteria might be decreased due to the accumulation of the metabolic H, which is one of
the end products of fiber degradation. Therefore, the BH might be hindered, resulting in
increased cis-9, trans-11 and trans-11 intermediates.

The major objective of my research was to assess the changes in the ruminal
fermentation and microbial population in vitro and in vivo when different rumen
modifiers were added to the diet. In the first experiment, different fat sources, including
corn oil, soybean oil, raw and roasted soybeans, and dried distiller's grain with solubles
(DDGS) with different particle sizes, were incubated for 24h in vitro. The BH patterns
consisting of the extent of BH at different incubation time points were assayed and
compared. The next experiment was for determining the effects of feeding Rumensin®
and Cinnagar® (essential oil from cinnamon and garlic) in diets on ruminal fermentation
characteristics using a dual flow continuous culture. Data are presented showing the
effect of the 2 modifiers on the protozoal pool size and generation time, ruminal
fermentation measurements, methane production, and BH. In addition, the protozoal pool
size and N per cell were found to be related to the microbial N flow. The final experiment
was conducted to determine if Megasphaera elsdenii (Lactipro®) administered to
transition dairy cows improves milk yield and reduces the risk of metabolic disease. Data
are presented for dry matter intake, body weight change, body condition score, urine
ketone, serum non-esterified FA and β- hydroxybutyrate, milk yield, and milk
components of the cows not dosed and those dosed with M. elsdenii. The population of
M. elsdenii in the cows and their persistence in the rumen using real-time PCR technique were examined.
CHAPTER 2
REVIEW OF LITERATURE
MANIPULATION OF RUMINAL BIOHYDROGENATION

Milk fat production and milk FA composition have attracted great interest because of their close relation with human nutrition. Besides their contribution to the texture and quality of dairy products and to the amount of dietary energy, different FA components in the ruminant milk, such as short- and medium-chain, mono- and polyunsaturated, cis and trans, conjugated FA, etc. exert potentially positive or negative effects on the health of consumers (Parodi, 2004).

Ruminal BH is the saturation of dietary UFA in the rumen. The trans FA are produced by the ruminal BH of linoleic and linolenic acids, and VA is the predominant isomer produced, as shown by the pathway for the BH of linoleic and linolenic acids (Figure 2.1). The BH limits the availability of PUFA, especially 18:2n-6, which serve as the major source of CLA in food derived from ruminants, with about 70 and 25% coming from dairy products and red meat, respectively (Ritzenhaler et al., 2001). The major CLA isomer of intermediates of BH, cis-9, trans-11 CLA, or rumenic acid, represents about 75 to 90% of the total CLA in ruminant products, such as milk, meat, and butter (Bauman and Grinari, 2003). Attempts have been made to increase the amount of cis-9, trans-11 CLA in ruminant fat because of their reported health benefits to reduce the risk
for cardiovascular diseases (Kritchevsky et al., 2004; Lock et al., 2004). Another intermediate of BH, *trans*-10, *cis*-12 CLA, was first identified as a potent inhibitor of milk fat synthesis by Bauman and Griinari (2001). They found that for many situations of diet-induced milk fat depression (MFD), the increase of *trans*-10, *cis*-12 CLA in milk fat due to the altered BH was correlated with reduction of milk fat yield. This accomplishment has helped people to better modify the BH pathway to avoid *trans*-10, 
*cis*-12 CLA production and increase the *cis*-9, *trans*-11 CLA in the milk.

Lipolysis releases the non-esterified fatty acids that form the substrates for BH. Ruminal pH affects the BH. Feeding rations with low forage to ruminants can decrease lipolysis of triglycerides and BH due to the decreased ruminal pH (Harfoot and Hazlewood, 1988; Van Nevel and Demeyer, 1996). The liberation of PUFA by lipolysis, however, seemed to be susceptible to inhibition as pH declined below 6.3 (Van Nevel and Demeyer, 1996). Consistently, Qiu et al. (2004) reported that low pH may increase intermediates of BH, such as CLA. Changing the diet from a high to a low forage type can hinder lipolytic and BH activities *in vitro* and decrease the number of ruminal lipolytic bacteria (Latham et al., 1972). However, Van Nevel and Demeyer (1996) reported that lipolytic activity was not affected by the presence of hay. They reported an inhibited lipolysis at pH ≤ 6.0, and the inhibition became stronger with decreasing pH. Incubations with 80 mg of soybean oil, at the same pH, had a stronger inhibition of lipolysis than with 40 mg. Griinari et al. (1998) showed that a low fiber diet decreased the rumen pH and increased proportion of propionate. They also reported that the low fiber diet and UFA increased concentration of *trans*-10 18:1 in milk fat. Lee (2013) showed
that BH pathways of linolenic acid partially switched under different pH conditions, with a strong influence on the cis-cis CLA at low pH.

The extent of BH is determined by the characteristics of the fat sources, passage rate of feedstuffs in the rumen, and BH capacities of the ruminal microorganisms. Supplementation of fat not only alters metabolic activity of ruminal microorganisms and increase FA flow from the rumen, but also increases energy content of the ration with minimal changes in the dietary forage to concentrate ratio, especially valuable for high producing cows (Mattias, 1982; Palmquist, 1984). However, unprotected fat, especially UFA added at high levels can negatively affect fiber digestion (Devendra and Lewis, 1974) and milk fat synthesis in the mammary gland. Remarkable increases in trans-10 18:1 concentration have been reported in ewes’ milk when the diet was supplemented with high levels of unprotected oil (Mele et al., 2006; Reynolds et al., 2006; Gómez-Cortés et al., 2008). The increased concentration of trans-10 18:1 in milk fat is generally attributed to a shift in the BH pathways, due the altered ruminal environment as a result of dietary changes (Chilliard and Ferlay, 2004; Palmquist et al., 2005).

Efforts have been focused on variable effects of fat sources on BH. One factor to cause the differences in BH is degree of saturation. The toxic effect of PUFA on BH bacteria (dominated by Butyrivibrio fibrisolvens and related taxa) and many other ruminal bacteria increased as the degree of UFA increased (Zhang et al., 2008). Thus, the toxicity of non-esterified PUFA (Maia et al., 2007) released by lipase in the rumen would have to be removed by BH, which seems to be a defense mechanism of the microbes. Also, having a free carboxyl group is critical for inhibition. Lipolysis is a prerequisite for
BH to proceed because the isomerase that catalyzes the initial step to form the \textit{trans}-11 isomer is not functional unless the FA has a free carboxyl group. Feeding of protected lipids, such as calcium salts of LCFA, fatty alcohols, fatty acyl amides, and triglycerides, is a common practice to achieve greater passage of UFA to the duodenum (Czerkawski et al., 1966; Jenkins and Palmquist, 1984; Fotouhi and Jenkins, 1992). Lundy et al. (2004) showed that calcium salts and amide derivatives of FA were both effective in enhancing omasal flow of UFA in lactating dairy cows, and amides were more effective than calcium salts for increasing the postruminal flow of oleic acid.

Duckett and Gillis (2010) suggested that fish oil addition altered ruminal formation of BH intermediates that is dependent on oil source supplemented in the diet. They observed that fish oil inclusion increased the outflow of n-3 FA, \textit{trans}-10 18:1, and the majority of CLA isomers, including \textit{cis}-9, \textit{trans}-11. \textit{Trans}-9 18:1 and \textit{trans}-11 18:1 flows to the duodenum were increased when fish oil was included in the canola oil-supplemented diet; however, no changes were observed when fish oil was included in the corn oil-supplemented diet. The higher BH of C18:2 for raw soybeans than for extruded and roasted soybeans reported in Reddy et al. (1994) is similar to the result presented by Troegeler-Meynadier et al. (2006). There are limited reports comparing the effects of particle size of soybeans on BH. Tice et al. (1994) reported minor effects of particle size of roasted soybeans on utilization of FA, but they suggested larger particles may limit availability of C18 FA for uptake by bacteria because concentrations of 12:0 and 16:0 in bacteria linearly decreased and concentration of 18:1 in bacteria tended to increase as particle size of soybean was reduced. Results of the experiment \textit{in vitro} by Whitney et al.
(2000) showed that among treatments of corn, soybean meal, soybean oil, and soybean, 18:3 was the only FA completely BH, therefore the BH of 18:1 and 18:2 was not complete. The milk fat from cows receiving coconut cake contained less 18:1 n-9 and more C12 and C14 than typical (Hilditch and Jasperson, 1943). Storry et al. (1971) demonstrated that the contents and yields of fat in milk tended to be higher on the coconut oil supplemented rations when compared with those on the basal ration. Another aspect with supplementing fat is protozoal suppression, which probably depends on dietary conditions. Firkins (1996) showed that the suppression was enhanced more in high grain rations than with high forage, which agrees with similar trends in more recent publications (Machmüller and Kreuzer, 1999; Eugene et al., 2004).

The ionophore monensin has been shown to decrease the rate of ruminal BH in vitro (Fellner et al., 1997) because monensin inhibits some of the lipolytic bacteria, which are important for hydrolyzing the FA from triglycerides (or other esterified forms) (McGuffey et al., 2001). This could lead to increased accumulation of intermediates during the BH, which is supported by AlZahal et al. (2008), who reported monensin increased the concentration of CLA in milk fat. However, higher grain rations have been associated with the formation of trans FA that increase the risk of MFD when monensin is fed, and protozoa likely help to reduce the formation of these trans FA (Karnati et al., 2009a).

Essential oils (EO) are volatile, aromatic compounds that can be extracted from plants by steam distillation. As the potential feed additive to manipulate bacterial populations involved in ruminal BH (Calsamiglia et al., 2007) due to its inhibition on
methanogenesis and BH of long-chain PUFA, EO have antimicrobial activity against gram-positive and gram-negative bacteria, protozoa, and fungi (McIntosh et al., 2003). The components of EO vary remarkably due to different ways of extraction and plant sources. The EO has been widely studied mostly in vitro with fairly inconsistent results, especially rumen methanogenesis (Benchaar and Greathead, 2011). The direct effect of EO on ruminal long-chain PUFA metabolism has not been extensively studied (Calsamiglia et al., 2007). Ethanolic extracts of EO from Australian plants selectively inhibited the growth of pure cultures of Clostridium proteoclasticum involved in the terminal step in the BH of linoleic acid, resulting in the accumulation of intermediates, such as CLA and VA in batch culture incubations (Durmic et al., 2008). Using a continuous culture fermenter system, Lourenço et al. (2008) found that cinnamaldehyde decreased apparent BH of 18:2n-6 and 18:3n-3 and shifted the BH away from the trans-11 pathway toward the secondary BH pathway of 18:2, leading to the accumulation of trans-10 C18:1 and trans-10, cis-12 CLA. This trans-10 “shift” has been documented to cause MFD in vivo (Jenkins et al., 2008). However, in a recent study (Benchaar and Chouinard, 2009), supplementation of TMR for lactating dairy cows with cinnamaldehyde (1 g/d) did not modify the FA profile of milk fat. The difference between the effects seen in continuous culture versus in vivo could be due to the absence of protozoa in continuous culture. Protozoa do not contribute to BH directly, but they could affect activity or populations of lipid-metabolizing bacteria by selective predation (Karnati et al., 2009b).
METHODS TO STUDY RUMINAL FERMENTATION AND METHANE PRODUCTION

A microbial system can be described based on (1) the types and populations of organisms concerned, (2) identifying what they do, and (3) observing how fast they do it (Hungate et al., 1971). The rates of many rumen activities have been measured in pursuance of step 3. Dual flow continuous culture systems have been used in such an analysis, which facilitates the study of the digestion of nutrients by the ruminal microorganisms, as well as their interrelationships and interactions. In the fermenters, a filtrate pump with a multi-stage filter system, described in Karnati et al. (2009a), can be used to achieve different flow rates for solids and liquids to remove only liquid and small particles from the vessel. The filter system retains most protozoa from passing with the filtrate so that they pass primarily with the overflow. This increases the residence time for the solids that pass out only in the overflow from the fermenter, which resembles the differential passages of solids and liquids and turnover rates of the materials in the rumen. The measurement of microbial activity and their integration with the amount and type of materials disappearing during the passage of feed have shown the fermenter’s resemblance to the physical environment of the rumen. Compared with batch culture in vitro and experiments in vivo, dual flow continuous culture systems allow researchers to measure volatile fatty acids (VFA) and methane production in millimoles per day, rather than concentration in millimoles per liter, which helps to better describe the ruminal fermentation.
Different *in vitro* and *in vivo* techniques have been developed to estimate the efficiency of strategies in mitigating ruminal methane formation, including the rumen simulation technique (RUSITEC) developed by Czerkawski and Breckenridge (1977) and modified by (Machmüller et al., 2002) to reproduce the ruminal environment in a culture vessel with a stable microbial community and similar fermentation pattern and product yield. RUSITEC is a semi-continuous culture system, but different from the continuous culture fermenters with lower feeding rates, absence of separate solid and liquid outflows, and lack of pH control.

The tracer technique (Johnson and Johnson, 1995) allows animals to move and graze and does not require sampling directly from an animal’s rumen or throat. It is useful under controlled conditions but is limited under production situations. A small permeation tube that releases an inert tracer gas, sulfur hexafluoride (SF6), at a known rate is inserted into rumen. This method assumes that the dilution rate of SF6 by the ambient air when it exits the animal is exactly the same as that of the methane. Methane emission rate is calculated from the release rate of the SF6 and the ratio of methane to SF6 concentration in the sample. The estimate of methane includes both the portion of ruminal methane and methane produced in hindgut that is absorbed into blood and respired. The major concern of this method is that SF6 might not mix exactly the same with the ambient air as methane when they exit the animal.

The Tunnel system offers measurement of methane production under grazing conditions and can be portable or fixed (Murray et al., 2007). Respiration chambers allow direct measurement of methane with more expensive cost to maintain and a limited
number of animals can be tested at one time. The animals are more restrained than those in the tunnel system. Estimates of methane from the chambers tended to be higher than the tunnels (31.7 ± 0.35 vs 26.9 ± 0.46 L/kg DM intake, respectively) because the different degree of restraining might affect the animal’s behavior (Murray et al., 1999). Another method is predicting methane production from measurement of VFA production using stoichiometric equations (Hegarty and Nolan, 2007). However, the limitations of this method make it the least appropriate measurement among those mentioned because of the requirement of cannulated animals and errors with assumption of constant rumen conditions over the experimental period. A newly patented system called GreenFeed™ system (C-lock Inc., USA) has been proposed for estimating CH$_4$ and CO$_2$ emissions from free-ranging ruminants (Storm et al., 2012; http://c-lockinc.com/introduction.php). The system installed with wheels is combined with an automatic feeding system. Animals fitted with a radio frequency identification device in their ear tags entering the system are recognized, and samples of CH$_4$ and CO$_2$ emitted during eating are taken and measured with high resolution. Airflow rates and other environmental measurements are calibrated with a propane sensor to estimate production of CH$_4$ and CO$_2$. Using the sensor information, a volumetric flux (liter per minute) of gases emitted by the animal is directly calculated. Once the volumetric flux is known, the mass flux in (grams per minute) can be calculated using the ideal gas law. This system can be used inside automatic milking systems, in tie-stalls, and for grazing animals. However, the methane measurement requires an animal’s head to be in the feeder during eating. Therefore, correlations with whole-day emissions must be examined thoroughly.
DIETARY ADDITIVES ALTER RUMINAL FERMENTATION AND NITROGEN METABOLISM

Dietary additives are typically non-nutritive compounds such as ionophores, fat supplements, probiotics, fibrolytic enzymes, methane inhibitors, and buffers that have been used to manipulate the ruminal fermentation (Nagaraja et al., 1997). Monensin decreased protozoal counts *in vivo* (McGuffey et al., 2001), but the sensitivity to monensin varies depending on the protozoal species (Nagaraja et al., 1997). Methanogens are associated closely with ruminal protozoa, including extracellular adhesion and intracellular symbiosis (Ushida and Jouany, 1996; Sharp et al., 1998). Protozoa produce a significant amount of H$_2$ that is utilized by methanogens inside or outside of protozoa to produce methane, which is referred to as interspecies H$_2$ transfer.

Monensin appears to target gram-positive bacteria, based on both *in vitro* and *in vivo* experiments. Those bacteria produce acetic, butyric, formic, and lactic acids and H$_2$, whereas the resistant gram-negative bacteria produce succinic and propionic acids as the main end products (Nagaraja et al., 1997). Monensin decreased methanogenesis as a result of a shift of bacterial population from gram positive to gram negative bacteria. Additionally, monensin improved N metabolism by inhibiting hyper ammonia producing bacteria, resulting in decreased ruminal ammonia N concentration and increased accumulation of non-ammonia non-protein N (Chen and Russell, 1991). Feeding monensin can decrease the incidence of bovine coccidiosis (Tyler et al., 1992), bloat, lactic acidosis, clinical and subclinical ketosis, and displaced abomasum. Monensin also can reduce loss of body condition and increase milk yield and milk production efficiency.
Monensin’s effect on ruminal N metabolism might be related with gram-positive BH bacteria. *B. fibrisolvens* (Wallace et al., 1997) and *Prevotella ruminicola* (Callaway and Russell, 2000) are the most important proteolytic and peptide-using bacteria that have been cultured, and they are susceptible to monensin, but the gram-negative *M. elsdenii* with strong capability for deamination is insensitive to monensin (Rychlik et al., 2002), which can affect the availability of rumen degradable protein (RDP) and its conversion to microbial protein. Kim (2011) reported that supplementary monensin to dairy cows did not shift bacterial populations via pyrosequencing analysis. Protozoa responds to monensin differently. Isotrichids are more insensitive to monensin (McGuffey et al., 2001) than entodiniomorphids, which have an crucial impact on increased microbial recycling (Oldick and Firkins, 2000) and decreased ruminal efficiency of microbial protein synthesis (EMPS) (Firkins and Yu, 2006).

Pure culture studies revealed that EO affected mostly ruminal hyper ammonia-producing bacteria (*Peptostreptococcus anaerobius, Clostridium aminophilum,* and *Clostridium sticklandii*) and fungi (McIntosh et al., 2003), hence significantly reducing the rate of deamination of amino acids. Other *in vitro* studies using batch or continuous cultures have reported variable effects of EO on deamination of amino acids (Newbold et al., 2004; Busquet et al., 2006; Castillejos et al., 2006). When a combination of cinnamaldehyde and eugenol was fed *in vivo* (Cardozo et al., 2006), acetate and NH$_3$-N concentrations were decreased, and propionate proportion, small peptides, and amino acids in ruminal fluid were increased. Even though they did not measure methane production in that study, the change in VFA profile was stoichiometrically consistent
with decreased methane production. If methanogenesis is inhibited by EO and if the electrons that are not used in methane formation are not efficiently relocated into other sinks, this could impair re-oxidation of cofactors and interspecies H transfer (Nagaraja et al., 1997). Because deamination releases one pair of reducing equivalents per mole of NH$_4^+$ released, the decrease in the redox potential caused by the inhibition of methanogenesis could perhaps inhibit deamination (Russell and Martin, 1984). The mechanism of EO on ruminal fermentation is elusive based on the research so far. Hart et al. (2008) suggested two modes of action of EO: (1) blend of EO affect the pattern of bacterial colonization of substrates, particularly starch rich substrates, as they enter the rumen, and (2) EO inhibit hyper ammonia-producing bacteria. Each of the above could possibly cause the inhibition on substrate incorporation of ruminal bacteria to produce microbial N, hence reducing the N efficiency. Patra and Yu (2012) reported that all the EO, including clove oil, eucalyptus oil, garlic oil, origanum oil, and peppermint oil, decreased the abundance of archaea, protozoa, and major cellulolytic bacteria (i.e., *Fibrobacter succinogenes*, *Ruminococcus flavefaciens*, and *R. albus*) linearly with increasing EO doses using real-time PCR.

Protozoal interactions with proteolytic and deaminating bacterial populations play important roles in the recycling of microbial N in the rumen, partially due to the protozoal engulfment and digestion of bacteria (Coleman, 1975). Microbial protein originates from a mixture of microorganisms flowing out of the rumen, including liquid- and particle-associated bacteria, fungi, and protozoa, and it accounts for about 2/3 of the protein reaching the duodenum (Cotta and Russell, 1997). Therefore, it is the most
important source of AA for the dairy cows, and the EMPS in the rumen has a profound effect on ruminant productivity (Firkins et al., 2006). However, there are variations in the prediction EMPS due to different experimental conditions, unrepresentative sampling from the rumen, and ineffective microbial markers (Firkins et al., 2006). Additionally, protozoal N that contributes substantially to the total microbial N reaching the duodenum is not included in most models (Dijkstra, 1994; NRC, 2001). Protozoa derive from 30 to 80% of their N requirements from the ruminal NH$_3$-N pool, which is obtained indirectly through the engulfment and incorporation of bacterial N (Mathison and Milligan, 1971; Steinhour et al., 1982). The contribution of intracellular α-amino N and peptides to the concentrations in ruminal fluid were constant in cattle, even with different diets (Ives et al., 2002). Ruminal bacteria derive from 50 to 80% of their N requirements from the ruminal NH$_3$-N pool and 20 to 50% from non-ammonia nitrogen (NAN) (Leng and Nolan, 1984; Koenig et al., 2000).

**METHODOLOGIES FOR DETERMINATION OF CHANGES IN MICROBIAL POPULATIONS**

Ruminants strongly depend on the complex microbial ecosystem within the rumen to utilize different feed stuffs; therefore, understanding the roles of ruminal microorganisms is crucial to facilitate the studies in ruminant nutrition. The ruminal microorganisms consist primarily of bacteria ($10^{10}$-$10^{11}$ cells/mL), protozoa ($10^4$-$10^6$ cells/mL), fungi ($10^3$-$10^5$ zoospores/mL), and methanogens ($10^9$ cells/mL) (Hobson, 1989). Culture-based techniques, including isolation, enumeration, and phenotypic methods, are very important for the investigation of the ruminal microbiology, and
microbiologists have relied heavily on agar media and plate count as an assay of viable cells. However, the population of total bacteria on a certain medium is much lower than the microscopic count of bacteria from a rumen sample. There is no single culture medium available that can support growth of all the ruminal bacteria, and some bacteria might require specific conditions for growth besides the plate surface (Pegden et al., 1998). Also, counts from the most probable number method, which allows microorganisms to grow in liquid medium, are often 10 to 100 fold greater than the plate counts (Stewart et al., 1997). The culture-based techniques are extremely limited due to the following aspects: (a) culturing inadequacies, only accounting for 10 to 20% of the ruminal microbial diversity (Zoetendal et al., 2004), and (b) similar species characteristics: Dehory (1994) found that 7 species of *Entodinium* with different morphological characteristics belong to a single species. Molecular biology research has revealed that *Butyrivibrio fibrisolvens*, *Prevotella ruminicola* and *Ruminococcus* represent phylogenetically diverse groups of bacteria, although culture-based efforts have shown that the species of these genera appear to have similar functions (Forster et al., 1996). Therefore, the information obtained from the cultured microbes is incomplete.

Culture-independent gene-based technologies typically utilize highly conserved and variable regions in the rDNA, primarily the gene coding for the rRNA of small subunits of ribosomes, for the rumen microbiology (Kocherginskaya et al., 2001; Koike et al., 2003; Shin et al., 2004). Numerous studies have taken advantage of the gene-based techniques to differentiate liquid- and solid-phase ruminal bacteria (Larue et al., 2005) and examine the ruminal bacteria under various dietary conditions (Tajima et al., 2000;
The variation of the 16s rRNA sequence is used to identify species of microorganisms. The increasingly use of the DNA-based techniques in studies on the ruminal bacteria has generated many sequences, which are publically available. By establishing clone libraries of 16S/18S rRNA of rumen microbes, more ruminal microbes can be identified through sequencing. However, due to the limited numbers of clones sequenced and biases because of techniques used and limited scopes of sampling in individual studies, such as diets, small numbers of animals, and geographic areas sampled, only predominant members of the ruminal microbiome have been identified (Kim et al., 2011). A meta-analysis was performed by Kim et al. (2011) to assess the current status of species richness that has been revealed in the rumen. All the 16S rRNA gene sequences (more than 10,000) of rumen origin found in public databases were included in the meta-analysis. They reported that the current coverage at the species level has reached 71% for bacteria and 65% for archaea.

There are different culture-independent techniques that can be used to study ruminal microbial species, such as fluorescent in situ hybridization (FISH), denaturing gradient gel electrophoresis (DGGE), and real-time polymerase chain reaction (PCR). The FISH, as the in vivo quantification with high specification, allows for the measurement of absolute numbers of mRNA molecules, thus providing quantitative data (Orjalo et al., 2011). The DGGE provides information to understand the relative abundance of microbial species. The PCR-DGGE has been used to study the shift in microbial communities in the rumen (Sylvester et al., 2004) and duodenum (Sylvester et al., 2005). The PCR-amplified 16S rRNA fragments, which are of approximately equal
size (McCracken et al., 2001), can be separated by DGGE based on the decreased electrophoretic mobility of a partially melted double stranded DNA molecule in polyacrylamide gel (Muyzer, 1999). However, these techniques are subject to the bias of PCR on which DGGE is based (Burr et al., 2006). One of the major problems with the PCR technique is humic acid that exhibits similar solubility properties to DNA (Lakay et al., 2007) and come in contact with samples during DNA purification. Also, different amplification efficiencies among microbial species and formation of chimera sequences contribute to the inaccuracy of the analysis.

Quantitative PCR technology for examining the ruminal microorganisms has given rise to large amount of publications in the recent years. Real-time PCR is used to amplify and simultaneously quantify a targeted gene by comparing the targeted gene copies to a standard curve generated from the same gene. Tajima et al. (2001) designed and revalidated PCR primers for 13 species of ruminal bacteria and quantified these bacteria using real-time PCR. They are the first group using real-time PCR to study the variations in the rumen microbial community due to dietary change, which appears to be the most important technique for quantification of bacterial isolates. *Streptococcus bovis* was confirmed with the real-time PCR in that its population was not affected when the ration was switched from forage to grain (Klieve et al., 2003). Although 34 years ago, this bacterium usually was considered to be starch utilizing bacteria in the rumen of animals fed a starch-rich ration (Mackie and Gilchrist, 1979). And instead, the lactate-utilizing *Megasphaera elsdenii* proliferated in response to the starch-rich ration and resulted in decreased lactate accumulation and increased propionate concentration in the
rumen (Klieve et al., 2003). Although the real-time PCR provides rapid quantification, the accuracy of this technique is limited to factors such as sample storage and efficiency of DNA extraction. Samples are usually stored at -80°C and DNA degradation is thought to be minimal at this low temperature. However, the loss from thawing of samples has not been well studied, and recovery probably depends on the sample volume and the freezing-thawing rate (Bellete et al., 2003; Nsabimana et al., 2003). A preliminary test in our lab found that the first round of freezing at -20°C and thawing at room temperature disrupted 30 to 50% of protozoa cells and didn’t disrupt protozoa further at a second round of freezing and thawing (data not published). The efficiency of DNA extraction is dependent on the protocol used to disrupt all the cells in the material but not degrade the DNA (Guthrie et al., 2000). Physical disruption, such as bead-beating, works well with bacteria that tightly adhere to or within feed particles. However, cells without a rigid cell wall, such as protozoa, may result in sheered genomic DNA and thus decreased yield.

TRANSITION COWS AND MEGASPHAERA ELSDENII

The transition period for a dairy cow is from 3 to 2 wk prepartum until 2 to 3 wk postpartum. It is very critical for the cow because of the physiological, metabolic, and nutritional changes that occur during this period, which can determine the success of the following lactation. For both heifers and multiparous cows, a gradual decrease in DMI generally begins 3 wk prepartum, reaching the most dramatic decrease with a typical DMI declined of 30% during the final week prepartum (Coppock et al., 1972; Johnson and Otterby, 1981; Kunz et al., 1985; Bertics et al., 1992; Emery, 1993). At the same time, the energy demands for milk production reach 1.3 to 1.5 times the maintenance
requirements by the end of gestation (Quigley and Drewry, 1998). The decreased DMI, combined with the increased energy demand, causes negative energy balance (NEB). As a result, a rapid mobilization of body tissues and concomitant loss of body condition score (BCS) compensates for the energy imbalance. A severe NEB in the transition period can partially contribute to the development of metabolic disorders, prolong the interval between calving and first ovulation, and decrease fertility (Butler et al., 1981). The metabolic disorders include ketosis, mastitis, hypocalcemia, and hypomagnesemia, as well as displaced abomasum, retained placenta, and laminitis (Mulligan and Dohery, 2008).

The change of ration from a low concentrate:high forage dry cow ration to a high concentrate:low forage lactating cow ration is crucial to meet the energy requirement for lactation. However, ruminal acidosis normally occurs due to the incapability to timely adapt to the increasing levels of non-structural carbohydrates (NSC) (Beauchemin and Penner, 2009). With the high level of fermentable carbohydrates, the VFA produced during the fermentation accumulate and exceed the capacity of the rumen to absorb them within a short time, leading to the subacute rumen acidosis (SARA), which has been defined as when ruminal pH remains below 5.6 for more than 3 h per 24 h (Plaizier et al., 2008), or below 5.8 for more than 5.2 h per 24 h (Zebeli et al., 2008). SARA was reported to be the result of lactic acid accumulation in the rumen (Nagaraja and Titgemeyer, 2007), which has been disagreed with by others, indicating that SARA was caused mostly by VFA accumulation with very little lactic acid detected in the rumen (Nocek, 1997; Krause and Oetzel, 2006; Aikman et al., 2011). SARA is usually
associated with reduced DMI and fiber digestibility, damaged rumen wall, and increased release of endotoxins into the bloodstream and inflammation (Gozho et al., 2006; Ametaj et al., 2009). The cellulolytic bacteria, such as Butyrivibrio fibrisolvens, F. succinogenes, Ruminococcus albus, and R. flavefaciens, are susceptible to the drop of pH during SARA and their decrease in population contributes to the overall reduction of fiber digestibility (Russell and Dombrowski, 1980; Hiltner and Dehority, 1983). Meanwhile, the activities of amylolytic and lactic acid-utilizing bacteria, such as S. bovis, Selenomonas ruminantium, Prevotella bryantii, and Megasphaera elsdenii, have been shown to increase (Fernando et al., 2010).

SARA has been associated with economic losses between $500 million to $1 billion each year in the US (Enemark, 2009). Proper feeding management practices may facilitate early recognition of the condition and limit economic losses. In recent years, the use of direct-fed microbial products (DFM), including probiotics, as an alternative to antibiotics has gained increasing attention (Zebeli et al., 2012). The DFM enhanced fiber digestion (Nocek and Kautz, 2006) during the transition period and shortened the time that ruminants spent transitioning (Klieve et al., 2003).

Megasphaera elsdenii is a gram-negative bacterium found in the rumen. It is a large coccus that produces a variety of VFA and is obligately anaerobic (Elsden et al., 1951; Elsden et al., 1956). Using culture-based techniques, Counotte et al. (1981) found that M. elsdenii was the predominant lactic-acid utilizer within the rumen, fermenting up to 73% of the ruminal lactate. In some in vitro studies, the lactate fermenting abilities of different strains of M. elsdenii have been studied in cultures for many years. Some
strains, such as *M. elsdenii* B159, are better at preventing lactic acid accumulation and excessive drop in pH than others in a mixed rumen culture with highly fermentable carbohydrates comprised of 55% starch and 26% glucose (Kung and Hession, 1995). Kim et al. (2002) reported that some *M. elsdenii* strains produced trans-10, cis-12 CLA, the inhibitor of milk fat synthesis. However, Maia et al. (2007) reported that the culture used by Kim et al. (2002) was likely contaminated. When a batch culture of rumen fluid is incubated with maltose as the substrate, strains CH7 and CH4 have shown the highest efficiency in utilizing lactic acid and maintained the lactic acid concentration below 10 mmol/L compared with another 9 strains, and the time that the culture stayed under pH of 6.0 was shorter for strains CH7, CH3, and CH4 (Henning et al., 2010b). Real-time PCR analysis by Fernando et al. (2010) detected significant fold increases in the *M. elsdenii* population in feedlot cattle during adaptation to a high-concentrate diet.

*M. elsdenii* has attracted growing interest as a DFM for grain-fed cattle. Hibbard et al. (1993) reported that oral drenching with *M. elsdenii* increased feed intake and decreased incidence of lactic acidosis in beef steers switched from a 50 to 90% concentrate diet. Klieve et al. (2003) has shown that *M. elsdenii* YE34 was not detectable in beef cattle without grain in the diet and established 5 to 7 d sooner in the rumen of beef cattle rapidly changed from a forage-based to a grain-based diet than the uninoculated cattle. The rumen population of *M. elsdenii* increased to about $10^8$ cell equivalents mL$^{-1}$ throughout the trial after the beef steers were inoculated via rumen cannula with $5.5 \times 10^{11}$ cfu of *M. elsdenii* (Klieve et al., 2003). McDaniel (2009) reported that the lactate concentrations were lower for steers administered with *M. elsdenii* of different dosing
amounts compared with the control steers. Henning et al. (2010a) found that the population of *M. elsdenii* tended to be higher in steers on 2 and 3 d after inoculation with $1.7 \times 10^{11}$ cfu/dose of *M. elsdenii* CH4 (NCIMB 41125) than that of the control steers. A follow up experiment was conducted by Henning et al. (2010b), who dosed 100 mL $10^8$ cfu/mL of strains CH4 or CH7 to 18 rumen-cannulated sheep. The rumen pH of the sheep dosed with strain CH4 increased to 6.0 at 24 h after the grain challenge, while the sheep dosed with strain CH7 remained at a pH of 5.5, and the rumen pH in the control sheep was below 5.0 (Henning et al., 2010b).

Cook et al. (1977) dosed intra-ruminally cultures of *M. elsdenii*, the amount of which was not reported, to crossbred heifers that were not adapted to a high-concentrate diet. Cattle were switched from an all-hay diet to an 85% concentrate diet and dosed with the culture. Heifers dosed with *M. elsdenii* gained less BW than did control animals over a 21-d period. Hagg et al. (2010) reported that the rumen pH was not affected by orally-dosing 250 mL of $10^9$ cfu/mL *M. elsdenii* to the Holstein cows fed with low concentrate (60% concentrate) and high concentrate diets (70% concentrate) on 2, 10, and 20 d post-partum. Also, lactic acid concentration was not affected by *M. elsdenii* in the cows fed with the high concentrate diet. Dosing *M. elsdenii* did not affect DMI; feed efficiency (kg milk/kg DMI); BW; BCS; yields of milk, protein, and lactose; or concentration of milk urea nitrogen. In the fresh cow study by Aikman et al. (2011), rumen pH was not affected by dosing via rumen cannula with either 250 mL of *M. elsdenii* ($10^{10}$ to $10^{13}$ CFU) or a placebo dose of an autoclaved suspension on d 3 and 12 postpartum. However, the length of time that rumen pH was below 5.6 was decreased immediately after dosing, and pH
fluctuated less from day-to-day. The milk fat concentration tended to be 5.2 g/kg lower in *M. elsdenii*-dosed cows than the placebo cows. Milk protein concentration was lower in *M. elsdenii*-dosed cows, which is consistent with the study on high yielding cows by Aikman et al. (2009). This reduction could be due to a dilution effect caused by the 2.2 kg/d increase in milk yield for cows on the acidosis-inducing diet. Zebeli et al. (2012) conducted a paired 2×2 crossover design for 2 periods of 21 d dosing 8 primiparous Holstein cows every day with either saline (control) or 35 mL (10^8 cfu/mL) suspension of *M. elsdenii*. The cows were fed a 55:45 forage-to-concentrate diet with 31% starch. Rumen pH for both treatments remained ≥ 5.8. Lactic acid and total VFA concentrations in the rumen, BHBA concentration in blood, and milk yield were not affected by *M. elsdenii*. However, ruminal acetate, isobutyrate, and isovalerate concentrations were lower, and valerate and butyrate were higher, for *M. elsdenii*-dosed cows. Lactose tended (P = 0.06) to decrease and somatic cell count (SCC) tended (P = 0.06) increase in *M. elsdenii*-dosed cows compared with control cows. The *M. elsdenii* treatment decreased the serum nonesterified fatty acids (NEFA). In a recent study with 162 primi- and multiparous Holstein cows orally drenched with *M. elsdenii* ~14 d prepartum, 1 to 3 d postpartum, or ~14 d prepartum and 1 to 3 d postpartum (Stevens, 2013), the author suggested dosing with *M. elsdenii* prepartum may improve ruminal conditions of high-producing (higher parity) transition cows because milk yield and peak milk yield increased for mature cows with ≥ 3 lactations. No overall differences in milk yield, peak milk yield, milk fat and protein percentages, SCC, BCS, or reproductive performance were observed.
SUMMARY

The forms and sources of fat in the feed can influence the extent of the ruminal BH, thus affecting FA composition in the milk. Understanding the manipulation of ruminal BH could enhance FA beneficial to human health. The ruminal fermentation can be improved by the use of fermentation modifiers, such as monensin, EO, and probiotics, to decrease methane production and excess N excretion into the environment. However, these strategies require accurate prediction of the effects on ruminal bacteria, protozoa, and methanogens, as well as their interactions. Culture-independent molecular techniques, such as real-time PCR, can allow the study of the microbial populations and their changes associated with fermentation modifiers to facilitate the interpretation of the physiological and metabolic changes, thus enhancing the understanding of the ruminal fermentation.
Figure 2.1. Pathways of the ruminal biohydrogenation of 18:2 and 18:3. CLA = conjugated linoleic acid. ①: Isomerase, ②: Reductase. Adapted from Chilliard et al. (2007).
CHAPTER 3
EFFECTS OF DIFFERENT SOURCES OF FAT ON THE
BIOHYDROGENATION OF FATTY ACIDS IN VITRO

ABSTRACT

The ruminal biohydrogenation (BH) limits the availability of polyunsaturated fatty acids (PUFA) for absorption, especially conjugated linoleic acids (CLA). An experiment was conducted to determine effects of different fat sources and particle sizes on the BH of unsaturated fatty acids (UFA) in vitro. Rumen fluid-buffer media solution was distributed to tubes prefilled with 7 substrates containing 10% FA (DMB): 1) SB: 0.25 g grass hay (GH) and 0.25 g raw soybean ground to pass a 1 mm screen; 2) RSB1: 0.25 g GH and 0.25 g roasted soybean ground to pass a 1 mm screen; 3) RSB2: 0.25 g GH and 0.25 g roasted soybean ground to pass a 2 mm screen; 4) DL: 0.1 g GH and 0.4 g dried distillers grains with solubles (DDGS) at original particle size; 5) DL1: 0.1 g GH and 0.4 g DDGS gound to pass a 1 mm screen; 6) CO: 0.45 g GH and 0.05 g corn oil; 7) SBO: 0.45 g GH and 0.05 g soybean oil. The incubation was stopped at 0, 1, 2, 4, 8, 12, 16 and 24 h on ice. Overall, DL had the highest BH among the treatments. The RSB1, RSB2, CO, and SBO had similar BH, which were lower compared with DL. Roasting and particle size did not affect overall BH. However, roasting process and particle size
affected the rate of disappearance of 18:2 in soybeans. Particle size exerted minimum effect on BH of FA, but the particle sizes differed at most by 1 mm in this study.

INTRODUCTION

Ruminal BH limits the availability of PUFA, 18:2n-6, which serve as the major source of conjugated linoleic acids (CLA) in food derived from ruminants, with about 70 and 25% coming from dairy products and red meat, respectively (Sebedio et al., 2003). The major CLA isomer of intermediates of BH is cis-9, trans-11 CLA, which represents about 75 to 90% of the total CLA in ruminant products, such as milk, meat, and butter (Bauman and Griinari, 2003; Pond and Bell, 2004). Attempts have been made to increase the percentage of cis-9, trans-11 CLA in ruminant fat because of their reported health benefits to reduce the risk for cardiovascular diseases (Lock and Bauman, 2004). The BH is determined by the characteristics of the fat sources, passage rate of FA in the rumen, and BH capacities of the ruminal microorganisms. Fat is fed to increase the energy density of the diet, and thus meet the energy requirements for maintenance and production by high producing cows. Studies have showed great variations in the response of dairy cows to fat supplementation, both among and within fat sources. Factors contributing to this inconsistent response include the amount of supplemental fat fed, FA profile of the fat source, and interactions between supplemental fats and feed ingredients of the basal diet (Smith et al., 1993; Onetti et al., 2002). Fats and FA were shown to alter the proportion of individual VFA in vitro (Chalupa et al., 1984; Jenkins, 1987).
Heat treatment is applied to denature the urease, trypsin inhibitor, and lipoxygenase that are naturally contained in raw soybeans (source of linoleic acid, or LA). Therefore, feeding roasted soybeans could increase rumen undegradable protein (RUP) and energy intakes of dairy cows, hence improving lactation performance (Faldet and Satter, 1991; Knapp et al., 1991). The less FA concentration in roasted soybeans than raw soybeans is likely due to loss of fat or chemical changes during the roasting process (Reddy et al., 1994; Petit et al., 2002). Hurrell (1980) suggested that the high temperature in the roasting process may affect FA release by binding peroxides of UFA with amino groups of proteins, hence inhibiting the isomerization during BH. The FA in roasted soybeans underwent less BH than those in raw or extruded soybeans. The lack of lipolysis in soybeans due to the roasting process was suggested to delay utilization of 18:2 in roasted soybeans compared with raw soybeans (Troegeler-Meynadier et al., 2006). Therefore, PUFA is protected from BH in roasted soybeans.

With 8 to 10% fat (Tjardes and Wright, 2002), inclusion of DDGS in the diet can substantially elevate total ration fat content. Grinding feed ingredients in the laboratory helps not only to obtain uniform samples for analysis but also increases the surface area accessible to ruminal microorganisms during fermentation \textit{in vitro}. Knott et al. (2004) reported that further grinding of DDGS may be warranted for optimized nutrient digestibility of DDGS in a complete mixed feed. However, limited studies on BH of DDGS with different particles sizes are available. Dietary supplementation of unsaturated vegetable oils has been found to increase the concentrations of CLA in milk fat (McGuire et al., 1996; Kelly et al., 1998). The FA profiles for corn oil, a source of LA, were similar
to those for DDGS (Leonardi et al., 2005; Pavan et al., 2007). The BH of dietary LA to stearic acid is sometimes incomplete, yielding several intermediates, including CLA isomers and trans or cis 18:1 (Bauman et al., 1999). Corn oil addition increased the flow of trans-10, cis-12 CLA by 3.4-fold compared to feeding high-oil corn diet (Duckett et al., 2002). Corn oil (Griinari et al., 1998) and soybean oil (Dhiman et al., 2000) decreased milk fat concentration of short- and medium-chain FA and increased milk fat concentration of long-chain FA. Trans 18:1 increased in rumen contents of steers fed high concentrate diets supplemented with increasing levels of soybean oil (Beaulieu et al., 2002). The effect of corn oil supplementation on milk yield is heavily debated. Corn oil addition significantly decreased milk yield with a low fiber diet (Griinari et al., 1998); whereas, it improved milk yield as shown by Leonardi et al. (2005).

Our hypothesis for this study was that roasting would decrease BH of FA in soybeans in vitro. Also, we hypothesized that BH would decrease as particle size of fat source increases. The objective was to determine the effects of raw and roasted soybean, corn oil, soybean oil, and DDGS, and different particle sizes on the BH pattern of FA in vitro, which can be reflective of the availability of the FA in the rumen for BH.

MATERIALS AND METHODS

Treatments and Particle Size Determination

An experiment was performed to determine differences in BH of FA in vitro from grass hay when supplemented fat sources and particle sizes were varied. Substrates containing 10% FA were formulated in the following 7 treatments: 1) SB: 0.25 g grass
hay (GH) and 0.25 g raw soybean ground to pass a 1 mm screen; 2) **RSB1**: 0.25 g GH and 0.25 g roasted soybean ground to pass a 1 mm screen; 3) **RSB2**: 0.25 g GH and 0.25 g roasted soybean ground to pass a 2 mm screen; 4) **DL**: 0.1 g GH and 0.4 g dried distillers grains with solubles (DDGS) at original particle size; 5) **DL1**: 0.1 g GH and 0.4 g DDGS ground to pass a 1 mm screen; 6) **CO**: 0.45 g GH and 0.05 g corn oil; 7) **SBO**: 0.45 g GH and 0.05 g soybean oil. Each treatment was incubated in two replicates (blocks) with one week of interval. The FA profiles from each treatment are shown in Table 3.1.

Grass hay was dried at 55°C for 48 h. All the feed ingredients, except for DL, were ground through a 1-mm or 2-mm screen (Wiley Mill, Arthur H. Thomas Co., Philadelphia, PA). The particle size of DDGS was determined with a series of US standard testing sieves (2.36, 1.18, 0.60, 0.43, 0.30, and 0.15 mm) stacked in a sieve shaker (Tyler Portable Sieve Shaker, model RX- 24, W.S. Tyler Co., Salisbury, NC). Fifty-five grams of DDGS were weighed, passed through the sieves by agitation for 20 min, and the materials remaining on each of the sieves were weighed.

**Incubation Procedure**

Two non-lactating, ruminally-cannulated Holstein cows were fed a 50:50 forage:concentrate TMR twice daily at 0800 and 1800 h. After over 9 d of adjustment to the diet, representative rumen contents were obtained from 5 different spots in the rumen before the morning feeding from both cows. The rumen contents were strained through two layers of cheesecloth to allow small particles to remain in the inoculum, mixed, and taken to the laboratory immediately under anaerobic conditions at 39°C. After mixed with the solution of media, the inoculum was gassed with CO₂, placed in 39°C water
bath, and distributed to 2 replicate tubes containing each treatment. The 7 treatments were incubated for at 0, 1, 2, 4, 8, 12, 16 and 24 h, and the tubes were placed on ice to stop fermentation. The tube contents were transferred to aluminum pans, frozen, freeze-dried, and stored at -80°C until analyses.

**Analysis of Fatty Acids**

A two-step methylation procedure was performed for the FA in treatments and residues of incubation. The FA were methylated with 2 mL of 0.5 M sodium methoxide solution, and incubated in 50°C water bath for 10 min, followed by 3 mL of 5% methanolic HCl at 80°C for 10 min, as described in Kramer et al. (1997). Nonadecanoic acid (19:0) was used as an internal standard. Methyl ester standards were purchased from Nu-Check Prep (Elysian, MN; cat. No. GLC-60), and Matreya, Inc. (Pleasant Gap, PA; FIM-FAME-7). The 18:1 FA that were not available commercially (trans-6/8, trans-9, trans-12, trans-13, trans-15, cis-12, and cis-15) were identified by order of elution (Molkentin and Precht, 1995). The FA methyl esters were separated by GLC using a HP 5890 Series II gas chromatograph (Hewlett Packard Co., Palo Alto, CA). The column was a fused silica capillary (SP-2560, 100 m x 0.25 mm id x 0.2-μm film thickness; Supelco Inc., Bellefonte, PA). Helium was used as carrier gas. The flame ionization detector and injector temperatures were set at 245°C, and the split ratio was 105:1. The oven temperature was set at 110 °C for 3 min, increased by 7.0 °C /min to 180°C, held for 1 min, increased by 10.0 °C /min to 230 °C, held for 15 min, and decreased by 20.0°C /min to 110°C. The BH of FA was calculated described by Tice et al. (1993).
**Statistical Analyses**

Data were analyzed as a randomized complete block design using PROC MIXED of SAS 9.3. The treatments were compared with F-test-protected LSD according to the following model:

$$Y_{ijk} = \mu + A_i + c_j + T_k + AT_{ik} + \epsilon_{ijk},$$

where

- $Y_{ijk}$ = dependent variable for treatment $i$ on block $j$ and time $k$;
- $\mu$ = overall mean;
- $A_i$ = fixed effect of treatment $i$; $i = 1, 2, 3, 4, 5, 6, 7$;
- $C_j$ = random effect of block $j$; $j = 1, 2$;
- $T_k$ = fixed effect of time $k$; $k = 1, 2, 4, 6, 8, 12, 16, 24$;
- $AT_{ik}$ = interaction of treatment $i$ with time $k$; and
- $\epsilon_{ijk}$ = residual error associated with the $ijk^{th}$ observation.

A single pool, first-order kinetic model was fitted (Ørskov and McDonald, 1979) with PROC NONLIN of SAS 9.3 to estimate the fractional rates of disappearance of 18:2 and 18:3. The model was as follows:

$$Y = C + Pe^{-k(T-1)} + \varepsilon_i$$

- $Y$ = amount (mg/tube) of FA at time $t$;
- $C$ = pool of unavailable FA;
- $P$ = pool of potentially available FA;
- $k$ = fractional rate of FA disappearance (h$^{-1}$);
\[ T = \text{incubation time (h)}; \]
\[ L = \text{lag (h)}; \]
\[ \varepsilon_i = \text{residual error associated with the ith observation}. \]

Significance was declared at \( P < 0.05 \), and data differing at \( 0.05 < P \leq 0.10 \) were considered trends.

**RESULTS AND DISCUSSION**

The fat acid profiles of the treatments and fat sources were listed in Table 3.1. The treatments were based on the same forage and differed only in the fat sources added. The formulation was made to contain 50 mg of total FA of 0.5 g of substrate in each treatment. The DM of the substrates averaged 90% across the treatments (data not shown). With the total FA for each treatment averaging 9.6% (Table 3.1), we had about 45 mg of total FA in each treatment, which met the goal of formulation. The FA profiles of the 5 fat sources and grass hay were within the range compared with other publications (Dupont et al., 1990; Orthoefer, 1996; Duckett et al., 2002; McNiven et al., 2004; Lancaster et al., 2007; Clemente and Cahoon, 2009). The FA of roasted soybean was 0.4% lower than that in soybean, which was consistent with Reddy et al. (1994) and Petit et al. (2002). The DL, DL1, and CO contained about half the amount of 18:0 compared with SB, RSB1, RSB2, and SBO. The 7 treatments contained 19 to 24% of total 18:1, and 51 to 55% of 18:2 of the total FA. The 18:3 content of the total FA varied from 2.2% for DL, DL1, and CO, to 8.4% for SB, RSB1, RSB2, and SBO.
Effect of Fat Sources and Particle Sizes on Biohydrogenation

The free FA and esterified FA in the incubated tubes were not separated in the current study; therefore, the BH in the results represented the BH of FA after their liberation from triglycerides by lipolysis. The term, fractional rates of disappearance (DR), was used to express the BH of 18:2 and 18:3 in this study. We used equations described in Tice et al. (1994), in which double bonds were considered, to calculate the overall BH of FA during the incubation. In the first-order kinetics model, we set lag time as zero because it was not feasible to collect sufficient samples within the first 1 to 2 h of incubation (Troegeler-Meynadier et al., 2003) where lag phase was expected. Zero lag times have been published with incubation of forages (Boufaied et al., 2003) and oil seeds (Enjalbert et al., 2003). Consistently, the lag times for both 18:2 and 18:3 were similar to zero in a study to determine differences in BH of FA from fresh alfalfa and alfalfa hay in vitro when supplemental sucrose and media pH were varied (Ribeiro et al., 2007). However, lag times were reported between 1 and 2 h for soybean oil in vitro (Troegeler-Meynadier et al., 2003). Beam et al. (2000) observed no lag time when rapid hydrolysis of galactoglycerides and triglycerides took place. They stated that lag times were related to fat source and the time needed to liberate the glycerides from their matrices.

The fractional rates of disappearance of 18:2 and 18:3 during incubation were listed in Table 3.2. There was an overall treatment effect (P < 0.01) in the DR of 18:2. The RSB1 had the highest DR of 18:2 compared to the other treatments, followed by SB, and DL and CO had the lowest DR of 18:2 compared with SB. The DR of 18:3 tended (P
to differ by treatment. Except for RSB2, the DR of 18:3 was higher than that of 18:2 across the treatments, which indicated higher BH of 18:3 than 18:2. Accordingly, Duckett et al. (2002) reported BH were 91% for 18:3, 80% for 18:2, and 70% for 18:1 when steers were supplemented with typical corn, high-oil corn, and corn oil. The overall BH of C18 during the incubation in vitro was reported in Table 3.2. There was a treatment effect on the BH. The BH for DL was 46.9%, which was the highest among the treatments. RSB1, RSB2, CO and SBO had lower BH compared with DL. The BH differed by hour of incubation, which was expected. Wu and Palmquist (1991) reported BH of FA in vitro averaged 47% in diets containing calcium soap and 71% with animal-vegetable blend. Tice et al. (1994) showed about 55% of BH with soybeans in vivo by using different equations. Qiu et al. (2004) reported 30% of BH for the low pH (5.8) treatment with 4%/h solid dilution rate (SDR) and 1% LA in the feed in a dual-flow continuous culture system, and 61% for the control treatment with pH 6.5, 1% LA, and 4%/h SDR. Other studies have provided BH values for 18:2 and 18:3 over 80% in vivo (Sackmann et al., 2003) and in vitro (Loor et al., 2003). Such large variations among experiments can be due to experimental conditions, because BH in vitro depends on the N content, the starch content, source and concentration of FA, DMI, the particle sizes of the substrate, and the pH (Gerson et al., 1985; Van Nevel and Demeyer, 1996). The BH did not differ by particle size within the same fat source. The RSB1 and RSB2 showed similar BH (40%), and BH for DL and DL1 averaged 45.9%. The particle size of DDGS in DL was 1.22 mm, and this was only slightly greater than 1 mm with DL1. Therefore, the similar results of BH for DL and DL1 were expected. The lack of
difference in BH for RSB1 and RSB2 was consistent with Tice et al. (1994), who reported minor effects of particle size of roasted soybeans on utilization of FA. The mean particle sizes of soybeans in their study were > 4.75 mm for whole soybeans and whole roasted soybeans, 2.65 mm for cracked roasted soybeans, and 0.63 mm for ground roasted soybeans. They suggested larger particles may limit availability of C18 FA for uptake by bacteria because the concentration of 18:1 in bacteria tended to increase as particle size of soybean was reduced. The BH of FA in SB did not differ from RSB1 and RSB2, averaging 40.8%. This would indicate that roasting did not affect the overall BH of FA in soybeans, which was opposite as we hypothesized. However, the source of the soybeans for RSB1 and RSB2 was different from SB, which might cause the lack of difference in BH in the current study, although roasting was also reported to have minor effects on FA utilization by Tice et al. (1994).

The BH increased generally as the incubation proceeded (Figure 3.1). The BH of CO and SBO appeared to decrease by 1 h of incubation, indicating that the presence of readily available PUFA in both treatments might inhibit the BH. The BH has been proposed to be a detoxification mechanism by ruminal bacteria (Kemp et al., 1984). Butyrivibrio has been identified as the principal importance in undertaking BH of linoleic acid (Polan et al., 1964) and is sensitive to trans-11 18:1. The most common product from linoleic acid was trans-11 18:1, produced by 3 Butyrivibrio strains and 2 strains of Clostridium proteoclasticum, out of the 26 predominant species of ruminal bacteria studied by Maia et al. (2007). Only C. proteoclasticum produced 18:0, which might be due to its sensitivity to trans-11 18:1 (Maia et al., 2007). The percentages of trans 18:1 in
both CO and SBO were relatively higher than RSB1, RSB2, DL, and DL1. The PUFA have been reported to hinder the growth of BH bacteria, especially *C. proteoclasticum* (Maia et al., 2007), which probably explains the decreased BH in CO and SBO during the first 2 h of incubation. A decrease in the BH of RSB1 and RSB2 was observed until 2 h of incubation, which did not occur in SB. The roasting process of soybeans seemed to affect the BH of soybeans in the first 2 h of incubation but was not enough to affect the overall BH of FA in soybeans. Hurrell (1980) suggested that the high temperature in the roasting process may affect FA release by binding peroxides of UFA with amino groups of proteins, hence inhibiting the isomerization during BH. Roasting was suggested to delay the utilization of FA in soybeans due to the lack of lipolysis (Troegeler-Meynadier et al., 2006), hence protecting the PUFA from BH.

The areas under curves (AUC) were determined for the presence of C18 (Table 3.3) to compare effects of fat source and particle size on the mean amount of C18 present during BH *in vitro*. The overall treatment effect was significant for AUC of 18:3. The RSB1, RSB2, and SBO had the highest AUC of 18:3, followed by SB, and DL, DL1, and CO had the lowest AUC of 18:3. The lower AUC of 18:3 in DL1 than SBO was consistent with the trend of higher DR of 18:3 in DL1 than in SBO, which reflected the lower amount of 18:3 in the substrate of DL1 (2.15% of total FA) than in SBO (7.38% of total FA). Bolte et al. (2001) reported lower duodenal flow of 18:3 for steers consuming less 18:3 from typical corn than high-oil corn diets. Boufaied et al. (2003) also detected higher 18:3 flow from the rumen with fresh grass than with hay that contained less 18:3 than fresh grass. The treatment tended (*P < 0.06*) to affect the AUC of 18:1. The CO had
the highest AUC of 18:1, and SB, RSB1, RSB2, DL1, and SBO had similar AUC of 18:1, which tended (P < 0.06) to be lower than CO. There was no overall treatment effect on AUC of 18:0 and 18:2. Particle size did not affect the AUC values for C18 in RSB1 and RSB2, DL, and DL1.

C18 Present During Incubation In Vitro

The presence of C18 during the 24-h incubation for each treatment averaged by replicate is shown in Figure 3.2. The curves of 18:0 and 18:1 in all the 7 treatments showed a pattern of increased accumulation of 18:0 and 18:1 over the time of incubation, and the curves of 18:2 and 18:3 in all 7 treatments demonstrated a decreased pattern over the time, indicating the BH of 18:2 and 18:3. However, the presence of 18:0 in RSB1 decreased at 2 h, and increased at 4 h of incubation. The decrease of accumulation of 18:0 at 2 h in replicate 2 of RSB1 contributed mainly to this change. Similar situations occurred for 18:0 of RSB2 at 1 h in replicate 1 and 2 h in replicate 2, 18:0 of DL1 at 1 h in replicate 1 and 1 h and 2 h in replicate 2, and 18:0 of CO and SBO at 1 h in replicate 2. According to Bauman and Griinari (2003), the accumulation of 18:0 as the final product of BH would occur over time; however, in our study, with a closed in vitro system where 18:0 cannot be removed, we speculated that the decrease of the presence of 18:0 was due to FA oxidation (Ribeiro, 2005). All the treatments, except for CO and SBO, had a higher amount of FA at 24 h of incubation compared with 0 h, indicating de novo FA synthesis by bacteria (Harfoot and Hazlewood, 1997).

The increase of the presence of 18:1 from 0 h to 24 h across the treatments suggested incomplete BH of 18:2 and 18:3 and possibly higher rate of BH of 18:2 and
18:3 than that of 18:1. The rate of BH and the concentration of the intermediate FA are affected by the concentration of UFA (mainly 18:2 and 18:3) in the medium (Beam et al., 2000). The 18:2 acts as a competitive inhibitor for BH of monoenoic acid (Polan et al., 1964) and was reported to prevent BH of 18:1 to 18:0 by irreversible inhibition instead of competing for metabolic hydrogen (Harfoot et al., 1973). Similar to our results, Enjalbert et al. (2003) reported that BH rates for PUFA in raw and extruded blends of ground canola seeds and canola meals both in vitro and in situ were higher than BH of cis-9 18:1. They also observed that BH of PUFA in situ was not complete at 24 h of incubation partially due to lower rates of BH than the study in vitro. Additionally, Boeckaert et al. (2007) showed an incomplete BH of 18:2n-6 and 18:3n-3 in vivo, resulting in a strong accumulation of tran-11 18:1, tran-10 18:1, and trans-11, cis-15 18:2.

CONCLUSIONS

Fat source affected BH of FA. Overall, DDGS had the highest BH among the treatments. RSB, CO, and SBO had similar BH, which were lower compared with DDGS. Roasting and particle size did not affect overall BH. However, roasting process and particle size affected the rate of disappearance of 18:2 in soybeans. Particle size exerted minimum effect on BH of FA, but the particle sizes differed at most by 1 mm in this study.
Table 3.1. Fatty acid profiles of the treatments with grass hay and different sources of fat.

<table>
<thead>
<tr>
<th>Treatments(^\text{1})</th>
<th>Fat sources(^\text{2})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SB</td>
</tr>
<tr>
<td>Total fatty acids (FA)</td>
<td>----</td>
</tr>
<tr>
<td>9.10</td>
<td>8.95</td>
</tr>
<tr>
<td>Individual FA</td>
<td>----</td>
</tr>
<tr>
<td>16:0</td>
<td>11.7</td>
</tr>
<tr>
<td>18:0</td>
<td>4.07</td>
</tr>
<tr>
<td>cis-9 18:1</td>
<td>20.8</td>
</tr>
<tr>
<td>cis-11 18:1</td>
<td>1.30</td>
</tr>
<tr>
<td>trans 18:1</td>
<td>0.18</td>
</tr>
<tr>
<td>18:2</td>
<td>50.9</td>
</tr>
<tr>
<td>18:3</td>
<td>8.47</td>
</tr>
</tbody>
</table>

\(^1\)SB: 0.25 g grass hay (GH) and 0.25 g raw soybean gound to pass a 1 mm screen; RSB1: 0.25 g GH and 0.25 g roasted soybean gound to pass a 1 mm screen; RSB2: 0.25 g GH and 0.25 g roasted soybean gound to pass a 2 mm screen; DL: 0.1 g GH and 0.4 g dried distillers grains with solubles (DDGS) at original particle size; DL1: 0.1 g GH and 0.4 g DDGS gound to pass a 1 mm screen; CO: 0.45 g GH and 0.05 g corn oil; SBO: 0.45 g GH and 0.05 g soybean oil.
Table 3.2. Fractional rates (k) of disappearance of 18:2 and 18:3 and biohydrogenation (BH) of C18 after incubation for 24 hours *in vitro*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>18:2</th>
<th>18:3</th>
<th>BH, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB</td>
<td>0.294&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.380</td>
<td>42.5&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>RSB1</td>
<td>0.459&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.503</td>
<td>40.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>RSB2</td>
<td>0.205&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.156</td>
<td>40.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DL</td>
<td>0.138&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.271</td>
<td>46.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DL1</td>
<td>0.193&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.620</td>
<td>44.8&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>CO</td>
<td>0.171&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.465</td>
<td>38.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SBO</td>
<td>0.190&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.216</td>
<td>39.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SEM</td>
<td>0.036</td>
<td>0.099</td>
<td>2.42</td>
</tr>
<tr>
<td>TRT</td>
<td>&lt;0.01</td>
<td>0.11</td>
<td>0.05</td>
</tr>
<tr>
<td>Time&lt;sup&gt;3&lt;/sup&gt;</td>
<td>NA</td>
<td>NA</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TRT*Time</td>
<td>NA</td>
<td>0.96</td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>SB: 0.25 g grass hay (GH) and 0.25 g raw soybean gound to pass a 1 mm screen; RSB1: 0.25 g GH and 0.25 g roasted soybean gound to pass a 1 mm screen; RSB2: 0.25 g GH and 0.25 g roasted soybean gound to pass a 2 mm screen; DL: 0.1 g GH and 0.4 g dried distillers grains with solubles (DDGS) at original particle size; DL1: 0.1 g GH and 0.4 g DDGS gound to pass a 1 mm screen; CO: 0.45 g GH and 0.05 g corn oil; SBO: 0.45 g GH and 0.05 g soybean oil.

<sup>2</sup>Average SEM across treatments.

<sup>3</sup>Time relates to replicate 1 and 2 for fractional rates of disappearance of 18:2 and 18:3; 0, 1, 2, 4, 8, 12, 16, and 24 hour of incubation for BH.

<sup>abc</sup>Values within a given row that do not share common superscripts differ (P < 0.05).
Table 3.3. Areas under the curve (mg x h) of C18 during incubation *in vitro* with various treatments.

<table>
<thead>
<tr>
<th>Item</th>
<th>SB</th>
<th>RSB1</th>
<th>RSB2</th>
<th>DL</th>
<th>DL1</th>
<th>CO</th>
<th>SBO</th>
<th>SEM²</th>
<th>TRT</th>
<th>Hour</th>
<th>TRT*Hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:0</td>
<td>16.9</td>
<td>16.3</td>
<td>15.4</td>
<td>18.5</td>
<td>17.3</td>
<td>15.7</td>
<td>16.3</td>
<td>1.42</td>
<td>0.77</td>
<td>&lt;0.01</td>
<td>1.00</td>
</tr>
<tr>
<td>18:1</td>
<td>13.6^v</td>
<td>13.7^v</td>
<td>12.8^v</td>
<td>14.4^{xy}</td>
<td>14.1^v</td>
<td>16.2^x</td>
<td>14.0^v</td>
<td>0.67</td>
<td>0.06</td>
<td>&lt;0.01</td>
<td>0.56</td>
</tr>
<tr>
<td>18:2</td>
<td>10.9</td>
<td>13.1</td>
<td>12.9</td>
<td>11.31</td>
<td>10.9</td>
<td>13.5</td>
<td>13.5</td>
<td>1.24</td>
<td>0.22</td>
<td>&lt;0.01</td>
<td>1.00</td>
</tr>
<tr>
<td>18:3</td>
<td>1.81^b</td>
<td>2.23^a</td>
<td>2.21^a</td>
<td>0.70^c</td>
<td>0.67^c</td>
<td>0.94^c</td>
<td>2.23^a</td>
<td>0.14</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.06</td>
</tr>
</tbody>
</table>

^1**SB**: 0.25 g grass hay (GH) and 0.25 g raw soybean ground to pass a 1 mm screen; **RSB1**: 0.25 g GH and 0.25 g roasted soybean ground to pass a 1 mm screen; **RSB2**: 0.25 g GH and 0.25 g roasted soybean ground to pass a 2 mm screen; **DL**: 0.1 g GH and 0.4 g dried distillers grains with solubles (DDGS) at original particle size; **DL1**: 0.1 g GH and 0.4 g DDGS ground to pass a 1 mm screen; **CO**: 0.45 g GH and 0.05 g corn oil; **SBO**: 0.45 g GH and 0.05 g soybean oil.

^2Average SEM across treatment.

^abcValues within a given row that do not share common superscripts differ (P < 0.05).

^xyValues within a given row that do not share common superscripts differ (P < 0.06).
Figure 3.1. Biohydrogenation of FA averaged by replicate during incubation of treatments in vitro with grass hay (GH) and different sources of fat. **SB**: 0.25 g grass hay (GH) and 0.25 g raw soybean ground to pass a 1 mm screen; **RSB1**: 0.25 g GH and 0.25 g roasted soybean ground to pass a 1 mm screen; **RSB2**: 0.25 g GH and 0.25 g roasted soybean ground to pass a 2 mm screen; **DL**: 0.1 g GH and 0.4 g dried distillers grains with solubles (DDGS) at original particle size; **DL1**: 0.1 g GH and 0.4 g DDGS ground to pass a 1 mm screen; **CO**: 0.45 g GH and 0.05 g corn oil; **SBO**: 0.45 g GH and 0.05 g soybean oil.
Figure 3.2. Presence of 18:0, 18:1, 18:2, and 18:3 during incubation *in vitro* for the 7 treatments. **SB**: 0.25 g grass hay (GH) and 0.25 g raw soybean ground to pass a 1 mm screen; **RSB1**: 0.25 g GH and 0.25 g roasted soybean ground to pass a 1 mm screen; **RSB2**: 0.25 g GH and 0.25 g roasted soybean ground to pass a 2 mm screen; **DL**: 0.1 g GH and 0.4 g dried distillers grains with solubles (DDGS) at original particle size; **DL1**: 0.1 g GH and 0.4 g DDGS ground to pass a 1 mm screen; **CO**: 0.45 g GH and 0.05 g corn oil; **SBO**: 0.45 g GH and 0.05 g soybean oil.

(Continued)
Figure 3.2. Continued

C  
RSB2

D

DL

(Continued)
Figure 3.2. Continued

(Continued)
Figure 3.2. Continued
CHAPTER 4
ESSENTIAL OIL AND RUMENSIN AFFECT RUMINAL FERMENTATION IN CONTINUOUS CULTURE

ABSTRACT

The combination of Rumensin® and essential oil could be beneficial for ruminal fermentation by suppressing protozoa and methane production while maintaining normal rumen function. The objective of this study was to determine the effects of feeding Rumensin and Cinnagar® (essential oil of cinnamon and garlic) in diets on ruminal fermentation characteristics. Four continuous culture fermenters were maintained in 4 periods in a 4 X 4 Latin square design. Four dietary treatments were arranged in a 2 x 2 factorial: (1) control diet, 40 g of a 50:50 concentrate:forage diet containing no additive; (2) Rumensin at 11g/909 kg of DM; (3) Cinnagar at 0.0043% (DM basis); and (4) a combination of Rumensin and Cinnagar at the levels in (2) and (3). There were no effects of treatment on protozoal generic distribution, concentration of NH₃-N, total N flow of effluent, production of total VFA, or flows of conjugated linoleic acid and total C18. Rumensin decreased acetate:propionate ratio and biohydrogenation of total C18 and cis-9 18:1. Rumensin increased protozoal generation time, concentration of peptide, and flow of trans-11 18:1. Rumensin tended to decrease protozoal counts in effluent flow and flow
of 18:0, and it tended to increase propionate production. Cinnagar decreased true OM digestibility and protozoal N flow of effluent and increased non-ammonia non-microbial N flow of effluent. Cinnagar tended to decrease protozoal counts, microbial N flow of effluent, NDF digestibility, and protozoal N per cell. Cinnagar tended to increase biohydronenation of total C18, 18:2, and 18:3. Cinnagar tended to increase isovalerate production. Cinnagar and Rumensin tended to interact for increased methane production and bacterial N flow. Under the conditions of our study, we did not detect an additive response for Rumensin® and Cinnagar® to decrease protozoal counts or methane production.

INTRODUCTION

Increasing public concern over global warming and increasing emphasis on rumen-derived methanogenesis have directed considerable research efforts to suppress ruminal protozoa because of their close association with methanogens (Williams and Coleman, 1992). By decreasing methanogenesis in the rumen, reducing equivalents would be converted to propionate (the major gluconeogenic precursor in ruminants) rather than being eructated as methane, or H₂ if methane is inhibited, capturing more metabolizable energy for the animal. This strategy to reduce methanogenesis by inhibiting protozoa is complicated because results have been inconsistent or not long-lasting and not always related to reduced methane production (Williams et al., 2009); thus, selective inhibition of protozoa likely is needed to consistently decrease methane
production while concomitantly providing other benefits, such as decreasing proteolytic and deminase activities, prior to transferring these programs successfully to industry.

The use of essential oil (EO) as a natural alternative to the use of antibiotics in animals is gaining wide attention. The antimicrobial activity of EO is likely due to their disruption of microbial cell membranes, leading to impaired electron transport, ion gradients, translocation (Ultee et al., 2002), and inactivation of bacterial enzymes (Gill and Holley, 2004). EO have been suggested as a potential means to manipulate bacterial populations involved in ruminal biohydrogenation (BH) of fatty acids (FA) (Calsamiglia et al., 2007). Ethanolic extracts of EO from Australian plants selectively inhibited the growth of pure cultures of Clostridium proteoclasticum involved in the terminal step in the BH of linoleic acid, resulting in the accumulation of intermediates such as conjugated linoleic acid (CLA) and vaccenic acid (VA) in batch culture incubations (Durmic et al., 2008). Using a continuous culture fermenter system, Lourenço et al. (2008) found that cinnamaldehyde decreased apparent BH of 18:2n-6 and 18:3n-3 and shifted the BH away from the trans-11, leading to the accumulation of 18:1 trans-10 and trans-10, cis-12 CLA. This trans-10 “shift” has been documented to cause milk fat depression in vivo (Jenkins et al., 2008). However, in a recent study (Benchaar and Chouinard, 2009), supplementation of TMR of lactating dairy cows with cinnamaldehyde (1 g/d) did not modify the FA profile of milk fat. The difference between the effects seen in continuous culture versus in vivo could be due to the absence of protozoa in continuous culture. Protozoa do not contribute to BH directly, but they could affect activity or populations of lipid-metabolizing bacteria by selective predation (Karnati et al., 2009b).
Supplementation of EO has been shown to have variable effects on the numbers of ruminal ciliate protozoa. Cardozo et al. (2006) observed that addition of a mixture of cinnamaldehyde (180 mg/d) and eugenol (90 mg/d) to the diets of Holstein heifers increased numbers of holotrichs and had no effect on entodiniomorphs. Patra et al. (2010) found that entodiniomorphs and holotrichs were increased by extracts of garlic added at 0.25 mL/100 mL in an in vitro incubation. Perhaps, results among these studies are too variable because of different types and concentrations of EO or because of carryover effects on microbial populations in their Latin square designed project (28-d periods).

Monensin has been shown to decrease the rate of ruminal BH of unsaturated FA (UFA) in vitro (Fellner et al., 1997) and increased the concentration of CLA in milk fat (AlZahal et al., 2008). The protozoa were found to adjust to monensin, apparently by inhibiting the function of their digestive vacuole (Karnati et al., 2009a; Sylvester et al., 2009), but the additional challenge of EO along with monensin might be needed to suppress protozoa long term while avoiding the need for large amounts of UFA that can promote milk fat depression. In that regard, the presence of protozoa increased the trans-11: trans-10 18:1 ratio (Karnati et al., 2009a). Moreover, suppression of protozoa-associated methanogens directly (with a specific inhibitor) would increase accumulation of hydrogen, resulting in affected protozoa metabolic activity (Wolin, 1975), which would make them less competitive and thus lower their biomass in the rumen. Thus, we hypothesized that Rumensin® and Cinnagar® (a blend of extracts from cinnamon and garlic) would interact for decreased protozoa counts and methane production, the benefit of which however must be characterized within the context of resulting changes in the
bacteria that interact positively or negatively with these microbial groups to assess the full efficacy of such a strategy. The objective of this study was to determine the effects of feeding Rumensin and Cinnagar in diets on ruminal fermentation characteristics.

**MATERIALS AND METHODS**

**Experimental Design**

A modified dual flow continuous culture system was used in the study. Four continuous culture fermenters in a 4 X 4 Latin square design were modified to retain protozoa and maintained in 4 periods of 10 d each (7 d of adaptation). The fermenters were fed once daily a meal of 40 g of a 50:50 concentrate:forage diet (38% NDF, 16% CP) containing either no additive, Cinnagar® provided by Provimi North America (Brookville, OH) at 0.0043% DM basis, Rumensin® provided by Elanco Animal Health (Greenfield, IN) at 11g/ 909 kg of DM, and Cinnagar® (0.0043% DM basis) plus Rumensin® (11g/ 909 kg of DM) (Tables 4.1 and 4.2).

**Continuous Culture Operation**

The dual flow continuous culture system was modified with liquid dilution rate of 7.0 %/h and the solid dilution rate of 5.0 %/h compared with standard conditions (Noftsger et al., 2003), based on the system initially described by Hoover et al. (1976). For each period, ruminal contents were taken from 2 cannulated Holstein cows maintained on a diet without EO or Rumensin, squeezed through 2 layers of cheesecloth into a container maintained at 39ºC. After being transported to the laboratory, the rumen fluid was strained through 2 layers of cheesecloth before inoculation. The 500 mL of
strained rumen fluid was inoculated into each fermenter with buffer filling up to the overflow level. As described in Karnati et al. (2009a), a multi-stage filter system was used on the pumps to retain protozoa and was cleaned when necessary. The volumes of the 4 fermenters ranged from 1630 to 1810 mL. The pH in the fermenters was monitored and adjusted between 6.4 and 6.8 during the feeding cycle. Temperature was maintained at 39 ºC, and agitation was set at 50 rpm. Flow rates were determined once a day during the adaptation period by weighing solid and liquid outflows from each fermenter and adjusted as necessary.

**Sample Collection and Analyses**

On day 5 of each period, 10 mL of 10 % enriched \((^{15}\text{NH}_4)_2\text{SO}_4\) as a microbial marker was added to make a 20 L buffer to be infused overtime to the fermenters for maintaining pH as described previously. At the same time, 1 mL of the \((^{15}\text{NH}_4)_2\text{SO}_4\) solution was primed into each fermenter. A sample of effluent was taken prior to the priming, and continuous infusion for background \(^{15}\text{N}\). On day 6, formalin was added to the solid and liquid overflows to reach 2% (v:v) final concentration of formalin. Formalin was divided to 3 aliquots, was added to the collecting containers at 0, 6, and 12 h. The 10 mL of fermenter samples were taken at 0, 4, 8, and 12 h for protozoa counting (Dehority, 1993; Oldick and Firkins, 2000). On day 7, the 10 mL of fixed effluent was collected for protozoa counting. The protozoal counts were used to calculate the protozoal generic distribution and generation time of protozoa according to the following formula:

generation time in hours = total protozoal counts in fermenter/flow of protozoa in effluent per hour (Sylvester et al., 2004). On days 8, 9 and 10 of each period, a sample of 20% of
total daily effluent was collected overnight on ice and composited by fermenter. Freeze-dried effluent samples were analyzed for DM, OM, N using the Kjeldahl method (AOAC, 1990), NDF in the presence of heat-stable amylase and sodium sulfite (Van Soest et al., 1991), and long-chain FA (LCFA) and ammonia using standard methods (Chaney and Marbach, 1962). The FA in the freeze-dried effluent samples were methylated, analyzed, and BH of FA calculated as previously described (Qiu et al., 2004). An aliquot of the effluent sample was strained through 4 layers of cheesecloth. The filtrate was acidified using 3 mL of 6 N HCl per 50 mL of filtrate to stop fermentation prior to analysis for VFA (Firkins et al., 1990).

An aliquot of the effluent sample was fixed in formalin for a pelleted bacterial sample and a protozoal sample for the protozoal N:cell ratio, which was determined in a new procedure based on the method in (Coleman, 1992) (Figure 4.1). The protozoal N:cell ratio was calculated by dividing the lost protozoal N through boiling in the water bath and sonication with an ultrasonic cleaning bath of average output 80 kHz by the loss of protozoal counts through boiling and sonication (BS). Aliquots of effluent and background samples to be used for 15N analysis were adjusted to a pH over 10 with 25% NaOH to volatilize ammonia from the sample (Noftsger et al., 2003) and dried in 90°C overnight. Then, the ammonia 15N samples by diffusion protocol (Hristov et al., 2001), bacterial, protozoal retentates recovered through the procedure in Figure 4.1, background fermenter samples before 15N infusion, and effluent samples were analyzed for 15N and non-ammonia nitrogen (NAN) by the Stable Isotope Laboratory at Pennsylvania State University. The gas collection and analysis were as described in (Karnati et al., 2009a).
After the analysis of $^{15}$N, the lost protozoal $^{15}$N through BS was calculated by difference of the 2 retentates. The lost protozoal N through BS was determined using the Kjeldahl method (AOAC, 1990) and calculated by difference. The protozoal N:cell ratio was multiplied by total outflow of protozoa cells to determine protozoal N flow. Protozoal $^{15}$N flow was calculated in 2 ways for comparison (Figure 4.1): (1) The protozoal $^{15}$N in retentate was calculated by multiplying weight of BS or not-boiled-or-sonicated (NBS) retentate by N% and protozoal $^{15}$N% of the retentate. The lost protozoa $^{15}$N through BS was determined by the difference of NBS and BS protozoal $^{15}$N and was divided by lost protozoal N and multiplied by protozoal N flow to calculate protozoal $^{15}$N flow; and (2) with only the protozoal $^{15}$N from NBS retentate, we back calculated with the dilution factor to yield protozoa $^{15}$N amount in the 3-day composited effluent sample, which was converted to protozoal $^{15}$N flow by multiplying effluent outflow per day. Then the NAP $^{15}$N flow was corrected for bacteria $^{15}$N flow by excluding the protozoal $^{15}$N flow.

The test for the correction factor of the protozoal N:cell ratio was done to determine whether any adjustment was needed. The correction factor was calculated by dividing the protozoal N:cell ratio calculated the way mentioned before by the one calculated with only NBS retentate.

We used a crossed stage micrometer as a horizontal and vertical scale (Type NE17 with 21 mm diameter from Electron Microscopy Sciences, Hatfield, PA) on the eyepiece of microscope to measure the length and width of about 100 Entodinium from each treatment of the NBS retentates to estimate the protozoal cell volume to determine
the effect of treatment on the *Entodinium* sizes. We compared 2 different calculations for protozoal cell volume: 1) volume = length( length/ 4)^2 \pi (Teather et al., 1984); and (2) volume = length( width/ 2)^2 \pi (Dehority, 2010).

**Statistical Analysis**

Data were analyzed using the MIXED procedure (SAS, 1999) according to the following model: 

\[ Y_{ijk} = \mu + F_i + P_j + T_k + e_{ijk}, \]

where:

- \( Y_{ijk} \) is the dependent, continuous variable,
- \( \mu \) is the overall population mean,
- \( F_i \) is the random effect of the \( i^{th} \) fermenter (\( i = 1, 2, 3, 4 \)) (3 df),
- \( P_j \) is the fixed effect of the \( j^{th} \) period (\( j = 1, 2, 3, 4 \)) (3 df),
- \( T_k \) is the fixed effect of the \( k^{th} \) treatment (\( k = 1, 2, 3, 4 \)) (3 df), and
- \( e_{ijk} \) is the residual error, assumed independent and \( \sim N(0, \sigma^2_e) \) (15 df).

Three contrasts were used to determine the main effect responses to Rumensin®, Cinnagar®, and interaction between Rumensin® and Cinnagar®. The treatments were compared by F-test-protected LSD. Means were tested by ANOVA. Significance was declared at \( P \leq 0.05 \) and trends were noted at \( 0.05 < P < 0.15 \).

**RESULTS AND DISCUSSION**

**Protozoa Counts and Generation Time**

The main effect of Rumensin did not interact with Cinnagar for decreasing the total counts of protozoa, which was contrary to our hypothesis (Table 4.3). The average
total counts of protozoa (17.3 x 10^3/mL) was similar to those observed in Karnati et al. (2009a) with the similar set up of continuous culture and multistage filter system to retain protozoa. The main effect of Cinnagar tended to decrease the total protozoal counts (P = 0.07) and therefore effluent flow of cells per day (P = 0.09). In contrast, the geometry-calculated volume for entodinia (comprising > 90% of cells) tended (P = 0.12) to be greater for the Cinnagar treatments. The generic distributions of *Isotrichidae* and *Diplodiniinae* in fermenter and effluent were similar relative to the control. Cinnagar tended to increase the *Entodidium* (P = 0.12) in fermenter. There is very limited research on the effect of cinnamaldehyde on the population of protozoa. The effect of cinnamaldehyde on the protozoa counts was first reported by Benchaar et al. (2008). Supplementing cinnamaldehyde at 1000 mg/d to Holstein cows had no effect on the total counts of protozoa, as well as numbers of *Dasytricha*, *Diplodinium*, *Entodinium*, and *Polyplastron*, and increased the number of *Isotricha*. Other essential oils were concluded to decrease the protozoa counts only at high doses, even results varied among different types of essential oils (Hart et al., 2008).

Besides the type of EO and the experimental set-up, such as continuous culture and animal trial that affect the results of EO supplementation, the microbial populations exhibit a remarkable capacity to adapt to or degrade the essential oils, particularly in low dosage rates *in vitro* (Benchaar and Greathead, 2011). Cardozo et al. (2006) reported that cinnamaldehyde (180 mg/d) plus eugenol (90 mg/d), together about 0.0036% (DMB) of the diet, fed to beef heifers increased numbers of holotrichs and had no effect on entodiniomorphs at 3 h after feeding. In the current study, the type of EO, higher dosage
of Cinnagar (0.0043%, DMB) and the fermenter experimental design may be the reason to cause different results, and there is possible different generic shift right after feeding compared with the population after the 7 d of adaptation, which we didn’t measure.

The average generation time (25 h) was shorter than that in Karnati et al. (2009a) (48 h). The protozoa adapted to the higher solid dilution rate in the current study (5.0%) than Karnati et al. (2009a) (2.0%), which resulted in shorter generation time in our study. Sylvester et al. (2009) documented that protozoa rapidly synchronized their generation time with the transfer interval. The solids passage rate of 5%/h is equivalent with an average retention time of 20 h. The settling of particles beneath the overflow port (which controlled solid passage rate) therefore allows sequestration (isotrichids were less numerous in effluent than fermenter except for control), which probably explains the slightly longer (22 to 28 h) generation times.

Rumensin significantly increased the generation time of protozoa because it was associated with an increase in energy requirements for maintenance at a low growth rate (Stouthammer and Bettenhaussen, 1973). There was a trend (P = 0.13) for Rumensin to decrease the effluent flow of cells per day. We previously noted that monensin increased generation time, but the generation time typically decreased to near the control diet by about 7 d, apparently as a result of adaptation to keep monensin from disrupting digestive vacuole function (Sylvester et al., 2009). The 7-d adaptation to Rumensin either was not quite complete by our sampling period or was energetically costly such that generation time was increased by monensin. Another possible explanation would be the way monensin was added. In Karnati et al. (2009a), monensin was infused as liquid, whereas
in the current study, Rumensin® was mixed with other feed components into pellets, by which Rumensin® is more readily available to interact with protozoa instead of interacting in the liquid phase with the dietary fat before reaching the protozoa to produce further effects.

**Digestibility of Nutrients**

Cinnagar did not affect the apparent OM digestibility, but it decreased true (corrected for microbial OM in effluent) OM digestibility by 6.62% (P = 0.05), and tended to decrease the NDF digestibility (P = 0.11) relative to the control treatment (Table 4.4). Rumensin didn’t affect the digestibilities of NDF or OM. Protozoa with lower fibrolytic enzyme activity compared with that in fibrolytic bacteria should indirectly increase the NDF digestibility in vitro by quenching oxygen to help avoid a decrease of ruminal pH, resulting in an optimized environment with more steady supply of growth factors for fibrolytic bacteria (Firkins and Yu, 2006). Thus, the tendency for reduced total protozoal counts could be part of the causes of decreased true OM digestibility with Cinnagar. The result in our study was inconsistent with Busquet et al. (2005), who reported that the cinnamaldehyde and garlic oil additions at both low and high levels didn’t affect the true OM or NDF digestibilities in the continuous culture fed with a 50:50 alfalfa hay:concentrate diet. In another trial of that report, monensin added at 12.5 mg/L of culture fluid tended to lower the true OM digestibility and decreased the NDF digestibility (P < 0.05), whereas the Rumensin in our study added at 11g/909 kg of DM, approximately 51 mg/L, didn’t affect the digestibility, which is consistent with the
previous study in our lab (Karnati et al., 2009a) when monensin was added at 2.5\(\mu\)mol/L in the continuous culture with a 70:30 forage:concentrate diet.

**N flows and NH\(_3\) Concentration**

The NH\(_3\)-N concentrations, effluent flows of total N and NAN, and N efficiency were not affected by treatments (Table 4.4). Rumensin increased the peptide-N concentration (\(P = 0.05\)). Unlike ruminally derived samples from which these small particles can generally be removed by flocculation (removal of floating particles), protozoa repeatedly co-filtered with small particles from our effluent samples. As a result, protozoal N per cell is overestimated (Sylvester et al., 2004). This was also shown in the current study, where protozoal N per cell calculated only with NBS retentates was higher than the one calculated with the by-difference procedure (Figure 4.1). We used protozoal N per cell calculated using the by-difference procedure for the other effluent N flow calculations. It was reported the boiling in the water bath and sonication disrupted 100% of the protozoa (Coleman, 1992). In our preliminary trial, we found about 95% of protozoa were disrupted (data not shown). Successful cryopreservation depends on controlled rates of freezing (Nsabimana et al., 2003), but freezing to disrupt protozoa was not effective in our situation, perhaps because of the formaldehyde treatment.

Evaluating the \(^{15}\)N enrichment ratios of protozoa relative to bacteria (ranging from 0.536 to 0.576) suggested that not correcting protozoa for feed N contamination underestimated this ratio compared with estimates that protozoa incorporate most of their protein from bacteria (i.e., a ratio closer to 1.0 being expected) (Williams and Coleman, 1992). However, our correction procedure yielded a higher ratio (\(\geq 0.835\)) that is more
consistent with expectations (explained above). However this ratio was more variable and resulted in an impossible value over 1.0 for the treatment containing both Rumensin and Cinnagar, which was within the confidence interval for 1.0 as verified by the large SEM. The current mixed population was predominantly *Entodinium* (Table 4.3). The resultant mean N/cell (3.05 to 9.80; Table 4.4) from our by-difference approach closely approximated the range of N/cell when calculated at 16 h post-feeding of cultures of *E. caudatum* (~ 3 ng/cell) or mixed entodinia (~ 9 ng/cell) in our previous study (Sylvester et al., 2009). Boiling and sonication also could disrupt bacterial cells, but we presume our protozoal samples had only minor contamination with bacteria based on our previous work.

Microbial N flow tended (P < 0.07) to be decreased by Cinnagar as a result of decreased (P < 0.05) protozoal N flow (Table 4.4). That decrease in protozoal N flow by Cinnagar without a corresponding increase in bacterial N flow would be inconsistent with expectations of increased bacterial N to compensate for the void from reduced protozoa (Firkins et al., 2007), although an alternative explanation is that protozoa still acquired substrate but did not convert it efficiently into protozoal biomass because they were using those nutrients to counteract the inhibition.

The flow of bacterial N tended to be increased (P = 0.15) as a non-additive response of the combination of Rumensin and Cinnagar. Cinnagar increased (P = 0.05) NANMN flow and the percentage of NANMN of the N intake. Rumensin decreased bacteria $^{15}$N:NH$_3$-$^{15}$N ratio (P < 0.01), and Cinnagar increased it (P = 0.03). Cinnagar tended (P < 0.09) to decrease N/cell, not cell counts, but tended (P < 0.09) to increase cell
volume, estimated as the volume of a cylinder (Table 4.3). However, we commonly observe that inhibitors make protozoa more translucent or sometimes appear to be shrunken. The environmental ciliate, *Paramecium*, responds to nutrient stress, as we expected in the current study would be introduced by Cinnagar, by decreasing the percentage of cells that are in a division state or increasing those that undergo autophagy (protracted recycling of cellular organelles to conserve nutrients) (Berger, 2001). Such a response might be enhanced because of the pressure from the relatively rapid solids passage rate in our fermenters. Thus, Cinnagar could be inhibiting protozoa such that they are still maintaining their digestion under the conditions of this experiment, but doing so as a result of inhibition that is rather influencing the usage of those digested nutrients. In support, Cinnagar could uncouple cellular energy through impaired membrane permeability (Benchaar and Greathead, 2011; McIntosh et al., 2003). The attempts for the protozoa cell volume calculations with 2 functions couldn’t offer an explanation. Teather et al. (1984) used the empirical formula assuming the protozoa were spherical. But, the length-to-width ratio in the current study averaged 1.56, indicating the sphere assumption was not realistic. Dehority (2010) considered *Epidinium* cell as a cylinder. Besides the accuracy of the function per se, the function in Dehority (2010) was used for *Epidinium*, which might be not suitable for other genera due to the different morphologies. Further research is needed on cell size change of other species of ruminal protozoa upon the cinnamaldehyde addition.

The protozoa N flow accounted for 41% of the microbial N with a 5.0% of solid dilution rate in the current study, but the number was 15% in Karnati et al. (2009a) with a
2.0% of solid dilution rate. In a preliminary experiment (data not shown), the generation time decreased as the solid dilution rate increased from 2 to 5%. Based on the protozoa retention control, 5.0% of solid dilution rate was set in the current study, and, as expected, the protozoa generation time was lower than that in Karnati et al. (2009a) resulting in increased protozoa N in the effluent outflow.

There is some research of the mode of action of cinnamaldehyde and garlic on microbes (Benchaar and Greathead, 2011). Kwon et al. (2003) reported that cinnamaldehyde caused uncompleted septa formation and cell division of the gram-positive bacterium, *Bacillus cereus*. Domadia et al. (2007) showed that cinnamaldehyde inhibited the GTPase function and polymerization of FtsZ, a bacterial homolog of tubulin that is essential for prokaryotic cytokinesis, to form the Z-ring, thus inhibiting the process of cell division and growth of the gram-negative bacterium, *Escherichia coli*. During a cell division, GTP-dependant polymerization occurs in the FtsZ protein to produce filaments, which assemble into Z-ring at the division site and later on decrease the size of the pole region as invagination proceeds. Both gram-positive and gram-negative ruminal bacteria may be susceptible to the cinnamaldehyde in the same way as reported due to the shared characteristics of cell division. Moreover, Burt (2004) suggested the antimicrobial properties of cinnamaldehyde on gram-positive and gram-negative bacteria were due to its carbonyl group binding and inactivating microbial enzymes. For ruminal microorganisms, Hart et al. (2008) suggested 2 modes of action of EO. One is EO affect the pattern of bacterial colonization of substrates, particularly starch rich substrates, as they enter the rumen. The other suggested mode of action is the inhibition of hyper
ammonia producing bacteria involved in deamination. All the above could possibly cause the inhibition on substrate incorporation of ruminal bacteria to produce microbial nitrogen, hence explaining the numerically reduced N efficiency in our study.

Non-ammonia non-microbial N (NANMN; an estimate of undegraded protein) was increased by Cinnagar (P = 0.05) but unaffected by Rumensin. The trend of decreased protozoa counts (Table 4.3) by Cinnagar explained the increased NANMN flow (decreased proteolysis), because a decrease in protozoal populations would be expected to decrease protease activity (Williams and Coleman, 1992; Walker et al., 2005). Furthermore, the increased NANMN flow by Cinnagar likely explains the decreased true OM digestibility (P = 0.05). Although Rumensin increased peptides, apparently as a result of inhibited hyperammonia-producing bacteria, but that was not enough to affect NANMN. But increased bacteria $^{15}$N:NH$_3$-$^{15}$N ratio by Cinnagar should have resulted in decreased NANMN flow. One explanation for the increased NANMN by Cinnagar is the nature of the dual flow fermenter system. The preprocessing of grinding and stirring for the diets provides more small particles to pass with the liquid phase than \textit{in vivo}, hence this allows greater efflux of NANMN than \textit{in vivo} (Karnati et al., 2009a). The protozoa limit the population of bacteria by predation and competition for substrate, thus the decreased protozoa population usually results in increased bacterial population (Dehority, 2003). Although there was no interaction between Cinnagar and Rumensin for the total protozoal counts (Table 4.3), the numerical decrease was consistent with the trend for increased bacteria N flow, possibly due to the increased bacterial population because protozoa contribute extensively to the ruminal proteolysis (Walker et al., 2005).
Volatile Fatty Acids

There was a trend for Cinnagar to increase the production of isovalerate ($P = 0.15$); however there was no main effect of Rumensin and Cinnagar on the production of total VFA, acetate, isobutyrate, butyrate, and valerate. Also, there was no interaction of Rumensin and Cinnagar for the production of total VFA or any individual VFA (Table 4.5). Rumensin tended to increase propionate production ($P = 0.10$), increased isovalerate production, and decreased acetate:propionate ratio from 4.6 to 3.8. The latter is expected based on previous research (Ipharraguerre and Clark, 2003). Also, Fellner et al. (1997) found that monensin added at 2 µg/mL of fermenter fluid decreased acetate:propionate ratio from 3.0 to 1.7. Jenkins et al. (2003) reported that feeding monensin (25 ppm/fermenter content) to fermenters with a diet of 70:30 concentrate:alfalfa hay decreased acetate:propionate ratio and increased molar proportions of acetate, propionate, and butyrate. However, Rumensin® fed at 12 g/909 kg DM to lactating Holstein cows didn’t show a decrease in acetate:propionate ratio (Mathew et al., 2011).

Supplementing cinnamaldehyde (0.6 g/d) and eugenol (0.3 g/d) to beef heifers decreased the branched chain VFA, such as isobutyrate and isovalerate (Cardozo et al., 2006). Patra and Yu (2012) reported decreased molar percentage of valerate by garlic oil added at 1g/L in vitro, but isovalerate was not affected at 3 doses (0.25, 0.5, and 1g/L). Besides the unconfirmed mode of action, cinnamaldehyde, as the main active component of cinnamon bark oil, may interact with other components present in low concentrations; thus, the component structure and the concentration might be the cause for inconsistent results (Macheboeuf et al., 2008).
Methane Production

Rumensin and Cinnagar tended \((P = 0.06)\) to interact for increased methane production (Table 4.5), which was contrary to our hypothesis. It was possibly due to the relative low dosage (about 0.91 mg/L/d) in the continuous culture experiment compared with those in batch culture in the studies reviewed by Benchaar and Greathead (2011), where cinnamon oil and garlic oil added at high concentrations (i.e., >300 mg/L of culture fluid) was effective at decreasing methane production \textit{in vitro}. Neither Rumensin nor Cinnagar affected the methane production, but Rumensin and Cinnagar numerically decreased methane. The numerical decreased methane by Rumensin is stoichiometrically consistent with increased propionate. Rumensin sometimes decreases methanogenesis (Odongo et al., 2007) but not consistently (Beauchemin et al., 2008). Perhaps, the slowing of growth rate by Rumensin (longer generation time, Table 4.3) combined with uncoupling of cellular energy by Cinnagar through impaired membrane permeability might have caused compensatory uptake and fermentation by protozoa to try to overcome the combined treatment. In a study with wortmannin, an inhibitor that decreases protozoal growth without toxicity, the disrupted digestive capacity and lower cellular energy were surmised to be responsible for the repeated observations of enhanced chemotaxis by entodiniomorphid protozoa (Díaz et al., 2008). Particularly during the first few hours after feeding when protozoal H\(_2\) production peaks and methanogens swarm to protozoa (Firkins and Yu, 2006; Ushida, 2010), the combined effects of these compounds could have promoted excessive protozoal H\(_2\) production during fermentation.
Moreover, EO could change the diversity of archaea and bacteria (Ohene-Adjei et al., 2008). Hart et al. (2008) observed the addition of allicin, the major component of garlic oil, at concentrations of 2 and 20 mg/L to show no effect on the total bacterial DNA, but it decreased methanogen DNA. They suggested the selective effect of allicin on ruminal microbial populations. However, Patra and Yu (2012) reported that the ruminal bacterial population changed by garlic oil at 0.25, 0.5, and 1 g/L \textit{in vitro}. Garlic oil decreased the populations of \textit{Ruminococcus flavefaciens}, \textit{R. albus}, and \textit{Fibrobacter succinogenes} by 2 log units, 1.7 log units and 0.8 log units, respectively. The \textit{R. flavefaciens} and \textit{R. albus} produce hydrogen as the substrate for methane production; whereas, \textit{F. succinogenes} produce formate (Russell and Hespell, 1981). Formate can also serve as a substrate for methanogenesis, but most of ruminal formate is converted to hydrogen and CO\textsubscript{2} (Hungate et al., 1970). Ohene-Adjei et al. (2008) supplemented cinnamaldehyde, garlic, and juniper berry oil at 0.02 g/kg DM to lambs, resulting in increased diversity of methanogenic archaea related to \textit{Methanosphaera stadtmanae}, \textit{Methanobrevibacter smithii}, and some uncultured archaea. This was claimed to support the adaptive response of the microbial community in the rumen after EO supplementation; also, the inhibition of protozoa was responsible.

Methane production can be uncoupled from methanogen abundance (Mosoni et al., 2011) and is not necessarily correlated with protozoal counts (Morgavi et al., 2012). Thus, inhibition strategies probably must reduce cellular metabolic capacity of protozoa (not necessarily cell counts). Further work is needed regarding how to inhibit protozoa enough to decrease their negative effects but not so much to disrupt normal microbial
ecology of the rumen. The inconsistent results may result from the nature of in vitro culture, the animal trial, and the dual flow continuous culture, the diet or feed, and the purity of the EO.

**Effluent Flow and Biohydrogenation of FA**

Rumensin tended (P = 0.07) to decrease the flow of 18:0 and increased (P = 0.02) the flow of trans-11 18:1 (vaccenic acid) (Table 4.6), thus increasing the ratios of this iromer relative to trans-10 18:1 or to total 18:0. Rumensin decreased (P < 0.01) the BH extent of total C18 FA, particularly the cis-9 18:1. Thus, Rumensin disrupted the complete BH of UFA, with such a shift being considered favorable for milk fat synthesis (Jenkins et al., 2008). When combined with diets containing high amounts of free oil and nonstructural carbohydrate, Rumensin might be expected to promote a trans-10 BH pattern (Oelker et al., 2009), but such is not always the case (Mathew et al., 2011). Interactions among protozoa and BH bacteria, as influenced by other environmental conditions, remain elusive (Firkins et al., 2008; Jenkins et al., 2008). Moreover, the faster turnover rate of particulates in this study compared with the previous study (Karnati et al., 2009a) would result in less extensive BH by bacteria (Qiu et al., 2004), which explains the generally lower extent of BH of FA than that in the study by Karnati et al. (2009a).

Cinnagar didn’t affect FA flow under the conditions of our study, but it increased (P = 0.03) the BH extent of cis-9 18:2 and tended to increase the BH extents of C18 (P = 0.10), 18:2 (P = 0.14), and 18:3 (P = 0.13). Cinnagar tended to decrease the total counts (Table 4.3) and numerically increased the generation time of protozoa, which contribute
minorly to BH (Singh and Hawke, 1979). The trend for increased BH extent of C18, 18:2 and 18:3 by Cinnagar relative to the control diet indicated a possible increase of bacterial population to BH the FA. Little research has been done on the influence of cinnamaldehyde and garlic oil on the FA profile and bacterial abundance involved in BH. Garlic oil infused through rumen cannula of goats inhibited the ruminal BH and didn’t affect the total abundance of Butyrivibrio group (Zhu et al., 2012). However, the abundance of Butyrivibrio proteoclasticus tended (P = 0.06) to decrease. Although not all bacteria involved in BH are known, these results suggested that Butyrivibrio is involved in BH of 18:2n-6 to cis-9 18:1. Also the garlic oil decreased protozoal biomass, thus reducing the incorporation of unsaturated or partially hydrogenated FA. This response, combined with decreased stearic acid producers, might have provided conditions for accumulation of bioactive FA. Cinnagar did not affect any ratios of those FA, except for a tendency to decrease the ratio of VA:trans-10 18:1 (P = 0.12). Because the VA:stearic acid ratio in bacteria was reported < 0.10 (Vlaemink et al., 2006), the increased ratio by Rumensin indicated a less complete BH, which was consistent with our BH results. Thus, Cinnagar’s apparent inhibition of protozoa would not necessarily spill over to bacteria, as evidenced by lack of effect on NDF digestibility and VFA production. Protozoa are not involved directly in BH, but they might influence those bacteria that are involved in BH (Karnati et al., 2009a).

Dietary lipids in the rumen are hydrolyzed by lipases originated from plants and microbes (Lourenço et al., 2010). Both the saturated and unsaturated free FA from lipolysis can be sequestered into microbial cells for lipid synthesis (Harfoot and
Hazlewood, 1997). Zhu et al. (2012) suggested that the effect of garlic oil on lipolysis may play a role in the modification of FA. The shift in bacteria diversity upon EO supplementation would be worth further investigation.

**CONCLUSIONS**

Under the conditions of our study, we did not detect an additive response for Rumensin® and Cinnagar® to decrease protozoal counts or methane production. Further research is needed on cell size change of ruminal protozoa upon the Cinnagar addition and how to inhibit protozoa enough to decrease their negative effects but not so much to disrupt normal microbial ecology of the rumen.
Table 4.1. Ingredient and nutrient composition of diets without (-) or with (+) Rumensin® and Cinnagar®

<table>
<thead>
<tr>
<th>Item</th>
<th>Rumensin®</th>
<th>Cinnagar®</th>
</tr>
</thead>
<tbody>
<tr>
<td>Item, % of DM</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Alfalfa meal</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Soybean hulls</td>
<td>20.4</td>
<td>20.4</td>
</tr>
<tr>
<td>Corn, ground</td>
<td>18.1</td>
<td>18.1</td>
</tr>
<tr>
<td>Distillers grains with solubles</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Soybean meal, 48 % CP</td>
<td>4.50</td>
<td>4.50</td>
</tr>
<tr>
<td>Corn oil</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>TM salt</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>Magnesium oxide</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>Rumensin®2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cinnagar®2</td>
<td>0</td>
<td>0.0043</td>
</tr>
<tr>
<td>Nutrients, % of DM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>93.1</td>
<td>93.3</td>
</tr>
<tr>
<td>OM</td>
<td>91.5</td>
<td>91.5</td>
</tr>
<tr>
<td>NDF</td>
<td>38.8</td>
<td>39.3</td>
</tr>
<tr>
<td>CP</td>
<td>17.0</td>
<td>16.7</td>
</tr>
</tbody>
</table>

1Diets were fed at 40 g/d.
2Rumensin® was provided by Elanco Animal Health, Greenfield, IN; Cinnagar® was provided by Provimi North America, Inc., Brookville, OH.
Table 4.2. Fatty acid (FA) composition of the dietary treatments without (-) or with (+) Rumensin® and Cinnagar®

<table>
<thead>
<tr>
<th>Item</th>
<th>Rumensin®</th>
<th></th>
<th>-</th>
<th>+</th>
<th></th>
<th>Cinnagar®</th>
<th></th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total FA, % of DM</td>
<td>2.03</td>
<td>2.07</td>
<td>2.07</td>
<td>2.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Individual FA, g/100 g FA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>17.3</td>
<td>17.4</td>
<td>17.5</td>
<td>17.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>3.12</td>
<td>3.11</td>
<td>3.11</td>
<td>3.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis-9 18:1</td>
<td>16.0</td>
<td>16.4</td>
<td>16.3</td>
<td>16.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis-11 18:1</td>
<td>0.78</td>
<td>0.80</td>
<td>0.79</td>
<td>0.79</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>trans 18:1⁴</td>
<td>0.40</td>
<td>0.44</td>
<td>0.48</td>
<td>0.49</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2n-6</td>
<td>37.9</td>
<td>38.2</td>
<td>38.8</td>
<td>37.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLA⁵</td>
<td>1.16</td>
<td>1.12</td>
<td>1.11</td>
<td>1.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3n-6</td>
<td>0.77</td>
<td>0.76</td>
<td>0.76</td>
<td>0.77</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3n-3</td>
<td>15.4</td>
<td>15.1</td>
<td>15.0</td>
<td>15.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Diets containing Rumensin® at 11g/909 kg or Cinnagar® at 0.0043% on a dry matter basis.
²Consisted of 18:1 trans-10, trans-12, and trans-13.
³Geometrical and positional isomers of conjugated linoleic acid.
Table 4.3. Protozoal counts, generation time, generic distribution, and protozoa cell volume in continuous cultures fed diets without (-) or with (+) Rumensin® and Cinnagar®

<table>
<thead>
<tr>
<th></th>
<th>Rumensin®</th>
<th></th>
<th>Contrasts²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>SEM</td>
</tr>
<tr>
<td>Total counts (10³ x mL⁻¹)</td>
<td>20.5</td>
<td>13.6</td>
<td>16.8 1.9</td>
</tr>
<tr>
<td>Effluent flow, cells/d (x 10⁶)</td>
<td>38.3</td>
<td>26.6</td>
<td>27.5 24.4 3.7</td>
</tr>
<tr>
<td>Generation time (h)</td>
<td>22.0</td>
<td>22.3</td>
<td>27.0 28.2 1.2</td>
</tr>
<tr>
<td>Genera in fermenter (% total)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entodinium</td>
<td>90.8</td>
<td>93.7</td>
<td>90.2 92.1 4.0</td>
</tr>
<tr>
<td>Isotrichidae⁴</td>
<td>5.93</td>
<td>5.02</td>
<td>6.65 5.15 1.4</td>
</tr>
<tr>
<td>Diplodiniinae⁵</td>
<td>1.93</td>
<td>0.83</td>
<td>1.90 1.83 0.47</td>
</tr>
<tr>
<td>Genera in effluent (% total)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entodinium</td>
<td>92.2</td>
<td>94.5</td>
<td>93.3 93.8 1.8</td>
</tr>
<tr>
<td>Isotrichidae⁴</td>
<td>6.65</td>
<td>3.40</td>
<td>2.74 3.33 1.7</td>
</tr>
<tr>
<td>Diplodiniinae⁵</td>
<td>0.60</td>
<td>2.08</td>
<td>2.17 1.41 0.95</td>
</tr>
<tr>
<td>Mean length:width</td>
<td>1.58</td>
<td>1.50</td>
<td>1.65 1.52 0.038</td>
</tr>
<tr>
<td>Protozoa cell volume (x 10⁴ µm³)</td>
<td>2.75</td>
<td>2.65</td>
<td>2.76 3.05 0.28</td>
</tr>
<tr>
<td>Protozoa cell volume (x 10⁴ µm³)</td>
<td>4.69</td>
<td>5.11</td>
<td>4.09 5.53 0.41</td>
</tr>
</tbody>
</table>

¹Diets contained Rumensin® at 11g/909 kg or Cinnagar® at 0.0043% on a DM basis.
²Main effects or interaction of Rumensin® (Rum) and Cinnagar® (Cin). NS= Not significant; P > 0.15.
³Total pool size of cells in fermenter/ effluent flow of cells x 24h/d.
⁴Family, including the genera Dasytricha and Isotricha.
⁵Subfamily, including the genera Diplodinium, Eudiplodinium, Enoploplastron, Metadinium, Ostracodinium and Polyplastron.
⁶Length( length/ 4)²π (Teather et al., 1984).
⁷Length( width/ 2)²π (Dehority, 2010).
Table 4.4. The neutral detergent fiber (NDF) and organic matter (OM) digestibilities, ammonia nitrogen, peptide nitrogen and N fractions and bacterial efficiency in continuous cultures fed diets without (-) or with (+) Rumensin® and Cinnagar®

<table>
<thead>
<tr>
<th></th>
<th>Rumensin®</th>
<th>Cinnagar®</th>
<th>SEM</th>
<th>Contrasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NDF digestibility (%)</td>
<td>58.8</td>
<td>57.1</td>
<td>58.2</td>
<td>52.8</td>
</tr>
<tr>
<td>Apparent OM digestibility (%)</td>
<td>48.0</td>
<td>47.8</td>
<td>48.5</td>
<td>46.5</td>
</tr>
<tr>
<td>True OM digestibility (%)</td>
<td>58.9</td>
<td>55.0</td>
<td>61.7</td>
<td>51.9</td>
</tr>
<tr>
<td>NH₃-N (mg/dL)</td>
<td>15.0</td>
<td>14.9</td>
<td>15.0</td>
<td>15.6</td>
</tr>
<tr>
<td>Peptide N (mg/dL)</td>
<td>7.47</td>
<td>7.85</td>
<td>8.41</td>
<td>8.44</td>
</tr>
<tr>
<td>Protozoa N (ng/cell)</td>
<td>16.1</td>
<td>28.2</td>
<td>22.6</td>
<td>17.2</td>
</tr>
<tr>
<td>Protozoa N (ng/cell)</td>
<td>5.68</td>
<td>4.63</td>
<td>9.80</td>
<td>3.05</td>
</tr>
<tr>
<td>Effluent N flows</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total N (g/d)</td>
<td>0.861</td>
<td>0.871</td>
<td>0.815</td>
<td>0.862</td>
</tr>
<tr>
<td>Microbial N (g/d)</td>
<td>0.504</td>
<td>0.369</td>
<td>0.478</td>
<td>0.334</td>
</tr>
<tr>
<td>Protozoal N (g/d)</td>
<td>0.250</td>
<td>0.119</td>
<td>0.254</td>
<td>0.071</td>
</tr>
<tr>
<td>Bacterial N (g/d)</td>
<td>0.259</td>
<td>0.251</td>
<td>0.226</td>
<td>0.263</td>
</tr>
<tr>
<td>NAN (g/d)</td>
<td>0.861</td>
<td>0.871</td>
<td>0.815</td>
<td>0.862</td>
</tr>
<tr>
<td>NAN, % of N intake</td>
<td>86.0</td>
<td>87.5</td>
<td>82.1</td>
<td>86.4</td>
</tr>
<tr>
<td>NANMN (g/d)</td>
<td>0.357</td>
<td>0.503</td>
<td>0.337</td>
<td>0.528</td>
</tr>
<tr>
<td>NANMN, % of N intake</td>
<td>35.7</td>
<td>50.4</td>
<td>33.9</td>
<td>52.9</td>
</tr>
<tr>
<td>N efficiency</td>
<td>26.9</td>
<td>22.6</td>
<td>24.6</td>
<td>20.5</td>
</tr>
<tr>
<td>Bacteria ¹⁵N:NH₃⁻¹⁵N</td>
<td>0.687</td>
<td>0.693</td>
<td>0.652</td>
<td>0.673</td>
</tr>
<tr>
<td>Protozoa ¹⁵N:Bacteria ¹⁵N¹¹</td>
<td>0.576</td>
<td>0.549</td>
<td>0.562</td>
<td>0.536</td>
</tr>
<tr>
<td>Protozoa ¹⁵N:Bacteria ¹⁵N¹²</td>
<td>0.908</td>
<td>0.885</td>
<td>0.835</td>
<td>1.108</td>
</tr>
</tbody>
</table>

¹Diets containing Rumensin® at 11g/909 kg or Cinnagar® at 0.0043% on a DM basis.
²Main effects or interaction of Rumensin® (Rum) and Cinnagar® (Cin). NS= Not significant; P > 0.15.
³Determined by the formula: Peptide N = Trichloroacetic acid-soluble N minus NH₃N.

(Continued)
(Table 4.4. Continued)

4 Calculated only with N in not boiled or sonicated (NBS) retentates (Figure 4.1).
5 Calculated by difference of N from NBS and boiled and sonicated (BS) retentates (Figure 4.1). All the other effluent flow calculations used this protozoa N.
6 The treatments control and Rum had missing values in period 1. The SEM is approximately 20% lower than the repeated SEM.
7 Nonammonia N (NAN) determined by the formula: NAN = Total N flow minus NH$_3$-N.
8 N intake was from feed and did not include N addition from buffer.
9 Nonammonia, nonmicrobial N (NANMN) determined by the formula: NANMN = NAN minus microbial N.
10 Grams microbial N produced/kilogram OM truly digested.
11 Calculated only with protozoa $^{15}$N from NBS retentates.
12 Calculated by difference of protozoa $^{15}$N from NBS and BS retentates.
Table 4.5. Fermentation characteristics in continuous cultures fed diets without (-) or with (+) Rumensin® and Cinnagar®

<table>
<thead>
<tr>
<th></th>
<th>Rumensin®</th>
<th>Cinnagar®</th>
<th>Contrasts</th>
<th>SEM</th>
<th>Cin</th>
<th>Rum</th>
<th>Cin*Rum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VFA, mmol/d</td>
<td>150</td>
<td>147</td>
<td>143</td>
<td>153</td>
<td>7.4</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Individual VFA (mmol/d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>96.4</td>
<td>95.6</td>
<td>89.2</td>
<td>95.9</td>
<td>4.6</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Propionate</td>
<td>31.7</td>
<td>30.7</td>
<td>33.4</td>
<td>35.5</td>
<td>1.9</td>
<td>NS</td>
<td>0.10</td>
</tr>
<tr>
<td>Isobutyrate</td>
<td>1.06</td>
<td>1.01</td>
<td>0.89</td>
<td>1.09</td>
<td>0.17</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Butyrate</td>
<td>15.0</td>
<td>14.7</td>
<td>14.0</td>
<td>14.4</td>
<td>0.75</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Isovalerate</td>
<td>2.50</td>
<td>2.61</td>
<td>2.74</td>
<td>3.17</td>
<td>0.16</td>
<td>0.15</td>
<td>0.05</td>
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<tr>
<td>Valerate</td>
<td>2.78</td>
<td>2.71</td>
<td>2.54</td>
<td>2.56</td>
<td>0.14</td>
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<td>NS</td>
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<tr>
<td>Acetate: Propionate</td>
<td>4.56</td>
<td>4.60</td>
<td>3.83</td>
<td>4.13</td>
<td>0.21</td>
<td>NS</td>
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<tr>
<td>Methane (mmol/d)</td>
<td>29.3</td>
<td>21.8</td>
<td>22.4</td>
<td>36.7</td>
<td>6.0</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

1Diets containing Rumensin® at 11g/909 kg or Cinnagar® at 0.0043% on a DM basis.
2Main effects or interaction of Rumensin® (Rum) and Cinnagar® (Cin). NS = Not significant; P > 0.15.
<table>
<thead>
<tr>
<th></th>
<th>Rumensin®</th>
<th></th>
<th></th>
<th>Contrasts²</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Flow, mg/d</td>
<td>18:0</td>
<td>325</td>
<td>321</td>
<td>267</td>
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<tr>
<td></td>
<td>cis-9 18:1</td>
<td>110</td>
<td>102</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>trans-10 18:1</td>
<td>13.1</td>
<td>13.5</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>trans-11 18:1</td>
<td>185</td>
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<td>224</td>
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<tr>
<td></td>
<td>18:2n-6</td>
<td>133</td>
<td>118</td>
<td>136</td>
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<td>17.7</td>
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<td>18:3n-3</td>
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<td>41.5</td>
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<td>873</td>
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<td>Total C18</td>
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<td></td>
<td>18:2</td>
<td>67.5</td>
<td>68.9</td>
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<td>18:3</td>
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<td>71.6</td>
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<td>VA:trans-10⁵</td>
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<td>13.4</td>
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<tr>
<td></td>
<td>VA:18:0</td>
<td>0.58</td>
<td>0.58</td>
<td>0.86</td>
</tr>
</tbody>
</table>

¹Diets containing Rumensin® at 11 g/909 kg or Cinnagar® at 0.0043% on a DM basis.
²Main effects or interaction of Rumensin® (Rum) and Cinnagar® (Cin). NS = Not significant; P > 0.15.
³Geometrical and positional isomers of conjugated linoleic acid.
⁴BH = Biohydrogenation, calculated as percentage of C18 FA that disappeared between intake and effluent flow (Tice et al., 1994); total C18 FA were weighted for the number of unsaturated bonds.
⁵VA = vaccenic acid (trans-11 18:1); UFA = total C18 unsaturated FA; trans-10 = trans-10 18:1.
Composited effluent

\[ \downarrow \]

Collected to container with a final concentration of 1% of formalin

\[ \downarrow \]

Centrifuge at 500 g for 10 min

\[ \downarrow \]

Collect the pellet

300 micron filter; collect filtrate rinsed with distilled water; evenly divided into 2 aliquots

Take samples for N, DM, and cell number

Filtrate 1

\[ \downarrow \]

10 micron filter; collect retentate

Take samples for N, DM, and cell number

Filtrate 2

Boil in water bath for 5 min; sonicate for 10 min; 10 micron filter; collect retentate

Take samples for N, DM, and cell number

Retentate

Figure 4.1. Procedure of boiling and sonication on protozoa
CHAPTER 5

EXAMINING THE EFFECT OF ORAL ADMINISTRATION OF
MEGASPHAERA ELSDENII ON PERFORMANCE OF JERSEY COWS DURING
EARLY LACTATION

ABSTRACT

Lactate utilizing *Megasphaera elsdenii* has been suggested to reduce the incidence of rumen acidosis. Administration of *M. elsdenii* could lessen the adverse impacts during the first 30 DIM of dairy cows when they are prone to metabolic diseases, thus improve the overall performance. The objective of this study was to determine if *M. elsdenii* orally administered to transition Jersey cows would improve milk yield and reduce the risk for metabolic disease, and to monitor the establishment and persistence of *M. elsdenii* in the rumen. Thirty primi- and multiparous Jersey cows, blocked according to parity and date of calving, were used in a randomized complete block design until 90 DIM. Within each block, the cows were assigned to 1 of 2 treatments: 1) control (no dose), or 2) 200 mL Lactipro by oral drenching at 1 to 2 d postpartum (*M. elsdenii*, 1 x $10^8$ cfu/mL; MSBiotec, Littleton, CO). Close-up cows were fed 60% forage (62:38 mix of grass and ryegrass balage:corn silage), and fed 50% forage (23:77 ryegrass balage:corn silage) for 30 DIM after calving, and then the cows were fed 50% forage (13:87...
The DMI, BCS, and BW change were similar between treatments, and no treatment by time interactions occurred. There was no difference in milk yield between the treatments (29.1 and 28.9 ± 0.89 kg/d). A treatment by parity interaction was found for milk yield (P < 0.01), and a trend (P = 0.06) of treatment by parity interaction was found for milk fat yield. For cows with ≥ 3 lactations, those dosed with *M. elsdenii* had higher milk yield and tended to have higher milk fat yield than the control cows. No treatment effect or treatment by time interactions were detected for milk fat and protein percentages. Based on urine ketones, 6.7% of the cows experienced clinical ketosis. The cows with ≥ 15 mg/dL urine ketones were 13.3% for the control cows and 6.7% for the dosed cows. *M. elsdenii* population appeared to be numerically greater in the 2 dosed cows than the 2 control cows, and apparent higher population may have persisted for about 2 d after dosing.

**INTRODUCTION**

The transition period is very critical for cows because of the physiological, metabolic, and nutritional changes and the change from low concentrate:high forage ration to a high concentrate:low forage ration. The success of the following lactation might be determined by the performance during the transition period. The increasing concentration of non-structural carbohydrates (NSC) with a high concentrate ration may lead to undesirable accumulation of VFA and lactic acid in the rumen, causing subacute rumen acidosis (SARA), which is defined when ruminal pH remains below 5.6 for more than 3 h/24 h (Plaizier et al., 2008) or below 5.8 for more than 5.2 h/24 h (Zebeli et al.,
The SARA has been associated with economic losses between $500 million to $1 billion each year (Enemark, 2009). Attempts have been made to prevent the incidence of SARA by using buffers, ionophores, and direct-fed microbials (DFM) (Yoon and Stern, 1995; Allen, 1997; Ghorbani et al., 2002; Enemark, 2009).

Using culture-based techniques, Counotte et al. (1981) found that \( M. \) \( elsdenii \) was the predominant lactic-acid utilizer within the rumen, fermenting up to 73% of the ruminal lactate. Real-time PCR analysis by Fernando et al. (2010) detected significant fold increases in the \( M. \) \( elsdenii \) population during adaptation to the high-concentrate diet. It has attracted growing interest as a DFM to prevent acidosis in grain-fed cattle (Kung and Hession 1995; Wiryawan and Brooker 1995; Owens et al., 1998). The inoculation of \( M. \) \( elsdenii \) in beef steers or lambs showed decreased lactic acid accumulation in the rumen (McDaniel, 2009; Henning et al., 2010a) or had no effect on the lactic acid concentration (Kliève et al., 2003; Hagg et al., 2010). Milk production and composition results are inconsistent for the dairy cows administered with \( M. \) \( elsdenii \) (Hagg et al., 2010; Aikman et al., 2011). A milk fat reduction by dosing \( M. \) \( elsdenii \) was shown by Aikman et al. (2011). However, the reduction in milk fat may be better explained by a reduction in mobilization of body fat stores (Aikman et al., 2011). Based on the current research available, the effect of \( M. \) \( elsdenii \) on milk fat concentration is still unclear. Very limited animal trials have evaluated the effect of \( M. \) \( elsdenii \) administration on blood metabolites and dairy cow health. Orally dosing cows with \( M. \) \( elsdenii \) prepartum or postpartum didn’t affect the health events and reproductive performance (Stevens, 2013).
Dosing with *M. elsdenii* prepartum may improve ruminal conditions of high-producing transition cows whereby milk yield is increased (Stevens, 2013).

The ability to both examine the population of *M. elsdenii* as probiotics and to then monitor the establishment and persistence of this population is essential to evaluate the efficacy of the organism (Ouwerkerk et al., 2002). The real-time Taq nuclease assay was used by Ouwerkerk et al. (2002) for the population of *M. elsdenii* in one rumen-cannulated steer at pasture and 4 rumen-cannulated steers in pens by intra-ruminal administration of $5.5 \times 10^{12}$ cfu of *M. elsdenii* YE34. They found that *M. elsdenii* was not detected in ruminal contents from the pasture-fed steer or the 2 un-inoculated steers. But, it was detected at $3.3 \times 10^6$ cells/mL and $3.1 \times 10^6$ cells/mL at 2 h after dosing, and $2.7 \times 10^6$ cells/mL and $7.4 \times 10^6$ cells/mL at 50 h after dosing. *M. elsdenii* in the 2 un-inoculated cows was not detectable. Klieve et al. (2003) has shown that *M. elsdenii* YE34 was not detectable in beef cattle without grain in the diet and established 5 to 7 d sooner in the rumen of beef cattle rapidly changed from a forage-based to a grain-based diet than the uninoculated cattle. Henning et al. (2010a) found that the population of *M. elsdenii* was higher in steers 2 and 3 d after inoculation with $1.7 \times 10^{11}$ cfu/dose *M. elsdenii* CH4 (NCIMB 41125) than that of the control steers. We know of no data available for the establishment and persistence of *M. elsdenii* in dairy cows up to now.

The hypotheses of this study were that dosing cows with *M. elsdenii* would reduce the risk for metabolic diseases and increase milk yield during early lactation. The objectives of the current experiment were to: (1) determine if *Megasphaera elsdenii* (Lactipro) orally administered to transition dairy cows would improve milk yield and
reduce the risk for metabolic disease, and (2) to monitor the establishment and persistence of *M. elsdenii* in the rumen using real-time PCR.

**MATERIALS AND METHODS**

**Experiments, Treatments, and TMR**

Thirty primi- and multiparous Jersey cows, including 4 rumen-cannulated Jersey cows (cannulated under Animal Use Protocol 2010A00000176) at Waterman Dairy Center (Columbus, OH) were used in this trial starting on December 13, 2012. Close-up cows and heifers were fed a diet with about 60% of forage beginning ~21 d prepartum, and the last cow calved on May 1, 2013. The trial ended on July 29, 2013 when the last cow reached 90 DIM. All 30 cows were followed until 90 DIM for milk production data. The experiment was performed as a randomized complete block design. The heifers and cows were blocked based on parity and date of calving. Within each block, the cows were randomly assigned to 1 of 2 treatments: 1) control (no dose, n=15), or 2) 200 mL Lactipro at 1 to 2 d postpartum (*M. elsdenii*, strain CH4, patented NCIMB 41125, 1 x 10⁸ cfu/mL; MSBiotec, Littleton, CO; n=15). The 200 mL dose of Lactipro was orally drenched while the cows were in headlocks during the morning feeding. Close-up dry cows and heifers were fed once daily at 0600 h for *ad libitum* intake. All cows were placed in a tie-stall barn at calving and fed a fresh cow TMR with 51:49 forage:concentrate for 30 d (Table 5.1). The TMR was mixed once daily for the lactating cows and fed at 2 intervals: 0700 h and 1700 h. After 30 DIM, cows were moved to free stalls and fed a lactating cow TMR with 50:50 forage:concentrate *ad libitum* (Table 5.1),
which was mixed and delivered every day at 1730 h. All the cows had *ad libitum* access to water. This study was approved under Animal Use Protocol 2012A00000158.

**Dry Matter Intake, Body Weight, BCS, and Health Events**

The feed refusals were recorded for 30 d after calving and the DM intake was calculated while the cows were in the tie stalls. Body weight was measured weekly within the 30 DIM, then at 60 and 90 DIM. Cows were body condition scored (1 to 5 on a quarter unit scale; 1 = thin, 5 = fat) prepartum (~14 DIM) and at 30, 60, and 90 DIM. Health events were monitored until 90 DIM. The parlor log recorded by the dairy personnel with health events and the log recorded by the author were combined into a full log for further analysis of health event.

**Urine and Blood Metabolites**

Urine ketones were measured using Ketostix (Bayer Corporation, Leverkusen, Germany) at 7 to 14 d prepartum and 7 to 14 DIM. Blood samples were taken at 3 to 5 days prior to the projected calving date and 7 to 14 DIM via the median coccygeal vein or artery for analysis of beta-hydroxybutyrate (BHBA) and non-esterified fatty acids (NEFA) to determine the metabolic status of the cows pre- and postpartum. The blood samples were collected in 10 mL Vacutainer® tubes (Becton Dickinson Vacutainer, Franklin Lakes, NJ) with clot activator. The blood tubes were immediately placed on ice until they were centrifuged at 2000 x g at 4°C for 20 min to obtain serum. The serum was then divided into three aliquots in 1.5 mL Eppendorf® tubes and stored at -20°C before BHBA and NEFA analyses. The BHBA concentrations were measured using an assay kit (Sigma-Aldrich Co. LLC, St Louis, MO). The NEFA concentrations were determined
using an enzymatic colorimetric assay with NEFA-HR 2 procedure (Wako Diagnostics, Richmond, VA). All samples were analyzed in duplicate for both BHBA and NEFA assays. The concentration of BHBA and NEFA were calculated using the absorbance values obtained from the microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA).

**Feed Sampling and Analyses**

Samples of the corn silage, wet wrapped ryegrass (WWR), grain mix, and TMR for the fresh cows and grass mix for close-up cow diet were collected weekly. The feed samples were dried at 55°C for 48 h and ground through a 1-mm screen (Wiley Mill, Arthur H. Thomas Co., Philadelphia, PA). The grain mix for the close-up cow diet was collected monthly from January to May, 2013, and ground through a 1-mm screen. The corn silage, WWR, and TMR for the fresh cow diet were composited bi-weekly for DM (AOAC, 1990), crude protein (N x 6.25) (AOAC, 1990; Kjeldahl N, Foss 220 Kjeltec Auto Distillation, Eden Praire, MN), and ash (AOAC, 1990). The NDF concentration was analyzed in the presence of heat-stable amylase and sodium sulfite (Van Soest et al., 1991). The TMR for the fresh cow diet and corn silage, grass mix and grain mix for close-up cow diet were composited by month for minerals, starch, and fat analyses. Mineral concentrations were analyzed using inductively coupled plasma emission spectrometry by The Ohio State University STAR laboratory (Wooster, OH). Starch content (Hall, 2009) and fat by ether extraction (AOAC, 2006) were analyzed at Cumberland Valley Analytical Services (Maugansville, MD).
Milk Collection and Analyses

Cows were milked 2 times daily (0500 and 1700 h) through 90 DIM using the Afimilk system (SAE Afikim, Kibbutz Afikim, Israel) at the farm, and daily milk weights were recorded by AfiFarm (SAE Afikim, Kibbutz Afikim, Israel) for data collection. Daily milk yield was averaged by week for each cow for milk production analysis. Milk samples were collected weekly for 4 consecutive milkings during the first 30 DIM. Milk samples were sent to DHI Cooperative, Inc. (Columbus, OH) for milk composition analysis. Milk fat and protein were analyzed by infrared spectroscopy (B2000 Infrared Analyzer, Bentley Instruments, Chaska, MN), and SCC were analyzed using a Bentley Somacount 300 (Bentley Instruments, Chaska, MN).

Ruminal Sampling and Analyses

Rumen samples were collected from the 4 cannulated cows, 2 of which were control and the other 2 were dosed with M. elsdenii postpartum. Samples were collected 7 to 10 d prior to the projected calving date, post-calving-pre-dosing (if applicable), 0h, 0h after dosing (if applicable), 2 h, 4 h, 8 h, 24 h, 48 h, 3 d, 5 d, 7 d, 60 d, 90 d after calving to monitor the presence of the M. elsdenii in the ruminal contents. The samples from one cow (486) were insufficient because of her death at 11 DIM due to the mucosal ulceration of the abomasum. Representative rumen samples were obtained from 5 different spots in the rumen at each sampling. A total of 46 samples were collected. The samples were blended (Waring Products, Division, Dynamics Corporation of America, New Hartford, CT) for 1 min at high speed to dislodge particle-associated bacteria. The rumen fluid was squeezed through 8 layers of cheese cloth. A known volume of the rumen fluid was
centrifuged at 4°C at 19,000 x g and the supernatant was removed. The pellet was collected with weight recorded and stored at -80°C before DNA extraction.

Extraction of DNA was conducted using repeated bead beating plus column (RBB+C) method described by Yu and Morrison (2004), with modification in Appendix A. The DNA concentration for each sample was determined by NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, DE), and 100 ng of DNA was used as the template in the real-time PCR, the protocol of which is described in Appendix B. Primers targeting 16s rRNA gene of M. elsdenii were used. The primer set was: forward primer MelsF: 5’-GACCGAAACTGCGATGCTAGA-3’ (E. coli numbering system 635-655); reverse primer MelsR: 5’- CGCCTCAGCGTCAGTTGTC-3’ (745-763) (Ouwerkerk et al., 2002).

**Statistical Analysis**

Data were analyzed using the MIXED procedure of SAS 9.3 (SAS, 1999) as a randomized complete block design with treatment, parity, and time as fixed effects and block as the random effect. The DMI, Milk yield, milk composition and BCS were analyzed as repeated measures. An autoregressive order 1 covariate matrix was used. Prepartum BCS, NEFA, and BHBA were used for covariate adjustment of BCS, NEFA, and BHBA data, respectively. The treatments were compared using PDIFF option of the LSMEANS statement. Significant treatment effects were declared at P ≤ 0.05 and values differed at the 0.05 < P ≤ 0.15 for trends. Nonsignificant 2- and 3-way interactions were pooled into the error term.
RESULTS

Animals, Sampling, and Feed Composition

Due to the limited stall availability in the tie stall barn, some of the cows had to be moved to the free stall barn before reaching 30 DIM. The average time that cows remained in the tie stall barn was 28 d, with the shortest of 19 d. The ingredients and chemical compositions of the close-up, fresh, and lactating cow TMR are listed in Table 5.1. Nutrient concentrations in the 3 diets met or exceeded NRC (2001) recommendations. However, the starch concentration of the fresh cow TMR was lower than the close-up and lactating cow TMR, which was likely due to the low starch concentration in the corn silage. The P concentrations in close-up and fresh cow diets were lower than 0.37%, which was recommended by NRC (2001). The Ca:P ratios for both diets, 4.00 and 3.06, respectively, were higher than the recommended maximum of 2.50 (NRC, 2001), which was probably due to the low P and the use of anionic salts in the close-up diet. Also, the P concentration in the ryegrass balage was found to be low (data not shown). In April 2013, the forage for the close-up TMR was changed from ryegrass balage to a mix of ryegrass balage and grass hay. However, the feed samples were composited by month, and the average P concentration was higher in the samples collected during the first 4 mo of the experiment.

DMI, BCS and Body Weight

The DMI was not affected by treatment (Table 5.2), but it differed by week of lactation (Figure 5.1). As expected, the DMI increased from 1 to 4 wk of lactation, but there was no treatment by week interaction for DMI. The BW was not affected by
treatment, DIM, or treatment by DIM interaction. Treatment did not affect the weekly BW change, with an average of 4.5 kg/wk decrease. There was a week effect on the BW change (Figure 5.2), which decreased by 56% from 1 to 2 wk of lactation (P = 0.02). The BCS did not differ by treatment (Table 5.2), but it tended to differ by DIM (Figure 5.3). No difference in BCS occurred between 30 and 60 DIM, or 60 and 90 DIM, but the BCS dropped at 30 DIM compared with prepartum, indicating an intense mobilization of body tissues for milk production. There was no treatment by day interaction for BCS (P > 0.15).

**Milk production and composition**

Milk yield from 1 to 13 wk of lactation was not affected by treatment, with an average of 29 kg/d. Also, milk yield from 1 to 4 week and from 5 to 13 wk of lactation did not differ by treatment. A difference by week of lactation was observed for milk yield from 1 to 4 week and 1 to 13 wk of lactation (Figure 5.4). The milk yield increased from 1 through 5 wk of lactation. Week of lactation tended (P = 0.08) to exert an effect on the milk yield from 5 to 13 week. There was no treatment by week interaction, but treatment by parity interaction occurred (P < 0.01) for milk yield. The treatment by parity interaction for the daily milk yield from 1 to 13 wk of lactation is shown in Figure 5.5. The control cows at 2nd lactation had a higher (P < 0.05) milk yield than the dosed cows; in contrast, the dosed cows with ≥ 3 lactations yielded more (P = 0.01) milk than the control cows.

There were no treatment, time, or interaction of treatment by time effects on milk fat percentage, which averaged 4.7%. No treatment or treatment by week interaction was found for milk fat yield. However, the milk fat yield differed by week, increasing by
37.9% from 1 to 2 wk of lactation (P < 0.01). Additionally, a treatment by parity interaction tended (P = 0.06) to occur for milk fat yield (Figure 5.6). Milk fat yield tended to be higher (P = 0.11) for 2\textsuperscript{nd} lactation control cows than the dosed cows and higher (P = 0.09) for dosed cows with \( \geq 3 \) lactations than the control cows. The milk protein percentage, averaging 3.54%, was not different between treatments, and no treatment by time interaction was observed. However, week of lactation affected the milk protein percentage, which decreased by 17.7% from 1 to 2 wk of lactation. No treatment effect or treatment by week interaction occurred for the milk protein yield. However, week of lactation affected the milk protein yield (P = 0.03), which decreased by 15.6% from 1 to 2 wk of lactation (P < 0.01). MUN did not differ by treatment but differed by time (P < 0.01). There was a trend for a treatment by time interaction (P = 0.10), which was mainly due to the higher (P = 0.04) concentration of MUN in dosed cows than the control cows at 4 wk of lactation. A treatment by parity interaction was observed for MUN (Figure 5.7). MUN concentration was higher for dosed cows at 2\textsuperscript{nd} lactation than the control cows (P \( \leq 0.01 \)). The SCC did not differ by treatment or week, and no interaction of treatment by week was observed.

**Urine and Blood Metabolites**

The sampling of blood occurred, on average, at 6 d prepartum, which was 1 d later than the targeted 3 to 5 d, due to the unpredictability of the actual calving date. The postpartum blood was sampled and urine ketone was measured both at 10 DIM, which was within the range of 7 to 14 DIM as planned. Urine ketones (acetoacetate) did not differ between treatments (Table 2). The average concentration of urine ketone
postpartum in our study was 11.7 mg/dL. Neither the NEFA nor the BHBA concentrations differed between the cows dosed with *M. elsdenii* and the control cows.

**Detection of *M. elsdenii* in Rumen Contents**

The population of *M. elsdenii* was expressed as 16s rRNA gene copies/mL rumen fluid in Figure 5.8. We did not perform the statistical analysis on the data due to the limited sample size. The *M. elsdenii* populations were $1.2 \times 10^6$ and $9.9 \times 10^5$ copies/mL for the 2 control cows at 0 h after calving. Due to the poor transition after calving, the largest population of *M. elsdenii* in 486 was detected at $1.5 \times 10^6$ copies/mL at 24 h, which was lower than for 492, whose greatest population of *M. elsdenii* reached $5.5 \times 10^6$ copies/mL at 4 h after calving. The population *M. elsdenii* in 492 remained for about 48 h and then on 3 d postpartum decreased to the level similar to the population precalving. For the cows dosed with *M. elsdenii* at 1 to 2 d postpartum, the population of *M. elsdenii* in 491 before dosing was $1.4 \times 10^6$ copies/mL, 2 h after dosing was $8.5 \times 10^6$ copies/mL, increased to $1.6 \times 10^7$ copies/mL, and then decreased at 3 d postpartum to a level less than prepartum. The population then increased to $1.6 \times 10^7$ copies/mL on 7 d after dosing. Cow 472 had a greater population of *M. elsdenii* of $9.8 \times 10^6$ copies/mL prepartum than the other 3 cows. The population increased to its largest amount at $1.0 \times 10^7$ copies/mL 2 h after dosing, and then decreased on 5 d after dosing.
DISCUSSION

DMI, BCS and Body Weight

The effect of dosing *M. elsdenii* on DMI has been inconsistent. When intra-ruminally dosing 2 to 3 x $10^{12}$ organisms of *M. elsdenii* (407A) in steers, Robinson et al. (1992) demonstrated that feed intake as a percentage of BW increased by 24% compared with the control group. Corresponding with that, Hibbard et al. (1993) reported that oral drenching with *M. elsdenii* increased feed intake in beef steers switched from a 50 to 90% concentrate diet. Also, Henning et al. (2010a) reported a significant and sustained 21% higher feed intake for *M. elsdenii* CH4 drenched steers than the control steers. However, *M. elsdenii* CH4 administration on the day of calving and again at 10 and 20 d postpartum showed no effect on DMI between day of calving and 80 d postpartum in a study on 60 multiparous Holstein cows (Henning et al., 2011). In the current study with 30 primi- and multiparous Jersey cows, *M. elsdenii* didn’t affect the DMI during the first 30 DIM. In addition, Zebeli et al. (2012) reported daily intra-ruminal administration of *M. elsdenii* ATCC 25940 (3.5 x $10^{9}$ cfu/dose) for 21 d had no effect on DMI in a study using 8 primiparous Holstein cows in a paired 2×2 crossover design.

Body condition score is a valuable tool to assess the energy status of dairy cows. The ideal BCS at calving is 3.5, but milk production differed little between a BCS of 3.0 and 3.5 (Roche et al., 2009). Ideally, cows should not lose more than 1 unit of BCS value during the first 100 DIM (Gallo et al., 1996). The cows in our study had a BCS of 3.10 at 14 d prepartum and only lost 0.2 unit by 60 DIM compared. The BCS at 60 and 90 DIM showed no difference from that of 30 DIM. Thus, the cows in our study met the
recommendation for sustaining milk production and minimizing health problems. Hagg et al. (2010) also did not find a difference in BCS when dosing high-producing multiparous Holstein cows with *M. elsdenii*. Consistently, no difference was observed in BCS when Stevens (2013) studied 162 primi- and multiparous Holstein cows with *M. elsdenii* dosed at ~14 d prepartum, 1 to 3 d postpartum, or ~14 d prepartum and 1 to 3 d postpartum. However, Henning et al. (2011) reported a trend of increased BCS by *M. elsdenii* in a study of 60 multiparous Holstein cows.

The effect of *M. elsdenii* on BW was inconsistent with other studies. Cook et al. (1977) dosed intra-ruminally cultures of *M. elsdenii*, the amount of which was not reported, to crossbred heifers that were not adapted to a high-concentrate diet. Cattle were switched from an all-hay diet to an 85% concentrate diet and dosed with the culture. Heifers dosed with *M. elsdenii* gained less BW than did control animals over a 21-d period. In contrast, Aikman et al. (2009) observed no effect of *M. elsdenii* on BW of 14 multiparous Holstein cows, which was consistent with our study. In contrast, the cows dosed with *M. elsdenii* had higher BW than control cows in Henning et al. (2011). They found that the differences in BW and BCS were greater in high-producing cows on a higher energy diet (70% concentrate) compared with a lower energy diet (60% concentrate). Although the BW differed by *M. elsdenii*, Stevens (2013) suggested the difference was due to the lack of covariate adjustment for BW in the statistical analysis and the measurement error associated with heart girth tape. Aikman et al. (2009) reported that cows dosed with *M. elsdenii* tended to either lose less or gain more BW than control cows, whereas the BW change reported in our study showed no treatment effect.
Milk Production and Composition

The intra-ruminally administered *M. elsdenii* exerted little effect on the milk production or composition in the current study. The results in other studies (Aikman et al., 2009; Hagg et al., 2010; Aikman et al., 2011; Zebeli et al., 2012; Stevens, 2013) also demonstrated no effect of *M. elsdenii* on milk yield when the cows were dosed immediately after calving. However, Aikman et al. (2009) observed an increase in milk production during the first 14 wk postpartum when the milk production of the cows consuming the high concentrate diet was compared with the low concentrate diet. Henning et al. (2011) reported that milk yield tended (*P* = 0.06) to increase by *M. elsdenii* CH4 when 40 high-producing cows on a 70% concentrate diet were orally dosed with $10^{11}$ cfu on the day of calving and again at 10 and 20 DIM in a trial lasting until 80 DIM. In our study, there was a treatment by parity interaction for milk yield in which mature cows (≥ 3 lactations) dosed at 1 to 2 d postpartum produced more milk than the control cows while the DMI did not differ (data not shown). This indicated increased feed efficiency by dosing with *M. elsdenii* for mature cows (≥ 3 lactations). However, feed efficiency was not affected by dosing *M. elsdenii* CH4 on the day of calving and again at 10 and 20 d postpartum in a study on 60 multiparous Holstein cows (Hagg et al., 2010). Stevens (2013) found there was no difference of milk yield for mature cows (≥ 3 lactations) between the control cows and the cows dosed on 1 to 3 d postpartum. In addition, they observed a treatment by parity interaction for milk yield in which mature cows (≥ 3 lactations) dosed prepartum produced more milk and peaked higher than control cows and cows dosed with *M. elsdenii* both pre- and postpartum. The lack of
response in milk production may be due to the concentrate level of the diets. The concentrates levels in the diets of recent studies, such as 50% concentrate for both the fresh and lactating diets in the current study, 60% concentrate in Hagg et al. (2010) and Henning et al. (2011), and 59% concentrate in lactating diets used by Stevens (2013), were at lower levels than used for finishing steer diets. Because of the lack of response in milk production by *M. elsdenii* in those dairy studies, cows fed with lower concentrate diets versus beef steers apparently benefited less from dosing with *M. elsdenii*. The higher milk yield in control cows at 2nd lactation than the dosed cows was likely due the the small sample size, because there were only 6 cows at 2nd lactation in the current study, and the difference was probably due to type I error. Similar results were also shown for milk fat yield and MUN.

Dosing the cows with *M. elsdenii* did not affect the milk fat percentage, which was similar to the studies by Zebeli et al. (2012) and Stevens (2013). On the contrary, Aikman et al. (2009) found that milk fat and protein percentages were reduced by *M. elsdenii* in high-producing cows on both their standard and high-energy diets. Hagg et al. (2010) reported that milk fat percentage differed between dosed and control cows fed a high concentrate (70%) diet, although no overall effect of *M. elsdenii* on milk composition was observed between control and dosed cows. Aikman et al. (2011) observed a trend for milk fat concentration to be reduced in cows dosed with *M. elsdenii*. These variable responses in milk fat are probably associated with differences in dietary compositions and feed processing (Henning et al., 2011). The primary concentrate source in our study was ground corn, whereas wheat and barley in Aikman et al. (2009), corn
meal only in Hagg et al. (2010), and steam-flaked sorghum and corn meal in Henning et al. (2011) were used as primary concentrate sources. These grain sources and their processing are well established to differ in ruminal fermentation rate, and consequently, in ruminal passage rate (NRC, 2001). Milk fat is the component of milk that reflects the ruminal fermentation, especially when the animal is fed a high-grain diet (NRC, 2001). This is the reason why milk fat content is often used as an indicator of the adequacy of fiber degradation and the risk of SARA in dairy cows (Zebeli et al., 2008). The milk fat and protein percentages in our study were 4.7% and 3.6%, respectively, which were consistent with the national average of 4.6% and 3.6%, respectively, for Jersey cows. And this indicated no high risk for SARA. Also, milk fat and protein percentages interpreted in series provided the highest potential as an indicator for ketosis when the risk for ketosis is the highest, such as the first 2 wk postpartum (Eastridge, 2012). In our study, the milk fat and protein percentages within the first 2 weeks after calving were 4.8% and 4.0%, respectively, which were 0.2 and 0.4 units higher than the general averages due to the intense mobilization of adipose tissue during that period. Additionally, no fat:protein inversion occurred in our study, also indicating good overall health status of the cows on trial. The result of no treatment effects on the milk fat yield and milk fat percentage, there was no milk fat depression detected in the current study, suggesting this strain is likely not associated with the strains producing trans-10, cis-12 conjugated linoleic acid found by Kim et al. (2002). However, Maia et al. (2007) reported that the culture used by Kim et al. (2002) was likely contaminated. The milk protein was not
affected by *M. elsdenii* in our experiment, which was consistent with the observations in other studies in dairy cows.

The lack of treatment effect on MUN was consistent for the dairy cows dosed with *M. elsdenii* (Hagg et al., 2010; Zebeli et al., 2012). However, the average concentration of MUN for the cows in the current study was 17.4 mg/dL, which was higher than the MUN concentrations reported by Hagg et al. (2010) and Zebeli et al. (2012), which were 15.1 and 13.9 mg/dL, respectively. Higher levels of readily fermentable carbohydrates increased efficiency of rumen N utilization for microbial crude protein and decreased MUN (Broderick, 2003; Hagg et al., 2010). We used a 50% concentrate ration, and Hagg et al. (2010) and Zebeli et al. (2012) used 60 or 70%, and 45% concentrate rations, respectively. Additionally, Fatehi et al. (2012) suggested that MUN concentration also differed by parity, DIM, and season.

There was no difference in SCC for control and dosed cows, similar to the results in Stevens (2013). Although Zebeli et al. (2012) reported a tendency for greater SCC in the cows intra-ruminally dosed with *M. elsdenii* compared with the control cows; both groups of cows had SCC lower than 150,000 cells/mL, indicating healthy udders. On the whole, the administration of *M. elsdenii* postpartum had minimal effect on milk yield and composition in our study. However, the treatment by parity interaction suggested mature, higher producing cows may benefit more from the administration of *M. elsdenii* than those in their first lactation.
Urine and Blood Metabolites

Urine ketones and concentrations of serum NEFA and BHBA are good monitors for negative energy balance (NEB) to predict the health status and productivity of the cows in early lactation. The concentration of urine ketones in our study indicated that the incidence of subclinical ketosis was low across all treatments. Carrier et al. (2004) reported a cut-off of 15 mg/dL when using Ketostix strip for detecting urine ketones with relatively high sensitivities and specificities to detect subclinical ketosis. Based on this cut-off, 6.7% of the cows experienced subclinical ketosis. The cows with ≥ 15 mg/dL urine ketones were 26.7% for the control cows and 13.3% for the dosed cows. In our study, 3.3% of the postpartum cows had NEFA concentrations between 0.7 to 1 mEq/L (Nydam et al., 2013), indicating subclinical ketosis. The NEFA are released via lipolysis from adipose tissues and are one of the major sources to form ketones during the states of severe NEB. Thus, the low NEFA concentration in our study indicated that the cows did not suffer from severe NEB. Subclinical ketosis is highly correlated with blood BHBA concentration from 12 to 14 mg/dL for postpartum cows (Duffield et al., 2009). In our study, the average BHBA concentration was 18.4 mg/dL. Zebeli et al. (2012) found *M. elsdenii* ATCC 25940 had no effect on the preprandial concentrations of NEFA or BHBA in dairy cows. However, the postprandial concentrations of NEFA in the plasma were lower in *M. elsdenii*-dosed than those in the control cows. We speculate the different strains of *M. elsdenii* used in Zebeli et al. (2012) compared with ours and Stevens (2013) probably contributed to the different responses of postprandial NEFA after the dosing. Henning et al. (2010b) reported that variability existed among 9 strains of *M. elsdenii* in
mixed rumen cultures, and certain strains were capable of affecting the magnitude of
decrease in rumen pH, thus preventing lactate accumulation during an acidosis challenge.
Sampling hour also affected the postprandial NEFA, which decreased until 4 h after
morning feeding and increased afterwards until 10 h after morning feeding. The average
concentration of BHBA in the current study was higher than the other dairy cow studies
dosed with *M. elsdenii*. Besides NEFA as a source for BHBA, ruminally produced
butyrate is also converted to BHBA in the rumen epithelium and liver. Butyrate might
have increased in the rumen due to the conversion from lactate by *M. elsdenii* and
contributed to the high concentration of BHBA (Counotte et al., 1981; Tsukahara et al.,
2006). Other measurements, including the NEFA concentrations and milk fat and protein
percentages during the first 2 weeks of lactation, indicated that the cows in our study had
low prevalence of subclinical ketosis. Combining our results with those from studies with
steers, the effect of dosing of *M. elsdenii* on NEFA and BHBA was not fully understood,
but it might help improve the energy status during the early lactation.

**Detection of *M. elsdenii* in Rumen Contents**

There are several studies monitoring the population of *M. elsdenii* in beef steers
when dosing them with *M. elsdenii*. Real-time PCR analysis by Fernando et al. (2010)
detected fold increases in the *M. elsdenii* population during adaptation to the high-
concentrate diet in a study with 4 ruminally cannulated beef steers. Henning et al. (2010a)
found that the population of *M. elsdenii* was higher in steers on 2 and 3 d after intra-
ruminally inoculation with $1.7 \times 10^{11}$ cfu/dose *M. elsdenii* CH4 than that of the control
steers. Ouwerkerk et al. (2002) detected $7.4 \times 10^6$ cells/mL of *M. elsdenii* 50 h after the
inoculation, indicating the persistence of the *M. elsdenii* population in the rumen for a certain length of time. We are the first to measure the population of *M. elsdenii* in dairy cows after the inoculation of the organism. The 2 dosed cows appeared to show a higher population of *M. elsdenii* in the rumen contents than the control cows, and the apparent higher population may have persisted for about 2 d after dosing. However, the availability of dairy cows limited the sample size to draw from conclusions. Further studies on dosing *M. elsdenii* to dairy cows using a greater number of cannulated cows and analysis on the lactate concentration of the rumen contents would be needed to better understand the benefit of the organism.

**CONCLUSIONS**

No treatment effect of orally dosing *M. elsdenii* at 1 to 2 d postpartum was observed on milk yield, milk composition, or overall cow performance. Mature cows with $\geq 3$ lactations (higher-producing cows) may have higher milk yield when dosed with *M. elsdenii*. 
Table 5.1. Ingredients and chemical compositions of the close-up, fresh, and lactating cow TMR (% of DM).

<table>
<thead>
<tr>
<th>Item</th>
<th>Close-up</th>
<th>Fresh</th>
<th>Lactating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient composition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ryegrass balage</td>
<td>NA</td>
<td>11.7</td>
<td>NA</td>
</tr>
<tr>
<td>Grass mix&lt;sup&gt;1&lt;/sup&gt;</td>
<td>37.3</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Legume/ grass balage</td>
<td>NA</td>
<td>NA</td>
<td>6.8</td>
</tr>
<tr>
<td>Corn silage</td>
<td>22.5</td>
<td>39.2</td>
<td>43.8</td>
</tr>
<tr>
<td>Whole cotton seed</td>
<td>NA</td>
<td>13.3</td>
<td>14.3</td>
</tr>
<tr>
<td>Corn, dry ground</td>
<td>26.2</td>
<td>12.9</td>
<td>9.78</td>
</tr>
<tr>
<td>Dry distillers grain with solubles</td>
<td>NA</td>
<td>NA</td>
<td>7.94</td>
</tr>
<tr>
<td>Soybean meal, 48% CP</td>
<td>2.47</td>
<td>9.46</td>
<td>5.97</td>
</tr>
<tr>
<td>Amino Plus&lt;sup&gt;2&lt;/sup&gt;</td>
<td>NA</td>
<td>7.26</td>
<td>1.58</td>
</tr>
<tr>
<td>Soybean hulls</td>
<td>NA</td>
<td>1.48</td>
<td>6.46</td>
</tr>
<tr>
<td>Fat&lt;sup&gt;3&lt;/sup&gt;</td>
<td>NA</td>
<td>0.71</td>
<td>0.70</td>
</tr>
<tr>
<td>Trace mineral salt</td>
<td>NA</td>
<td>0.88</td>
<td>0.39</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>NA</td>
<td>NA</td>
<td>0.78</td>
</tr>
<tr>
<td>Limestone</td>
<td>2.24</td>
<td>2.02</td>
<td>1.02</td>
</tr>
<tr>
<td>Selenium, 90 mg/lb</td>
<td>0.11</td>
<td>0.23</td>
<td>0.11</td>
</tr>
<tr>
<td>Magnesium oxide</td>
<td>0.27</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>Copper sulfate</td>
<td>0.01</td>
<td>0.002</td>
<td>0.003</td>
</tr>
<tr>
<td>Zinc sulfate</td>
<td>0.02</td>
<td>0.002</td>
<td>0.009</td>
</tr>
<tr>
<td>Bio-chlor&lt;sup&gt;4&lt;/sup&gt;</td>
<td>7.83</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SelenoSource AF 600&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.03</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Vitamin mix&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.38</td>
<td>0.15</td>
<td>0.08</td>
</tr>
<tr>
<td>Biotin, 220 mg/kg</td>
<td>NA</td>
<td>0.53</td>
<td>NA</td>
</tr>
<tr>
<td>Chemical composition</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM, %</td>
<td>59.5</td>
<td>52.5</td>
<td>51.6</td>
</tr>
<tr>
<td>CP, % of DM</td>
<td>14.6</td>
<td>17.9</td>
<td>17.5</td>
</tr>
<tr>
<td>NDF, % of DM</td>
<td>36.0</td>
<td>38.2</td>
<td>31.0</td>
</tr>
<tr>
<td>Starch, % of DM</td>
<td>24.9</td>
<td>17.9</td>
<td>28.5</td>
</tr>
<tr>
<td>Fat, % of DM</td>
<td>3.94</td>
<td>5.75</td>
<td>5.34</td>
</tr>
<tr>
<td>Ash, % of DM</td>
<td>10.2</td>
<td>8.65</td>
<td>7.79</td>
</tr>
<tr>
<td>P, %</td>
<td>0.31</td>
<td>0.33</td>
<td>0.39</td>
</tr>
<tr>
<td>K, %</td>
<td>1.55</td>
<td>1.21</td>
<td>NA</td>
</tr>
<tr>
<td>Ca, %</td>
<td>1.24</td>
<td>1.02</td>
<td>0.96</td>
</tr>
<tr>
<td>Mg, %</td>
<td>0.35</td>
<td>0.26</td>
<td>0.26</td>
</tr>
</tbody>
</table>

<sup>1</sup>Composted from ryegrass balage in the early period of the experiment and a mix of sawmill grass and ryegrass balage in the late period of the experiment.

<sup>2</sup>AMINOPLUS<sup>®</sup>, Ag Processing Inc<sup>®</sup>, Omaha, NE

<sup>3</sup>True Energy Formula “A”, G.A. Wintzer and Son Co., Wapakoneta, OH

<sup>4</sup>BIO-CHLOR<sup>®</sup>, Church & Dwight Co., Inc., Princeton, NJ

<sup>5</sup>SelenoSource<sup>®</sup> AF 600, Diamond V Mills, Cedar Rapids, IA

<sup>6</sup>Vitamin mix included the following: Vitamin A, 30,000 IU/kg; Vitamin D, 3,000 IU/kg; and Vitamin E, 44,093 IU/kg

<sup>7</sup>NA = Not applicable
Table 5.2. Least square means of dry matter intake, body weight, body condition score, urine ketones, serum NEFA and BHBA, milk production, and milk composition in cows not dosed and dosed with *Megasphaera elsdenii*.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>P value</th>
<th>SEM</th>
<th>TRT</th>
<th>Time²</th>
<th>TRT x Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Dose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMI, kg/d, wk 1-4</td>
<td>14.1</td>
<td>14.4</td>
<td>0.57</td>
<td>0.79</td>
<td>&lt;0.01</td>
<td>0.65</td>
</tr>
<tr>
<td>BW, kg</td>
<td>424</td>
<td>414</td>
<td>10.1</td>
<td>0.50</td>
<td>0.59</td>
<td>0.25</td>
</tr>
<tr>
<td>BW change, kg/wk</td>
<td>-4.86</td>
<td>-4.14</td>
<td>1.12</td>
<td>0.67</td>
<td>&lt;0.01</td>
<td>0.61</td>
</tr>
<tr>
<td>BCS</td>
<td>2.94</td>
<td>2.93</td>
<td>0.03</td>
<td>0.47</td>
<td>0.09</td>
<td>0.78</td>
</tr>
<tr>
<td>Milk yield, kg/d, wk 1-4</td>
<td>26.6</td>
<td>25.4</td>
<td>0.80</td>
<td>0.33</td>
<td>&lt;0.01</td>
<td>0.34</td>
</tr>
<tr>
<td>Milk yield, kg/d, wk 5-13</td>
<td>30.9</td>
<td>30.5</td>
<td>0.95</td>
<td>0.76</td>
<td>0.08</td>
<td>0.26</td>
</tr>
<tr>
<td>Milk yield, kg/d, wk 1-13</td>
<td>29.4</td>
<td>28.7</td>
<td>0.88</td>
<td>0.60</td>
<td>&lt;0.01</td>
<td>0.40</td>
</tr>
<tr>
<td>Fat, %, wk 1-2</td>
<td>4.90</td>
<td>4.77</td>
<td>0.24</td>
<td>0.70</td>
<td>0.82</td>
<td>0.74</td>
</tr>
<tr>
<td>Fat, %, wk 1-4</td>
<td>4.69</td>
<td>4.67</td>
<td>0.20</td>
<td>0.95</td>
<td>0.38</td>
<td>0.94</td>
</tr>
<tr>
<td>Protein, %, wk 1-2</td>
<td>3.91</td>
<td>4.00</td>
<td>0.16</td>
<td>0.70</td>
<td>&lt;0.01</td>
<td>0.62</td>
</tr>
<tr>
<td>Protein, %, wk 1-4</td>
<td>3.55</td>
<td>3.54</td>
<td>0.12</td>
<td>0.98</td>
<td>&lt;0.01</td>
<td>0.55</td>
</tr>
<tr>
<td>Milk fat yield, g/d, wk 1-4</td>
<td>1261</td>
<td>1204</td>
<td>58.4</td>
<td>0.48</td>
<td>&lt;0.01</td>
<td>0.68</td>
</tr>
<tr>
<td>Milk protein yield, g/d, wk 1-4</td>
<td>928</td>
<td>876</td>
<td>46.6</td>
<td>0.47</td>
<td>0.03</td>
<td>0.51</td>
</tr>
<tr>
<td>MUN, mg/dL</td>
<td>16.8</td>
<td>17.9</td>
<td>0.83</td>
<td>0.26</td>
<td>&lt;0.01</td>
<td>0.10</td>
</tr>
<tr>
<td>SCC1, 10^3 cells/mL</td>
<td>88.9</td>
<td>94.6</td>
<td>53.9</td>
<td>0.94</td>
<td>0.45</td>
<td>0.55</td>
</tr>
<tr>
<td>Urine ketone, mg/dL</td>
<td>13.1</td>
<td>10.3</td>
<td>5.64</td>
<td>0.63</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>NEFA1, mEq/L</td>
<td>0.51</td>
<td>0.58</td>
<td>0.17</td>
<td>0.79</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>BHBA1, mg/dL</td>
<td>18.5</td>
<td>18.6</td>
<td>1.34</td>
<td>0.95</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

1DMI= dry matter intake, BW= body weight, BCS= body condition score, MUN= milk urea nitrogen, SCC= somatic cell count, NEFA= nonesterified fatty acids, BHBA= β-hydroxybutyrate, and NA = not applicable
2Time relates to the 30, 60, and 90 DIM for BCS and BW, 1, 2, 3, 4, 9, and 13 wk for BW change; week for DMI, milk yield, milk fat, milk protein, and SCC.
Figure 5.1. Dry matter intake from week 1 to 4 of lactation for control cows and cows dosed with *Megasphaera elsdenii*. Letters indicate difference by week of lactation within the treatment (P < 0.01; SEM = 0.48).
Figure 5.2. Body weight change by week of lactation. Letters indicate difference by week of lactation within the treatment (P < 0.01; SEM = 2.22).
Figure 5.3. Body condition score from 2 weeks prepartum to 90 DIM for control cows and cows dosed with *Megasphaera elsdenii*. Letters indicate difference by week of lactation within the treatment (P < 0.01; SEM = 0.03).
Figure 5.4. Milk production from week 1 to 13 of lactation for control cows and cows dosed with *Megasphaera elsdenii*. Letters indicate difference by week of lactation within the treatment (P < 0.01; SEM = 0.87).
Figure 5.5. Treatment by parity interaction (P < 0.01) for milk yield from week 1 to 13 of lactation for control cows and cows dosed with *Megasphaera elsdenii* (*Treatments differed at P < 0.05; **Treatments differed at P ≤ 0.01; SEM = 1.6*).
Figure 5.6. Treatment by parity interaction ($P = 0.06$) for milk fat yield from week 1 to 4 for control cows and cows dosed with *Megasphaera elsdenii* ($^\dagger$Treatments tended to differ at $0.09 \leq P \leq 0.11$; SEM = 71).
Figure 5.7. Treatment by parity interaction (P < 0.05) for milk urine nitrogen (MUN) from week 1 to 4 for control and cows dosed with *Megasphaera elsdenii* (**Treatments differed at P ≤ 0.01; SEM = 1.0).
Figure 5.8. Populations of *Megasphaera elsdenii* in cannulated control cows and cows dosed with *Megasphaera elsdenii*. 
CHAPTER 6

CONCLUSIONS

The one-stage *in vitro* incubation allows us to assess the BH of total FA and fractional rates of disappearance of certain FA in a closed system, avoiding other factors, such as passage rate of diverse feeds from the rumen and ruminal pH variations, which might confound the results. The replicates of incubation with one week of interval account for the variations in rumen conditions of the two cannulated cows. As expected, fat source affected BH. Overall, DDGS had the highest BH among the treatments. Roasted soybean, corn oil, and soybean oil had similar BH, which were lower compared with DDGS. Roasting and particle size did not affect overall BH; however, the roasting process and particle size affected the rate of disappearance of 18:2 in soybeans. Particle size exerted minimum effect on BH, but the particle sizes differed at most by 1 mm in this study.

The dual flow continuous culture is a well-controlled two-stage *in vitro* system with inflow adding artificial saliva and outflow removing the wastes of fermentation. All fermenters were inoculated from the same starting source and at the same time, the animal and time effects on microbial populations were minimized. The development of the by-difference procedure involving boiling and sonication of effluent samples helped to determine the protozoal N per cell when reducing the feed contamination due to the
similar particle sizes of protozoa and feed. There were no effects of Rumensin or Cinnagar on protozoal generic distribution, concentration of NH$_3$-N, total N flow of effluent, production of total VFA, and flows of CLA and total C18. Rumensin decreased acetate:propionate ratio and BH of C18 and cis-9 18:1. Rumensin increased protozoal generation time, concentration of peptide, and flow of trans-11 18:1. Rumensin tended to decrease protozoal counts in effluent flow and flow of 18:0, and it tended to increase propionate production. Cinnagar decreased true OM digestibility and protozoal N flow of effluent and increased non-ammonia non-microbial N flow of effluent. Cinnagar tended to decrease protozoal counts, microbial N flow of effluent, NDF digestibility, and protozoal N per cell. Cinnagar tended to increase BH of total C18, 18:2, and 18:3. Cinnagar tended to increase isovalerate production. Cinnagar and Rumensin tended to interact for increased methane production and bacterial N flow. Under the conditions of our study, we did not detect an additive response for Rumensin and Cinnagar to decrease protozoal counts or methane production. Further research is needed on cell size change of ruminal protozoa upon the Cinnagar addition and how to inhibit protozoa enough to decrease their negative effects but not so much to disrupt normal microbial ecology of the rumen.

The dairy cow trial provided information on cow performance when dosed with $M. elsdenii$, which in vitro experiments can not achieve. Our study was the first to take advantage of molecular techniques to examine the establishment and persistence of $M. elsdenii$ population in the rumen of dairy cows. No treatment effect of orally dosing $M. elsdenii$ at 1 to 2 d postpartum was observed on milk yield and composition, or overall
cow performance. Based on our study and that of Stevens (2013), mature cows with \( \geq 3 \) lactations (higher-producing cows) may have higher milk yield when dosed with \textit{M. elsdenii}. Further studies on dosing \textit{M. elsdenii} to dairy cows using a greater number of cannulated cows and analysis of the lactate concentration of the rumen contents would be needed to better understand the benefit of the organism. Although the research available for dairy cows dosed with \textit{M. elsdenii} is limited and inconsistent up to now, additional research is needed to determine if transition cows may respond to administration of \textit{M. elsdenii} under more diverse dietary conditions that used in recent studies.
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120


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Appendix A: Protocol of Repeated Bead Beating Plus Column (RBB+C) Method

Prepare:

1. Weigh 0.4g of sterile zirconia beads (0.3g of 0.1 mm and 0.1g of 0.5mm) into 2-mL screw-cap tubes and autoclave them at least one day before use and dry them in 55°C oven.

2. a. If the raw sample is rumen content with liquid, blend it for 1 min and strain through 8-layer cheesecloth. Centrifuge at 4°C at 14000 rpm and aspirate the supernatant. The pellet will be the sample to start with for the DNA extraction. B. If the raw sample is a pure culture, transfer 1mL into a 1.5 mL tube and centrifuge for 1 min at 8000 rpm, discard the supernatant, add 200 uL of Tris-EDTA (TE) buffer into the tube, pipette to suspend the pellet, and transfer into a sterilized 2-mL screw-cap tube with beads.

3. Right before start of extraction, heat the water bath at 70°C. Make fresh lysis buffer. Dissolve 4% sodium dodecyl sulfate (SDS) into premade buffer (500 mM NaCl, 50 mM Tris- HCl, pH 8.0, 50 mM EDTA). For example: If 2 samples will be extracted, make lysis buffer for 3 samples (1 extra). Each sample will need 1.3 mL of lysis buffer (see step 1 and 5 below). The total volume will be 3*1.3= 3.9 mL. Weigh 3.9* 4%= 0.16 g of sodium dodecyl sulfate (SDS) into a tube with rest of premade buffer. Leave the tube in 70°C water bath to dissolve when necessary.

I. Cell Lysis

1. Transfer 0.5 g of sample into a sterilized 2-mL screw-cap tube with beads. Add 1 mL of lysis buffer.

2. Homogenize for 3 min at maximum speed on a Mini-Beadbeater™ (BioSpec Products, Bartlesville, OK, USA), with a maximum of 8 tubes at one time.
3. Incubate at 70°C for 15 min, with gentle shaking by hand every 5 min. Do not shake too hard because DNA at this point is unprotected.

4. Centrifuge at 4°C for 5 min at 13,200 rpm. Transfer the supernatant which includes the nucleic acid to a fresh 2-mL Eppendorf® tube. Cell wall and other part are in the pellet.

5. Add 300 uL of fresh lysis buffer to the lysis tube with settled pellet and repeat steps 2 through 4, and then pool the supernatant.

II. Precipitation of nucleic acids:

6. Add 260 uL of 10 M ammonium acetate (stored in the walk-in cold room) to each lysate tube, invert to mix well, and incubate on ice for 5 min. Ammonium acetate is to remove SDS.

7. Centrifuge at 4°C for 10 min at 13,200 rpm.

8. Transfer the supernatant to two 1.5-mL Eppendorf® tubes, and one volume (0.6 to 0.65 mL) of isopropanol (stored in the walk-in cold room) and invert to mix well, and incubate on ice to settle DNA down for ≥ 30 min, or overnight at -80°C when necessary.

9. Centrifuge at 4°C for 15 min at 13,200 rpm, remove the supernatant using aspiration, wash the nucleic acids pellet with 1 mL of 70% ethanol, invert to mix well and centrifuge for 1 min at 13,200 rpm. Remove the supernatant with aspiration, use Kimwipes to help remove supernatant when necessary, and do not touch the pellet. Dry the pellet under vacuum for 7 to 10 min.

10. Dissolve the nucleic acid pellet in 100 uL of TE buffer and pool the two aliquots. Store at 4°C for 10 min, or overnight when necessary. Centrifuge for 1 min at 13,200 rpm before step 11.

III. Removal of RNA, protein, and purification:

If the QIAamp DNA stool Mini Kit is new, make sure ethanol is added to Buffer AW1 and AW2. Instruction is on the bottle of the buffer.
11. Add 2 uL of DNase-free RNase (10 mg/mL, in -20°C) and incubate at 37°C for 15 min. Centrifuge for 1 min at 13,200 rpm.
12. Add 15 uL of proteinase K and 200 uL of Buffer AL (from the QIAamp DNA stool Mini Kit), invert to mix well, and incubate at 70°C for 10 min. Centrifuge for 1 min at 13,200 rpm.
13. Add 200 uL of ethanol (-20°C) and invert shortly to mix well. Transfer to a QIAamp column and centrifuge at 13,200 rpm for 1 min. Discard the flow through.
14. Add 500 uL of Buffer AW1 (Qiagen), and centrifuge for 1 min at 13,200 rpm. Discard the flow through.
15. Add 500 uL of Buffer AW2 (Qiagen), and centrifuge for 1 min at 13,200 rpm. Discard the flow through. Change to a new collection tube when necessary.
16. Dry the column by centrifugation for 1 min at 13,200 rpm.
17. Add 100 uL of Buffer AE (Qiagen) and incubate at room temperature for 10 min. Change the collection tube to a new 1.5-mL Eppendorf® tube.
18. Centrifuge at room temperature for 1 min at 13,200 rpm to elute the DNA. Aliquot the DNA solution into two tubes: One as backup stored at -80°C and the other one stored at -20°C for PCR, gel, etc. All the trash goes to the biohazard waste container.
19. NanoDrop for DNA concentration in the teaching lab of Plumb Hall, or run 2 uL on a 0.8% gel to check the DNA quality.
Appendix B: Protocol of Real-Time PCR

1. Turn on quantitative PCR (qPCR) machine (Stratagene Mx3000P). Then open the Mx 3000P software in the computer. Click the “Sybr green with dissociation curve”, “warm up the bubble”, then OK.

2. Put the fresh Tris-EDTA (TE) buffer bottle, di H2O bottle, PCR strip rack and 1.5 mL tube rack into the hood. Turn on the UV light in the hood for at least 20 min before making master mix. Switch is on top right of the hood. Always use supplies in the hood. Always use filtered pipette tips for PCR. Take the ingredient tubes for master mix and DNA samples out of the stocking box from -20°C freezer. Thaw them on a rack in room temperature. Take MgCl2 and 10x PCR buffer out of fridge. Put Taq DNA polymerase (Taq enzyme), MgCl2 and 10x PCR buffer on ice. The Taq enzyme is recommended to keep on ice as much as possible except when adding the Taq enzyme to the PCR tube.

3. Prepare template
The template is the DNA sample. Make sure it is 100 ng/uL. If not, use the data from NanoDrop achieved after DNA extraction to dilute to 100 ng/uL.

4. Prepare standard
Use a tweezer to pick up two strips of PCR tubes for standard dilution and put on the rack. Label the tubes when necessary. Add 9 uL of di H2O to 10 tubes. Add 1 uL of PCR standard to the first tube, and pipette gently up and down to mix well. That is the 10⁻¹ dilution. Change the pipette tip, take 1 uL out of the 10⁻¹ tube and add to the next tube and repeat for 9 times to create a dilution of 10⁻¹ to 10⁻¹⁰. The solution from 10⁻³ to 10⁻¹⁰ of the dilution will be needed as the qPCR standard.

5. Prepare master mix
Make 25 uL per reaction. Triplicates are needed in the qPCR. Add 2 extra reaction mixes for the master mix calculation. Add SYBR® green dye, ROX reference dye and Taq enzyme at last. After Taq enzyme is added to the master mix tube, finger flick the tube for several times and then briefly spin on the PMC-060 Capsulefuge on the right of the hood because the glycerol in the Taq enzyme is hard to mix by inverting. Load 24 uL of the master mix into the negative well and add 1 uL of di H2O. Load 24 uL of the master mix and 1 uL of 8 standard dilutions to each standard well. Load 24 uL of the master mix and 1 uL of template to each sample well. Repeat for three times.

6. Put lids on the wells and use the blue plate in the drawer on the right of the hood to press the lids down flat. Finger flick the bottom of well plate to mix. Then load the plate into the Jouan BR4i centrifuge on the right of the hood. Turn on the machine. The switch is on the lower left. Make sure the mode is 3. Press the “start” button. After it’s done, take the PCR plate out gently and check the lids if they are flat and dry. The light goes from the top of the lids when qPCR is undergoing. Make sure the lids are flat, and scratch- and liquid- free.

7. Load the PCR plate onto the PCR machine. Use Kimwipes to wipe the top of lids to make sure it’s clean. Set up the condition and plate lay out in the Mx 3000P software, check “turn off the lamp when it’s done”, and click “run”. All the trash goes to the biohazard waste container.