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THE EFFECTS OF A BLEND OF ESSENTIAL OILS ON RUMEN EFFICIENCY OF LACTATING DAIRY COWS

BY

KALI LINVILLE

A thesis submitted in partial fulfillment of the requirements for the

Masters of Science

Major Biological Sciences

Specialization in Dairy Science

South Dakota State University

2017

THE EFFECTS OF A BLEND OF ESSENTIAL OILS ON RUMEN EFFICIENCY OF LACTATING DAIRY COWS

KALI LINVILLE

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Biological Sciences with a specialization in Dairy Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance for this does not imply that the conclusion reached by the candidate are necessarily the conclusion of the major department.

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This thesis is dedicated to Louis, Flora and Denise Leverett.

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LIST OF ABBREVIATIONS

AA	Amino acids
AIA	Acid insoluble ash
ADICP	Acid detergent insoluble crude protein
ADF	Acid detergent fiber
A: P	Acetate to propionate ratio
BCS	Body condition score
BCFA	Branch chain fatty acids
BW	Body weight
Ca	Calcium
Cl	Chloride
CP	Crude protein
Cu	Copper
d	Days
DM	Dry matter
DHIA	Dairy herd improvement association
DIM	Days in milk
DMI	Dry matter intake
EO	Essential oils
FCM	Fat corrected milk
FE	Feed efficiency
Fe	Iron
g	Grams
g	Gravitational acceleration

hd	Head
HAP	Hyper ammonia producing
HMC	High moisture corn
H_2SO_4	Sulfuric acid
IVDMD	In vitro dry matter digestibility
kg	Kilogram
K	Potassium
L	Liter
ml	Milliliter
Mg	Magnesium
µm	Micro liter
mM	Millimolar
Mn	Manganese
MUN	Milk urea nitrogen
N	Nitrogen
Na	Sodium
NH_3-N	Ammonia nitrogen
NEFA	Non-esterified fatty acids
NDICP	Neutral detergent insoluble crude protein
NDF	Neutral detergent fiber
NH_3-N	Ammonia
P	Phosphorus
ppm	Parts per million

PUN	Plasma urea nitrogen
RCBD	Randomized complete block design
RF	Rumen fluid
S	Sulfur
SCC	Somatic cell count
SS	Stay Strong
TMR	Total mixed ration
TRT	Treatment
VFA	Volatile fatty acids
wk	Week
Zn	Zinc

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ABSTRACT

THE EFFECTS OF A BLEND OF ESSENTIAL OILS ON RUMEN EFFICIENCY OF LACTATING DAIRY COWS

KALI DANIELLE LINVILLE

2017

The objective of this study was to evaluate the effects of SS a blend of commercially available essential oils (EO) on rumen microbial efficiency and kinetics, and consequently, effects on production parameters of dairy cows fed a low-starch diet and *in vitro* analysis of varying doses of SS on ruminal fermentation at different stages of lactation. The study consisted of two experiments, the *in vivo* Experiment 1, was conducted on a commercial robotic dairy in southwest Minnesota outfitted with two Lely Astronaut A4robotic milking units (Goter's Clay & Dairy Equipment of MN, INC., Pipestone, MN). One hundred-seven Holstein cows were randomly assigned to either the control diet (CON) (25% starch) or the low-starch (LS) diet (22% starch) in a randomized complete block design (RCBD), where cows were blocked by body weight (BW) and body condition score (BCS). All cows were fed 28g/hd/d of SS throughout the trial. The experiment included 14 d of dietary adaptation and 28 d of data collection. A tendency for higher pellet intake was observed for the LS treatment. Milk production, fat, and protein yield were similar among treatments. Total volatile fatty acid concentrations were similar between treatments, with a tendency observed for an increase in acetate percentage for the LS treatment. BUN and MUN concentrations were similar between treatments, ammonia concentrations were significantly lower for LS (5.84 vs. 9.69mM),

and pH values tended to be higher for LS treatment. In Experiment 2, an *in vitro* analysis was carried out as a 2 × 3 factorial arrangement of treatments with two doses (i.e., single and double) of SS and rumen fluid (RF) from Holstein cows at three stages of lactation (i.e., early, mid, and late). The latter was done with the aim to evaluate the effects of EO at different stages of lactation on ruminal fermentation characteristics and kinetics. Each treatment was replicated in 3 gas fermentation bottles plus a control treatment with RF only, and 3 batches containing all treatments were repeated 3 times on different days. RF was collected from 9 lactating multiparous cows (n = 3/group) at early [days in milk (DIM) 32-36], mid (DIM 144-157), and late (DIM 277-290) stages of lactation via esophageal tube at approximately 4 h after feeding. Gas production was significantly lower with the addition of the double dose of SS, but no treatment differences were observed. Total VFA concentrations were similar among treatments. Propionate percentage was significantly greater for the early lactation and single dose of SS treatment. Acetate: propionate ratio was significantly greater for the late lactation, and both single and double dose of SS. Ammonia concentrations were significantly lower in the early lactation and single dose of SS treatment, and early, mid and late lactation and double dose with mid lactation having the greatest concentration and early lactation having the lowest concentration. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were not affected by experimental treatment effects.

CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

In recent years, consumer interest in management practices used to produce food has drastically increased, leading to concerns over the development of bacterial resistance to antibiotics and potentially harmful consequences to human health (Benchaar et al., 2008). Due to consumer pressures, changes in legislation on the use of in-feed antibiotics and growth promoters in the animal feed industry has made it extremely challenging to produce the amount of food needed to meet the requirements of an ever-growing world population and maintain a reasonable cost (Lekshmi et al., 2017). Restrictions on the use of in-feed antibiotics can considerably decrease feed efficiency and negatively impact animal health, leading to an increase in food prices. Essential oils (EO) have gained popularity because of the current trend to replace in-feed antibiotics and growth promoters for natural compounds (Benchaar et al., 2006). EO are considered safe for animal consumption and animal products such as meat and milk derived from animals fed EO are categorized and recognized as safe for human consumption in the USA (Benchaar et al., 2008). EO have a wide range of applications including antimicrobial properties, reduction in methane emission by ruminants as well as improvements to growth, milk production, and feed efficiency making them a useful replacement to in-feed antibiotics and growth promoters. (Hallier et al., 2013).

With the rising demand for food from an ever-growing world population and the negative connotations associated with the use of antibiotics and growth promoters, efforts are being made to maximize animal productivity while maintaining or lowering the input

i.e., improved feed efficiency. The primary focus for improving feed efficiency in dairy cows involves manipulation of rumen fermentation which has several aims such as minimizing energy losses through methane production and improving fiber digestibility. (Khiaosa-ard and Zebeli, 2014). Monensin is an ionophore, and an effective antibiotic commonly utilized in a prophylactic manner in ruminant nutrition, which has been successfully used to improve feed efficiency since 1977 (Petersson-Wolfe et al., 2007), but due to current efforts to minimize the use of antibiotics in the animal feed industry an alternative tool will need to be implemented in the near future. First reports of the potential benefits of EO on ruminal fermentation were provided by Borchers (1965), who observed a reduction in NH_3- N and an accumulation of AA when thymol was added at 1mg/mL *in vitro.* Since then the well documented antimicrobial activities of EO has attracted the attention of the research community, primarily ruminant physiologists with the aim to understand the potential use of EO as a tool to improve feed efficiency (FE) and nutrient utilization by ruminants (Calsamiglia et al., 2007, Benchaar et al., 2008). Along with the efforts to improve feed efficiency, dairy producers are also trying to lower input cost, such as feed costs (Chase, 2007). From a nutritional standpoint, energy as a nutrient is one of the most expensive nutrients in a dairy cow's ration. The implementation of feeding strategies that minimize the inclusion of high-energy ingredients such as, grains, may improve farm profitability, particularly if cow performance is not compromised.

Essential oils

Contrary to their name EO are not true oils, EO are blends of secondary metabolites obtained from the volatile fraction by steam distillation (Benchaar et al., 2008). The term essential is derived from “essence”, which means smell or taste, and relates to the property of these organic compounds providing specific flavors and odors to many plants. The characteristics they possess are very diverse in composition, nature, and biochemical activities (Benchaar et al., 2007b). Antimicrobial properties of EO has been attributed to several active compounds that have been divided into two main chemical groups the terpenoids and phenylpropanoids, based on their different precursors mainly from the primary metabolism and synthesis through separate metabolic pathways (Chao et al., 2000, Calsamiglia et al., 2007, Benchaar et al., 2008). Terpenoids are the most numerous and diversified EO within the plant secondary metabolites; these compounds are derived from a basic 5 carbon structure (C_5H_8), commonly called an isoprene unit, and are classified depending on the number of these units in their skeleton (Calsamiglia et al., 2007). Phenylpropanoids are the second group of EO, these compounds are not the most common in EO, but plants have them in significant proportions. These compounds are characterized as having a 3-carbon chain bound to an aromatic ring of 6 carbons, they are mainly derived from phenylalanine by the shikimate metabolic pathway, which is only present in plants and microorganisms (Calsamiglia et al., 2007).

Mode of action. The antibacterial effects of terpenoids and phenylpropanoids are associated with their interaction with the cell membrane of these microorganisms (Griffin et al., 1999, Dorman and Deans, 2000, Calsamiglia et al., 2007), where part of this activity is due to the hydrophobic nature of the cyclic hydrocarbons, which allow the interaction with cell membrane and accumulation in the lipid bilayer of bacteria,

occupying space between the chains of fatty acids (Sikkemat et al., 1994, Ultree et al., 1999, Calsamiglia et al., 2007). This interaction causes conformational changes in the cellular membrane resulting in its destabilization (Griffin et al., 1999). The loss of membrane stability leads to the leakage of ions across the cell membrane, causing a decrease in the transmembrane ionic gradient. In most cases, bacteria counterbalance these effects by using ionic pumps, avoiding cell death, but large amounts of energy are diverted to this function, resulting in slowed bacterial growth (Griffin et al., 1999, Ultree et al., 1999, Cox et al., 2001). In regards to the rumen, changes in ruminal bacteria growth rates result in the changes of the fermentation profile (Calsamiglia et al., 2007). In most cases, the antimicrobial activity is highest in oxygenated cyclic hydrocarbons, and particularly in phenolic structures, in which the hydroxyl group and the dislocated electrons allow for the interaction with water though hydrogen bridges at the main active site, making them active against microorganisms (Griffin et al., 1999, Dorman and Deans, 2000, Cox et al., 2001). Another mechanism has been proposed in which the hydroxyl group of phenols acts as a transmembrane carrier of monovalent cations and protons, which is a similar mode of action to the ionophore antibiotics. Previous work testing this hypothesis confirmed that hydroxyl groups of aromatic compounds are the only ones with such effect, in fact, Ultee et al. (2002) observed that other non-aromatic compounds such as menthol do not result in the same inhibitory effects. These mechanisms of action should be more effective when the cell membrane can interact directly with hydrophobic compounds of EO, such as in the case of gram-positive bacteria (Smith-Palmer et al., 1998, Chao et al., 2000, Cimanga et al., 2002, Calsamiglia et al., 2007). The hydrophilic nature of the gram-negative bacteria cell wall inhibits

lipophilic substances to enter and penetrate the cell membrane (Cox et al., 2001, Cimanga et al., 2002, Calsamiglia et al., 2007). However, the external membrane of gram-negative bacteria is not completely impermeable to hydrophobic compounds. Low molecular weight lipophilic compounds (i.e., carvacrol and thymol) and water can form hydrogen bridges and cross the cell wall by diffusion through the layer of lipopolysaccharides or membrane proteins and interact with the lipid bilayer of cells (Helander et al., 1998, Griffin et al., 1999, Dorman and Deans, 2000, Calsamiglia et al., 2007).

Although the proposed mechanisms of EO are centered around their activity on the cellular membrane, it is likely that their antimicrobial activity is not due to one specific mode of action but involves several, since EO are comprised of a large number of components (Calsamiglia et al., 2007, Benchaar et al., 2008). Other mechanisms of action for EO antimicrobial activity have been proposed including their ability to coagulate some cell constitutes, most likely by denaturation of proteins (Gustafson and Bowen, 1997), the capacity of some phenolic and nonphenolic compounds of EO to interact with chemical groups of biologically active molecules such as proteins and enzymes. Phenols interact through hydrogen bridges, and ionic or hydrophobic interactions and non-phenolic groups interact through another functional group such as the carbonyl group of cinnamaldehyde (Ouattara et al., 1997).

Ruminal fermentation.

The beauty of the ruminant lies in their ability to utilize energy sources unavailable to humans such as cellulose to produce high-quality food products such as milk and meat. This ability is due to the symbiotic relationship the ruminant shares with

the large microbial population within the rumen. This microbial population can digest and ferment plant cell-wall polymers (cellulose, hemicellulose, and pectin) otherwise indigestible, the end product of this process is the formation of volatile fatty acids (VFA), the major source of energy for ruminants (Greathead, 2007). Ruminal fermentation of carbohydrates and amino acids (AA) is accompanied by losses of energy and amino N, respectively. From the digestible energy ingested by dairy cows, 8-12% is lost in the rumen as methane production and 75-80% of N ingested is excreted in feces and urine (Tamminga, 1992, Busquet et al., 2006). Thus, the efficiency of rumen fermentation and kinetics can have a substantial effect on the production efficiency of the animal (Greathead, 2007). The effects of EO on ruminal fermentation up to date have been inconsistent, most likely due to the dose-dependent bactericidal and bacteriostatic effects on microorganisms (Smith-Palmer et al., 1998). Many commercial products available are a blend of one or more essential oil, and the potential synergy among them is unknown and may be contributing to the inconsistent results observed to date. (Calsamiglia et al., 2007).

Digestion. As mentioned above, the symbiotic relationship in ruminants between the host and the microbes allows ruminants to utilize plant materials, such as cellulose, otherwise indigestible to other non-ruminant animals. The diverse nature of several organic compounds in EO confer these compounds the potential to further enhance efficiency. Benchaar et al. (2007b) reported that the addition of a commercial mixture of EO containing thymol, eugenol, vanillin, and limonene to the diet of lactating dairy cows at the rate of 0.75g/d did not influence apparent digestibility of dry matter (DM), crude protein (CP), NDF, and ADF. Similar effects were observed by Benchaar et al. (2006)

when increasing the dose of the same mixture of EO (Benchaar et al., 2007b) from 0.75 g/d to 2 g/d, with no influence on apparent digestibility of DM, CP, and NDF, however, ADF digestibility was increased. Castillejos et al. (2006) observed no effect on DM, NDF and ADF digestion in a continuous-culture fermentation system with the addition of various doses (5, 50, and 500 mg/L) of a phenylpropanoid EO (i.e., eugenol). In the same study a 0.5 g/L dose of a terpenoid EO (i.e., thymol) significantly reduced digestion of DM (7.9), NDF (-1.96), ADF (0.49), with no effect being reported at lower doses (5 and 50 mg/L) (Castillejos et al., 2006). Matloup et al. (2017) observed an increase in digestibility of organic matter (OM), ether extract (EE), NDF, and ADF when 14mL/d/cow of coriander (comprised of both phenylpropanoid and terpenoid compounds) was added to the diet of lactating dairy cows. Information in the literature on the effect of EO on starch digestion is limited. Previous studies had reported no changes in ruminal degradability of starch when lactating cows were supplemented with either 0.75 or 2g/d of a mixture of thymol, eugenol, vanillin, and limonene (CRINA; Gland, Switzerland) (Benchaar et al., 2006; Benchaar et al., 2007b). EO have shown their ability to influence fermentation in both positive and negative ways, further research is needed to evaluate the most beneficial dose and type of EO for achieving more efficient digestion in the rumen.

Volatile fatty acids. VFA are the main end products of rumen fermentation, they provide up to 80% of the metabolizable energy used by ruminants (Owens and Goetsch, 1988) therefore, a reduction in VFA production is nutritionally unfavorable. Busquet et al. (2006) tested 12 plant extracts (anise oil, cade oil, capsicum oil, cinnamon oil, clove bud oil, dill oil, fenugreek, garlic oil, ginger oil, oregano oil, tea tree oil, and yucca) at doses

of 3, 30, 300, and 3000mg/L in a rumen microbial batch fermentation system to evaluate the effects of EO on ruminal fermentation. In general, rumen microbial activity was affected by the use of plant extracts such as anise oil, cinnamon oil, clove bud oil, garlic oil, oregano oil, and tea tree oil, when supplied at high levels (3,000mg/L) with an overall reduction of VFA concentrations (Busquet et al., 2006). However, cade oil, capsicum oil, dill oil, fenugreek, ginger oil, and yucca had no effect on VFA concentrations at any concentration (Busquet et al., 2006). The effects of EO on individual VFA production is more variable. For instance, acetate proportions increased with the use of cinnamon oil at 3,000 mg/L and decreased with the use of anise and clove bud oil at 300 mg/L and garlic and tea tree oil 3,000 mg/L. Propionate proportions increased with low concentrations (i.e., 30 and 300 mg/L) of garlic oil and high concentrations of fenugreek and oregano oil (at 3,000 mg/L), in contrast, a propionate decrease was observed with anise oil, dill weed oil, and tea tree oils at 3,000 mg/L. Castillejos et al. (2006) reported differences in effects for dose and each type of EO such as thymol, eugenol, vanillin, and limonene were evaluated on ruminal fermentation using an *in vitro* batch culture system. Thymol, eugenol, vanillin, guaiacol, and limonene at a dose of 500mg/L resulted in lower total VFA concentrations. Thymol at a rate of 500 mg/L reduced total VFA concentrations (-28.5%) and specifically propionate (-18.4%), while increasing acetate (+1.8%) and the acetate to propionate ratio (+35.5%). In contrast to the latter, no changes were observed when thymol was provided at lower doses of 5 and 50 mg/L. Eugenol at amounts of 5, 50, and 500 mg/L did not influence total VFA concentrations but did influence individual VFA proportions. In fact, eugenol at concentrations of 5 mg/L tended to reduce acetate % (-1.7%) and the acetate to propionate ratio (-5.4%), and at 500 mg/L eugenol reduced

propionate % (-3.9%) (Note: the negative percent values indicate a percent reduction). Vanillin at doses of 5, 50, and 500 mg/L did not affect total VFA concentrations. Total VFA concentrations were reduced with guaiacol at 5 or50 mg/L, however, total VFA concentrations were not altered at 500 mg/L. Limonene at 50 and 500 mg/L reduced total VFA concentrations but at 5 mg/L had no effect. Benchaar et al. (2006) reported no effects on total ruminal VFA concentrations nor molar proportions of individual VFA, when 2g/hd/d of EO as CRINA were offered in the diet of lactating dairy cows. This agrees with Newbold et al. (2004) who used 0.11g/hd/d of the same CRINA mixture of EO in sheep with no effect on total VFA concentration or individual VFA. Benchaar et al. (2007b) reported a decrease in total VFA concentration with the addition of 0.75g/d of the same CRINA mixture used by Benchaar et al. 2006 in a corn silage based ration, whereas it increased when an alfalfa silage ration was fed to lactating dairy cows. This resulted in an interaction between EO and the fiber source of the diet. Taken these data together it appears that the effects of EO are dependent on the chemical composition of the EO, dosage use, and composition of the diet. Higher doses of EO tend to have negative effects on VFA production while lower doses show potential to increase VFA production and alter VFA proportions in a positive way. Further research is needed to find the correct dose, EO composition, and diet types to maximize fermentation.

Ruminal NH_3-N. Ruminal NH_3-N is an important element for efficient rumen microbial fermentation, however, there is a need to fine-tune the exact concentration of dietary nitrogen necessary for maximal microbial growth (Satter and Slyter, 2007). In contrast, inefficient N utilization and retention by rumen microorganism leads to the excretion of excessive N in fecal matter and methane emissions which negatively impacts the

environment (Greathead, 2007), and excessive N fermentation by bacteria can considerably imbalance the final AA profile in the metabolizable protein (Satter and Slyter, 2007). Miyazawa et al. (1998) previously reported that EO could inhibit enzyme activity, this could be useful in limiting rumen ammonia concentrations consequently, increasing the amount of high-quality dietary protein that escapes into the small intestine (Busquet et al., 2006, Greathead, 2007). Benchaar et al. (2007b) observed no effect on the ruminal concentration of NH_3-N when the diet was supplemented with 0.75 g/d of a mixture of EO as CRINA. Borchers (1965) observed the addition of 0.001mg/L of thymol to rumen fluid *in vitro* resulted in decreased NH_3-N concentration and increase of AA, suggesting inhibition of deamination. This agrees with Busquet et al. (2006) who also observed a decreased concentration of NH_3-N when carvacrol (natural isomer of thymol) was added at a rate of 3,000 mg/L. McIntosh et al. (2003) observed supplementation of 1 g/d of a mixture of EO as CRINA inhibited growth of pure cultures of ruminal bacteria, with some of these species being “hyper NH_3-N producing” species that are detrimental to the efficiency of nitrogen retention in ruminants. Newbold et al. (2004) observed that NH_3-N concentrations were unaffected by the supplementation of 0.11g/d of the same CRINA mixture of EO. Overall these data indicate that EO have the ability to inhibit deamination of AA, this could be useful in increasing the amount of bypass of high-quality dietary protein and consequently increasing the ruminal bypass rate of limiting essential AA.

Gas production. Gas production is a product of digestion in the rumen, an increase in gas production is often associated with an increase in digestion. Hungate et al. (1954) reported using carbon dioxide and methane production measurements during

fermentation are essential to determine microbial activity. Feed ingredients, such as grains in the diet of dairy cows, have a high fermentation rate in the rumen due to their readily available carbohydrate content, resulting in greater differences in gas production when compared to those having a slower rate of digestion such as forages. This is supported by Maccarana et al. (2016), who reports an increase in gas production when hay was replaced with corn silage and cereal grains. Given the ease of measuring gas production, it is a useful tool when evaluating the effects of EO on microbial activity *in vitro*. Oh et al. (1967) reported inhibition of microbial activity based on lower gas production when a 0.12 ml/L dose of terpenoid EO from Douglas-fir needles leaves (α-pinene, (β-pinene, limonene, myrcene, camphene, A3-carene, and terpinolen) was used in an in vitro study. Benchaar et al. (2007a), reported a decrease in gas production when carvacrol (400 mg/L), thymol (200 mg/L), and eugenol (800 mg/L) (phenolic EO) were studied *in vitro*. The decrease in gas production is most likely associated with a decreased in microbial activity leading to a concomitant reduction in DM and NDF digestibility. The measurement of gas production has been proven a useful tool to evaluate microbial activity. Assessing the proportions of gasses produced may be useful in evaluating the microbial populations targeted by EO, since different types of bacteria may produce different gasses.

Comparison to Ionophores.

Ionophores such as monensin, are carboxylic polyethers provided to ruminants to increases the production of propionate by modification of ion transport across the cell wall of gram-positive bacteria resulting in a shift in rumen microbiota (Duffield et al.,

2008). The latter leads to increased efficiency of energy metabolism and improved nitrogen metabolism by enhancing the production of the main glucose precursor, propionate, in ruminants and minimizing excessive protein degradation in the rumen (Duffield et al., 2008, Scharen et al., 2017). At the onset of lactation, the cow requires a substantial amount of energy that cannot be met by the energy contained in the feed ingested (i.e., energy intake). Therefore, it is common to observe a state of lipolysis in the adipose tissue of cows transitioning from pregnancy to lactation. This mechanism will mobilize body energy reserves in the form of NEFA from adipose tissue to the liver, where NEFA will be utilized as an energy fuel for cells, as a result of this mobilization a decrease in body condition and severe cases lead to ketosis and fatty liver (Drackley, 1999). Ionophores have been used as a successful feed additive for the prevention of ketosis and a production enhancer for decades (Petersson-Wolfe et al., 2007). Feeding EO is a natural alternative to the use of antibiotics or rumen modifiers such as monensin, studies have shown a similar effect for EO but results are inconsistent (Khiaosa-ard and Zebeli, 2014). Busquet et al. (2006) observed an increase in propionate % with the addition of carvacrol, cinnamaldehyde, eugenol, fenugreek, and oregano at 3,000 mg/L and garlic oil at 30 and 300 mg/L in an *in vitro* system. Khorrami et al. (2015) reported an increase in propionate % relative to other VFAs after supplementation with 6.50g/d and 5.365 g/d thyme and cinnamon respectively, and consequently confirming the potential of EO to be considered as an alternative for monensin. This is not in agreement with Drong et al. (2016), who reported the supplementation of 1 g/hd/d of a mixture of EO as CRINA failed to elicit any effect on VFAs when compared to monensin (0.34 g/hd/d). It is unclear from the currently available data to associate a specific EO with an

ionophore-like response, but thymol, carvacrol, cinnamaldehyde, eugenol, fenugreek, oregano and garlic oil have shown promise. Further research is needed to assess the relationship of blends of EO in vivo and their effect on ruminal fermentation.

The rumen ecosystem.

The effects of EO on the rumen microbiota has recently been investigated in several studies (Benchaar et al., 2007b, Patra and Yu, 2012, Scharen et al., 2017). McIntosh et al. (2003) reported an inhibition of most pure cultures of ruminal bacteria when a mixture of EO as CRINA was added at less than 100 ppm in a culture medium growth. McIntosh et al. (2003) identified two of the most sensitive bacterial species with "hyper-ammonia producing" (HAP) characteristics, which are detrimental to the efficiency of N retention in ruminants. Additionally, McIntosh et al. (2003) observed that bacteriolytic activity of ruminal protozoa was not affected by EO, but ruminal fungi activity was almost completely inhibited at the concentration of 40 ppm of CRINA as an EO mixture. There is a high variation in terms of resistance strength and resilience across bacterial species, where some can adapt and grow in higher concentrations (100 ppm) of EO (McIntosh et al., 2003). The influence of EO may be greater on solid associated microorganism species as opposed to the liquid associated species. (McIntosh et al., 2003). Klop et al. (2017) reported that rotating the supplementation of various EO *in vivo* resulted in a decline of methane production along with DMI, the extent, and persistency of methane production was not improved when compared to a continuous feeding of EO. This supports the idea of species being adaptive, and, therefore, if EO supplementation is halted for a time, then bacteria growth rate will start to increase again. Busquet et al.

(2005) observed effects on VFA % due to EO disappearance after 6-7 days in a dual flow continuous culture system, indicating rumen microorganisms can adapt to EO. Taken these data together, consistent increase in fermentation efficiency may be plausible by alternating periods of feeding EO with periods of non-EO feeding or rotate types of EO such as thymol and eugenol because the chemical compositions are different, and they display different effect on rumen fermentation.

Milk production.

Most research conducted on the effects of EO have been *in vitro*, and the few *in vivo* studies on EO compounds, dosage, and diets have produced inconsistent responses (Benchaar et al., 2008). Santos et al. (2010) reported that supplementation of EO which, contained eugenol, geranyl acetate, and coriander oil (Agolin Ruminant from AGOLIN SA, Bière, Switzer-land), at the rate of 0.85g/hd/d to early lactation cows had no effect on milk production, however, it had a positive impact on milk fat yield and percent. The authors attributed this increase in milk fat to a shift in energy partitioning away from body condition. No rumen fermentation parameters were measured in this study, therefore it remains unknown if there was a shift in energy partitioning. Another plausible explanation is that the EO influenced the bacteria involved in biohydrogenation of unsaturated fatty acids thereby reducing the levels of trans fats (i.e., trans-10, cis 12 CLA) which depresses milk fat yield (Ma et al., 2015). The effect of EO supplementation on milk fat profile is not understood and is an area of further research.

Although Benchaar et al. (2006) did not observe an effect on milk production when supplementing mid-lactation cows with 2 g/hd/d of CRINA, a decrease in fat corrected

milk (FCM) was observed. Tassoul and Shaver (2009) reported similar observations, with no treatment effect on milk yield when dairy cows were supplemented from 3 wk before calving to 15 wk postpartum with a mixture of EO at the rate of 1.2 g/h/d of CRINA. However, Kung et al. (2008) reported a tendency for greater milk production and no effect on milk fat yield for early to mid-lactation cows supplemented with 1 g/hd/d of a CRINA. It is unclear as to why milk yield responses would differ depending on stage of lactation (early vs mid-lactation), but this may be associated with the changes in microbial profile and nutritional needs as the cow transitions through the stages of lactation. Few studies have been published on the effects of EO on milk production and composition of dairy cows, but until now the general conclusion is that EO have no effect on milk production. EO have been proven to manipulate ruminal fermentation *in vitro,* but the lack of response *in vivo* may be due to the vast and intricate ecosystem of the rumen that is yet to be understood.

Dietary starch

The corn prices for the next 10 years are projected to steadily increase (USDA-Economic Research Service, 2017). Consequently, lower starch-feeding strategies that minimize inclusion of corn and other grains may improve farm profitability, particularly if cow performance is not compromised (Dann et al., 2014). Chase (2007) summarized surveys of highly productive dairy herds in the United States and found the dietary starch content typically ranged between 20 and 27% DM, with few high-producing herds less than 20% DM of starch. Starch is an important source of fuel for lactating dairy cows and provides a supply of ruminally fermentable carbohydrates necessary for microbial growth

and protein synthesis. (Firkins et al., 2001). A recent meta-analysis (Ferraretto et al., 2013) concluded that DMI is not affected by dietary starch content, whereas milk yield tends to increase 0.08 kg/d for each percentage unit increase in starch content of the diet. For every unit increase in dietary starch % there is a reduction of 0.61% dietary NDF digestibility (Ferraretto et al., 2011). Dann et al. (2014) evaluated production performance of lactating dairy cows fed diets with lower starch concentrations than would be found in a commercial herd normally (14.6, 16.2, and 18.2 % DM of starch) (Chase, 2007). No response in DMI, BW, BCS, milk yield or component production was observed, concluding that cows can be fed lower-starch diets and maintain production performance. Additionally, Dann et al. (2014) observed no effects on ruminal pH, total VFA concentrations, or acetate: propionate ratio, but butyrate concentration decreased as starch levels increased. Ferraretto et al. (2011), also evaluated the effects of a reduced starch diet (22 vs. 27% DM) on performance parameters of lactating dairy cows, where trends for reduced milk yield, protein % and yield, and decreased feed conversions (kg of milk/kg of DMI) were observed for the 22% DM starch treatment. These findings suggest that at certain concentrations > 20%, dietary starch can produce a significant impact on animal performance, primarily by enhancing the readily available energy for microbial protein synthesis.

Effects of EO supplementation on low starch diets.

As mentioned above, energy is an important nutrient in the diet of dairy cows, but is also an expensive nutrient. Fiber in forages is a cheap source of energy to the ruminant, therefore, with a reduction in the dietary starch level there also needs to be an increase in

fiber digestion to maintain a suitable energy level for the cow. Matloup et al. (2017) reported an increase in NDF and ADF digestibility when cows were supplemented 14 mL/h/d of coriander oil confirming the plausibility that supplementation of coriander oil can increase fiber digestion, consequently increasing the energy availability to the cow, allowing a reduction in the need for inclusion of high-energy ingredients. Benchaar et al. (2007b) evaluated the effects of EO supplementation and silage source in the diet (alfalfa vs. corn) on ruminal fermentation and production parameters. Given the different silage sources, the starch content in the diet also varied, the alfalfa silage diet contained 17.6% DM of starch whereas the corn silage based diet contained 25% DM of starch. Milk production and DMI were not affected by the dietary treatments, however, cows fed the corn silage based diet (25% DM starch) produced milk with lower fat content, higher protein, and urea N concentrations when compared to cows fed alfalfa silage-based diets (17.6%DM starch). Fiber digestibility based on NDF and ADF was lower when cows were fed corn silage based diets (25% DM starch). The study concluded that the effects of EO supplementation on nutrient utilization, ruminal fermentation, and milk production are limited when cows were fed diets containing either alfalfa silage (17.6% DM starch) or corn silage (25% DM starch). This study eludes to the supplementation of EO having a positive effect on digestion, therefore, cows may be able to maintain production performance even with low starch levels in the diet. In fact, there is a current lack of data available on the effects of EO on low starch diets fed to lactating dairy cows, therefore, this is an area of further research.

Nutritional requirements for the stages of lactation.

The amounts of nutrients required by the cow are greater during times of increased production or impending production. The nutrients requirements of the cow also change as she advances through the various stages of lactation (1.61, 1.47, 1.36 NE_L Mcal/kg for cows in early, mid, and late stages of lactation, respectively) (Hutjens, 2008). A critical period for a dairy cow is the time from parturition to peak milk production also known as early lactation (0-70DIM) (Ingvartsen, 2006). For instance, it has been determined that for every 1 kg of increase in milk yield during peak milk, this represents an additional 200 kg of total milk production during the entire lactation (Schingoethe et al., 1988). Early lactation is the most challenging period during the lactation cycle of a dairy cow, where the high nutritional demand cannot be met by the intake of energy from the diet, this leads to the cow being in a negative energy balance (Drackley, 1999). This nutritional deficit is made up by metabolizing physiological tissues resulting in a loss of 90-135 kg of BW during this period (Schingoethe et al., 1988). During mid and late lactation cows are in a positive energy balance because the intake is higher than the energy requirements for maintenance and lactation. Consequently, the cow can replenish her body fat stores for the next lactation (Vazquez-Anon et al., 1977). However, in high-yield dairy cows, replenishing fat stores during lactation is more difficult due to the higher energy requirements than average or low-yield dairy cows. Therefore, high-energy diets may be beneficial to high-yield dairy cows to help restore body fat stores for the next lactation (Vazquez-Anon et al., 1977). An approach to increase the energy content of the diet is to increase grain content (i.e., starch) of the diet. This approach influences metabolism by increasing propionate production in the rumen, eliciting an insulin response resulting in either partitioning of nutrients toward body fat during mid and late

lactation or towards milk production during early lactation cows (Vazquez-Anon et al., 1977). Products such as ionophores which alter ruminal fermentation and improve feed efficiency are useful to help the cow overcome the negative energy balance during early lactation and help to provide the additional energy needed throughout the lactation to replenish fat stores. The current research carried out on EO set these organic compounds as a viable alternative tool for dairy producers to help increase the dietary energy by improving feed efficiency and alter fermentation patterns.

Effects of EO on production parameters of cows in various physiological stages.

As the cow progresses through a given lactation cycle, there are several physiological and metabolic adaptations that occur such as returning to a positive energy balance, starting the estrus cycle, and eventually the onset of pregnancy. Therefore, it is conceivable that such changes might alter the animal responsiveness to EO effects. The main physiological stages of an adult cow are lactating non-pregnant, lactating pregnant, and non-lactating pregnant. The latter could also be expanded to immature lactating cows (i.e., 1st lactation cows) and mature cows (i.e., 2nd lactation and over), by the former having additional nutrient requirements for growth. During all these stages, the dairy cow undergoes different nutritional demands that might affect the metabolism, and the way nutrients are utilized. The physiological state of the animal could affect the responses from supplementation with EO (Wall et al., 2014). For example, Wall et al. (2014) detected a parity by treatment effect with an increase in milk yield in multiparous cows compared to primiparous cows supplemented with 0.35g of EO (17% cinnamaldehyde and 28% eugenol; Pancosma SA, Geneva, Switzerland). This may be explained by the

change in nutritional requirements due to the physiological state (growth vs. mature) as the cow progresses through several lactation cycles. Wall et al. (2014) also reported an increase in DMI in multiparous and primiparous cows supplemented with EO. The latter could be associated with greater nutrient requirements of primiparous cows compared to multiparous since primiparous cows have an additional energy requirement for growth which is greater than the energy required for maintenance in multiparous cows, therefore greater milk production was only achieved in multiparous cows. The energy from the increase in DMI in primiparous cows was likely diverted towards growth instead of lactation and the multiparous cows most likely used the energy for milk production. Growth may not be the only physiological state affecting the efficacy of EO to improve milk production, but also stage of lactation may also play a role. Tassoul and Shaver (2009) reported an increase in milk production during week 15 of the lactation, along with an increase in feed efficiency during 56-98 DIM, the increase in efficiency provided the cow with more energy resources allowing a faster return to a positive energy balance, consequently more energy was diverted towards milk production. No other studies to our knowledge have reported and/or researched the possible mechanisms behind the effects of EO on physiological states of the dairy cows, thus this question needs further study.

CONCLUSION

The bactericidal and bacteriostatic effects of EO, due to their diverse composition, nature, and biochemical activities, warrant the investigation into their potential to manipulate ruminal fermentation. Although the current data acquired in lactating dairy cows have been inconsistent, EO have been proven to influence microbial fermentation and production response in dairy cows. The promising results on increased animal

productivity observed when feeding specific EO confer these organic compounds the potential ability to replace conventional antibiotic and growth promoters. A need for further research is warranted to fine-tuning the adequate supplementation of EO that could not only maintain greater animal productivity but also maximize the use of low-starch diets with the aim to expand the profit margin in dairy operations.

Chapter 2: The Effect of Feeding a Low Starch Diet to Dairy Cows Being Supplemented with a Blend of Essential Oils

INTRODUCTION

Consumer pressures and changes in legislation on the use of in-feed antibiotics and growth promotors have made it extremely challenging to produce the amount of food needed to sustain a growing population at a reasonable cost (Lekshmi et al., 2017). Limited availability of farming land and water have driven the increase in input costs, such as feed, to higher levels than before. Thus, there is a need for increased efficiency in animal productivity through alternative technologies than previously used. Therefore, research utilizing alternative avenues to lower feed cost is underway. The diverse composition, nature, and biochemical activities of essential oils (McIntosh et al., 2003, Calsamiglia et al., 2007, Benchaar et al., 2008), make them a prime candidate to help improve feed efficiency and animal productivity. Increases in milk yield, components, and feed efficiency have been reported with the supplementation of EO to a dairy cow diet (Tassoul and Shaver, 2009, Santos et al., 2010, Matloup et al., 2017). However, results vary in responses (Benchaar et al., 2007), and further investigation into optimal dosage and specific mechanisms of EO are warranted.

The average milk production in dairy herds continues to increase in the US (Chase, 1993), along with a drastic increase in corn prices in the past ten years and projections of further increase (USDA-Economic Research Service, 2017), a challenge exists to maintain a profitable margin. Thus, alternative ways of increasing profitability, such as implementing high-forage diets to minimize the inclusion of corn and other

grains and maximize digestion by altering passage rates in the rumen, have been utilized. The use of high starch diets to replace forage often reduces total tract digestibility (Beckman and Weiss, 2005), which may be due to an increase in digestion rate and passage rate through the rumen (Van Soest, 1982, Merchen, 1988). The implementation of high-forage diets at the expense of starch can decrease the passage rate through the rumen and to some extent improve feed efficiency of the cow (Van Soest, 1982, Merchen, 1988). This feeding strategy, when adequate, could maintain milk production, and considerably improve profitability in dairy operations.

Research supports the idea that EO can improve feed efficiency (Tassoul and Shaver, 2009, Santos et al., 2010), which could allow cows fed low starch diets to maintain the same efficiency of milk production as cows fed high-starch diets (Dann et al., 2014). Regardless of the economic potential of the combinatory effect of EO and LS diets, there is a lack of research on this topic. In theory, EO supplementation in combination with LS diets could further improve production efficiency. Therefore, the objective of this study was to evaluate production parameters of dairy cows fed a low starch diet with the supplementation of EO. We hypothesize there will be no difference in performance of the cows fed a low starch diet in combination with essential oils when compared to those fed a greater amount of starch.

MATERIALS AND METHODS

Experimental Design. The trial was conducted at a commercial robotic dairy farm in southwest Minnesota outfitted with two Lely Astronaut A4robotic milking units (Goter's Clay & Dairy Equipment of MN, INC., Pipestone, MN). All procedures were approved

(Protocol 17-029E) by the Institutional Animal Care and Use Committee at South Dakota State University. One hundred and seven lactating Holstein dairy cows were randomly assigned to either the control diet (CON) (25% starch; n = 59) or the low-starch (LS) diet (22% starch; n = 48). The trial was set up as a randomized complete block design (RCBD) which included 14 d as adaptation period and 28 d of data collection. Each treatment group was housed in a separate pen containing free stalls and a robotic milker. Cows were fed once a day, between 1000 h and 1200 h, and were fed to a “slick bunk”, meaning the cows ate all the feed they were given and there were no refusals. A pellet containing Stay Strong (SS) (a commercially available blend of EO) was dispensed robotically at each milking. The amount of pellet offered during each milking was depended on previous day milk production for each cow but did not exceed 5.45 kg/d. A maximum of 4 milking times was allowed per cow over a 24 h period.

Feed and milk samples. Individual ingredients, TMR, and pellet samples were collected once a week during the trial and submitted to Analab (Agri-King, inc, Fulton, IL) for digestibility analysis by using acid insoluble ash (AIA) used as an internal marker for determining nutrient digestibility coefficients for dry matter (DM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), starch hemicellulose, and minerals (Van Keulen and Young, 1977). Samples were analyzed by the AOAC (1998) methods: DM (935.29), CP (90.03), NDF (2002.04), ADF (973.29), acid detergent insoluble crude protein (ADICP) (973.18), neutral detergent insoluble crude protein (NDICP) (2002.04), ash (942.05), lignin, (973.18), calcium, phosphorus, magnesium, sodium, iron, copper, potassium, zinc and manganese (985.01) chloride (915.01) and sulfur (923.18). Other parameters measured were soluble protein (Krishnamoorthy et al.,

1982), starch (glucose reagent set, AMRESCO, Solon, OH and ALPKEM Corporation, 1990), fat (Damon, 1966), in vitro dry matter digestibility (IVDMD) (Marten and Barnes, 1980), neutral detergent fiber digestibility (Van Soest et al., 1991), ammonia nitrogen (NH_3-N) (United States Environmental Protection Agency, 1993, method 351.2 and International Organization for Standardization, 2013, method 11732), non-fiber carbohydrate (NRC, 2001) and net energy for lactation (NRC, 2001). The diet fed to LS cows (Table 1) consisted of 60.94% forage and 39.06% concentrate, and the CON diet (Table 1) consisted of 59.02% forage and 40.98% concentrate. The LS diet was formulated by removing 0.45kg of HMC (high moisture corn) and replacing it with corn silage resulting in a 2.59 % difference in dietary starch levels when the diets were compared. Milk yield and components as well as BW, rumination activity (min), and pellet intake was recorded at each milk time by the robotic milkers [Lely Time for Cows (T4C) milking software, Goter's Clay & Dairy Equipment of MN, INC., Pipestone, MN] and downloaded remotely daily throughout the trial.

Milk samples were collected biweekly for the duration of the trial using the Lely Shuttle milk sampling unit, the samples were analyzed by DHIA (Zumbrota, MN) for fat, protein, lactose MUN, and SCC. The BCS was recorded weekly by two individuals based on a scale of 1 (i.e., thin) to 5 (i.e., obese).

Rumen fluid and plasma urea nitrogen. Rumen fluid was collected from 15 randomly selected cows from each treatment during the last week of the trial using the esophageal tubing method. Rumen fluid pH was taken immediately to ensure the quality of the sample. Ten mL of rumen fluid sample were dispensed into a vial containing 2 ml of 25% meta-phosphoric acid and frozen at -20°C until analysis for volatile fatty acid (VFA). Ten

additional mL of rumen fluid sample were dispensed into a vial containing 200 μL of 50% sulfuric acid (H_2SO_4) and frozen at -20°C until analysis for ammonia N. For NH3 analysis in rumen fluid, the samples were thawed and centrifuged at 3,000 × *g* for 20 min at 20°C (Eppendorf 5403, Eppendorf North America, Hauppauge, NY), and then analyzed by using Chaney and Marbach (1961) procedures with a colorimetric assay performed on a microplate spectrophotometer (Cary 50, Varian Inc., Walnut Creek CA). Rumen fluid samples preserved with metaphosphoric acid were prepared according to Hamada et al. (1968) methods. Acetate, propionate, butyrate, isobutyrate, isovalerate, and valerate concentrations were analyzed by automated gas-liquid chromatography (model 6850, Agilent, Santa Clara, CA). Blood was taken from the tail vein of the same 15 randomly selected cows above during the last week of the trial, and centrifuged at 1000 × *g* for 20 min at 4°C (Centrifuge CR412 Jouan Inc.,Winchester, VA) and transferred into polystyrene tubes using plastic transfer pipette, and frozen at -20°C until later analysis. Plasma urea nitrogen concentration (PUN) was analyzed with commercially available colorimetric assay kits on a microplate spectrophotometer (Cary 50, Varian Inc., Walnut Creek CA) (Procedure 0508; Stanbio Laboratory, Boerne TX).

Statistical analysis. Data were analyzed using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). Fixed effects in the model were diet, time, and diet × time. Random effect was cow within diet. Individual cow data from 2 wk prior to start the experiment was used as a covariate and was maintained in the model if it was significant ($P < 0.05$). Only cows that completed the trial were used.

RESULTS

Feed analysis. Diet formulation and nutrient composition for CON and LS diet are presented in Table 1. The CON diet contained 2.6% more starch then the LS diet. Both diets had similar contents of metabolizable energy, crude protein, forage, fat, NDF and ADF. Diets were formulated to meet the nutritional requirements set by the NRC (2001).

Ruminal fermentation and blood components. Plasma urea nitrogen and ruminal fermentation parameters are presented in Table 3 and 4. Among ruminal fermentation parameters a significantly (P <0.01) lower NH_3-N concentration was observed for the LS treatment. The LS treatment tended (P = 0.08) to have higher pH values than the CON. A tendency (P = 0.09) was observed for lower molar concentration of isovalerate in the LS treatment. No differences between the treatments were observed for total VFA (P = 0.29), acetate to propionate ratio (P = 0.160, or molar concentrations of acetate (P = 0.44), propionate (P = 0.25), isobutyrate (P = 0.58), butyrate (P = 0.16), or valerate (P = 0.14). No differences between percentages of propionate (P = 0.36), butyrate (P = 0.11), isobutyrate (P = 0.42), isovalerate (P = 0.33), or valerate (P = 0.13) were observed between the treatments. A tendency (P = 0.06) for higher acetate percentages was observed for the LS treatment. No differences (P = 0.92) in BUN concentrations were observed between the treatments.

Body conformation, pellet intake, and rumination. A significant (P < 0.01) TRT × Time was observed for BCS and rumination. The latter effects were associated with greater (P < 0.01) BCS for LS cows during 2 and 3 wk of trial (Figure 1), while greater (P < 0.05) rumination was observed in LS cows at 8 and 13 d on trial (Figure 2). However, these effects on BCS and rumination did not translate into treatment effects ($P \geq 0.12$). A

tendency ($P = 0.07$) for greater pellet intake was observed for the LS treatment. The BW was not affected ($P \geq 0.16$) by experimental effects.

Milk Production and Composition. Milk production and composition parameters are presented in Table 5. A significant ($P \leq 0.01$) interaction of TRT × D was observed in milk production and composition parameters. However, with the exception of milk protein, most of these effects can be associated with transient changes over time ($P < 0.01$) rather than actual treatment differences over time, which is confirmed by the lack ($P \geq 0.46$) of overall treatment effects. While MUN concentrations were only affected by Day effect ($P < 0.01$), milk yield was the only parameter affected ($P < 0.01$) by stage of lactation

Stage of lactation. Because of the overall stage of lactation effect on milk yield, we further evaluated this parameter within each stage of lactation and observed that late lactation was the only stage with a significant treatment effect, where the LS diet had greater ($P = 0.02$) milk yield in comparison to the control. A treatment × time interaction was observed for protein and fat percentage, and rumination time. Rumination and protein percentage were mainly by a time effect ($P < 0.01$), while fat percentage had a treatment effect, where late lactation cows on the LS treatment had the lowest ($P < 0.01$) fat percentage. A tendency ($P = 0.07$) was observed for greater BW in late lactation cows on the LS treatment as well when compared to the CON group.

Although the blood and rumen fluid sampling was performed randomly across cows in both pens, we were able to determine that a significant number of those cows sampled were in early lactation for CON (n = 5) and LS (n = 9) treatments. Dietary

treatment effects on early lactation cows are presented in Table 7. Ruminal NH_3-N concentrations were lower ($P < 0.01$) in cows fed the LS treatment group. Ruminal pH ($P = 0.07$) and PUN ($P = 0.08$) both tended to be greater in early lactation cows in the LS when compared to the CON early lactation cows. Total VFA concentrations, individual VFA concentrations, and proportions, as well as acetate to propionate ratio, were not affected ($P \geq 0.12$) by dietary effects.

DISCUSSION

Low starch diets. There have been several studies evaluating the effects of a reduced starch ration on the performance parameters of lactating dairy cows (Ruppert et al., 2003, Dann et al., 2014, Luo et al., 2016). Luo et al. (2016) compared dietary starch levels of 21.4 and 22.6 % DM, and observed that this small reduction in starch levels did not cause any change in production performance. Ferraretto et al. (2011) evaluated production performance of dairy cows fed starch levels of 22 and 27% (similar to the ones in this study), trends for decreased milk yield, and milk protein % and yield, were observed in the 22% starch treatment when compared to the 27% starch diet.

Ruminal fermentation and blood components. The significantly lower NH_3-N concentrations in the LS treatment, when compared to the CON treatment, (Table 3) agrees with Benchaar et al. (2007) who reported a trend for lower NH_3-N concentrations when cows were fed a 17.6 % DM dietary starch in comparison to 25 % DM dietary starch. The decrease in NH_3-N concentrations for the LS diet could be due to inhibition of proteolytic bacteria thus, potentially increasing the dietary protein available to the animal (McIntosh et al., 2003). Ruppert et al. (2003) reported greater NH_3-N concentration in

cows fed greater starch levels (32.3 vs. 27.9 % DM starch) than in the current study, this could be due to the greater starch concentration providing greater energy to the microbes leading to increased microbial growth resulting in the use of more NH_3-N for production of microbial proteins (Hristov and Ropp, 2003). An alternative theory could be a decoupling between the ruminal degradation of carbohydrates and proteins given the feeding schedule at this dairy, which renders cows in LS treatment with a suboptimal microbial efficiency (Firkins et al., 2001). Because of the higher percentage of starch in the TMR fed to the CON group, the cows on this treatment were ingesting more starch more frequently than the LS treatment cows, therefore, we do not believe the CON was affected by the decoupling between ruminal degradation of carbohydrates and proteins. The lack of dietary effect on PUN (Table 3) or MUN (Table 5), is in contrast with the findings of Dann et al. (2014) and Benchaar et al. (2007), both observed lower MUN concentrations as dietary starch increased. These results suggest that increasing starch concentrations will produce greater N utilization (Luo et al., 2016). Our observation of no change in MUN concentration may be due to the effect of the SS in the rumen resulting in the same response as increases in starch concentration would. A higher ruminal pH (7.02 vs 6.68) for the LS treatment is in agreement with Ruppert et al. (2003) who reported higher rumen pH (6.09) when a 27.9% starch diet was fed in comparison with 32.2% dietary starch. However, Benchaar et al. (2007) observed no influence on rumen pH between 25 % and 17.6 % starch levels diets supplemented with EO. Both diets in the current study were adequate in physically effective fiber, and the % of starch in the diets was likely not enough to change rumination patterns between treatments as compared to Ruppert et al. (2003).

Dann et al. (2014) observed no significant changes in total VFA production when varying levels of starch were fed from 17.7% to 24.6%. In contrast, Benchaar et al. (2007) reported a tendency for a decrease in total VFA concentrations for cows fed a 25 % starch diet compared to those fed 17.6 % when supplemented with a blend of EO. The trend observed for greater acetate % for the LS diet (Table 3) was expected because of the increase in forage in the diet. Benchaar et al. (2012) also observed a significant increase in acetate production when cows were fed diets on 35 and 65% starch, but when eugenol was added to the diets, no effect was observed on acetate production. Benchaar et al. (2007) and Dann et al. (2014) also reported similar findings of no effect on acetate to propionate ratio. While Benchaar et al. (2012) reported a significant increase in acetate: propionate ratio in low starch diets (35 vs. 65%), the addition of eugenol to the diets had no effect on the acetate: propionate ratio. Our results are in agreement with Benchaar et al. (2007), where no treatment differences were observed for molar concentrations of acetate, propionate, isobutyrate, and butyrate. Benchaar et al. (2012) reported eugenol supplementation did not have an effect on acetate, propionate, and butyrate, but reported a significant decrease in branch chain fatty acids (BCFA) in low starch diets (35 vs. 65%) supplemented with eugenol. Isovalerate in the current study was significantly decreased in the LS diet. This is only in partial agreement with Benchaar et al. (2012) because we did not observe an increase in isobutyrate. The decrease in isovalerate suggests the EO supplementation improved nitrogen utilization (Benchaar et al., 2012).

Body conformation and intake. Body conformation parameters such as BW and BCS have been observed to be not responsive to dietary starch levels (17.7, 21.0, 24.6%

starch) (Dann et al., 2014), which is in agreement with our results in BCS and BW (Table 4). Alterations of DMI have been observed when cows are fed high-starch diets, for instance, Ruppert et al. (2003) observed greater DMI for cows consuming a 27.9% starch diet when compared with cows fed a 32.3 % starch diet. In contrast to Ruppert et al., (2003), Benchaar et al. (2007) reported similar DMI between cows fed a 25 % or 17.6% starch diet. Although the pellet intake provided in the robotic unit during the milking time do not represent the actual intake of TMR, it is interesting that our results on a trend for greater pellet intake in LS treatment follow the same pattern as observed by (Ruppert et al., 2003). The latter effect could be to some extent associated with a need for higher energy levels to maintain production when cows are switched to a lower starch diet.

Milk Production and Composition. Benchaar et al. (2007) and Dann et al. (2014), reported no change in milk production when cows were fed varying levels of dietary starch, which is in agreement with our observations. Our results indicate that the overall dietary starch level effect did not alter milk components, which corresponds to results reported by Dann et al. (2014). In contrast to our results, Benchaar et al. (2007) reported a trend for lower milk fat and greater protein yield for cows fed a 25 % starch diet (corn silage based) in comparison to a 17.6 % starch diet (alfalfa silage based). A significant effect of stage of lactation was observed on milk production with no effects on milk components or MUN (Table 5). The stage of lactation effect was likely driven by a greater milk production (≥ 2.81 kg/d) in early lactation cows (DIM ≤ 120) in comparison to mid (DIM 120 to 240) and late lactation (DIM ≥ 240) cows. Interestingly, an effect of dietary starch concentration was only observed in late-lactation cows, that resulted in LS cows having greater (ca. 3.13 kg/d) milk production. The latter effect could be associated

with a shift in ruminal VFA production towards greater propionate production, resulting in a similar rumen fermentation pattern produced by the rumen modifier, monensin (Granzin and Dryden, 2005). No changes in the VFA concentrations, proportions, acetate: propionate ratio in RF from early lactation cows (Table 7), agrees with Benchaar et al. (2012) who reported no changes in acetate, propionate butyrate and acetate: propionate ratio when early lactation cows fed high and low levels of starch (35 vs. 65%) supplemented with eugenol. The lower NH_3-N concentration in LS cows in early lactation suggests that protein digestion in the rumen was more efficient. The latter could be a result of the low starch content resulting in slower growth of proteolytic bacteria allowing higher levels of protein escaping into the small intestine (Poos et al., 1979, Recktenwald et al., 2014). The latter effect is partially supported by the greater PUN concentration in LS cows. In ruminants, blood urea nitrogen concentration is associated with dietary CP intake, since the latter is ruminally degraded resulting in greater production of ruminal ammonia that is transported into the bloodstream. Once ammonia reaches the liver, it will be metabolized into urea and secreted into the blood (Broderick and Clayton, 1997). Constant recycling of N to the rumen from the blood stream (Owens and Zinn, 1988) is occurring, excess ammonia is absorbed via the rumen wall and converted into urea in the liver, where it returns in the blood, saliva or is excreted by the body. The amount of N recycled to the rumen is reduced when ruminal NH_3-N concentrations are high (Owens and Zinn, 1988). This also many be a driving factor for the greater PUN concentration in LS cows. MUN observations of this study agree with (Benchaar et al., 2007, Benchaar et al., 2012), both reported the supplementation of EO

had no effect on MUN concentrations when cows were fed diets with varying starch levels (17.6 vs. 25, 35 vs. 65% respectively).

CONCLUSION

Regarding production parameters, the lack of treatment effects indicates that low starch diets supplemented with SS might not have a significant impact on dairy cow performance. Overall fermentation characteristics were similar except for the significant reduction in NH_3-N concentrations in cows fed the LS diet, indicating an increase in dietary protein bypassing the rumen fermentation and reaching the small intestine where if absorbed will improve the dietary protein available to the animal. To our knowledge no other studies have investigated the effect of low-starch diets during EO supplementation on performance parameters of cows in various stages of lactation, this may be an area of future research.

Table 1. Ingredient and nutrient composition of the TMR for each treatment. All values are %DM unless noted otherwise.

	TMR	
Ingredient	Low starch	Control
Alfalfa Grass Hay	0.84	0.84
Alfalfa haylage	18.47	18.43
Corn Silage	35.06	33.09
Alfalfa hay	6.58	6.57
High moisture corn	6.42	8.34
Dairy sweet	2.05	2.05
Corn/cottonseed	12.69	12.67
Soybean meal	2.21	2.20
Energy booster[2]	0.78	0.78
Protein mix[3]	14.92	14.89
Chemical analysis		
CP	16.92	16.95
Starch	22.44	25.03
Forage	60.94	59.02
EE	4.12	4.07
NDF	31.05	31.34
peNDF	24.60	24.12
ADF	18.88	19.13
ME (Mcal/kg)	0.51	0.51
Ash	8.20	8.24
Na	0.46	0.50
Mg	0.36	0.35
P	0.35	0.35
S	0.21	0.21
K	1.21	1.25
Ca	1.01	1.00
Cl	0.66	0.67
Mn ppm	100.67	100.67
Fe ppm	323.33	313.33
Cu ppm	24	22.33
Zn ppm	125	130.67

[1] The corn/cottonseed blend contained ground corn (88.19) and fuzzy cottonseed (11.81) on a %DM basis.

[2] Milk Specialties Global, Eden Prairie, MN.

[3] The protein mix contained the following ingredients on a percent DM basis: soybean meal (18.92), dry distiller grains (10.98), canola meal (11.15), SoyPlus (Landus Cooperative) (9.91), sodium bicarbonate (6.15), meat/bone meal (5.19), limestone (5.14), MetAAein blood meal (4.62), fat tallow porcine (4.77), soy hulls (3.81). salt (2.58), corn gluten meal (2.26), starch corn (1.70), magnesium oxide (1.47), calcium phosphate (1.23), urea (1.22), Omnigen (Phibro Animal Health Corporation, Teaneck, NJ) (0.69), mineral mix (6.21), vitamin premix (0.63), DCAD plus (0.36), XPC (Diamond V) (0.33), Bio-Mos (Alltech, Lexington, KY) (0.25) Vitamin E 20000 IU/kg (0.14), biotin 1% (0.052)

Table 2. Nutrient composition of the pellet, corn silage, high moisture corn, alfalfa haylage. All values are %DM unless noted otherwise.

	Feed Ingredient			
	Pellet	Corn Silage	High Moisture	Alfalfa
Nutrient				
DM, %	87	33.62	67.40	41.22
RFV[1]	- -	- -	- -	147
TDN[2]	- -	- -	- -	64
CP	24.98	8.68	7.98	22.10
Starch	22.44	32.44	63.68	0.09
EE	1.98	2.73	3.26	2.4
NDF	20.69	37.71	18.985	40.46
ADF	9.90	21.52	- -	31.94
ADIP[3]	- -	0.33	- -	1.16
NDIP[4]	4.40	0.61	- -	1.61
Ash	4.13	3.66	- -	10.01
Lignin	- -	1.80	- -	6.54
NH_3N	- -	1268.5	- -	1254
Mg	0.3	0.17	0.1	0.27
Na	0.05	0.03	- -	0.1
P	0.5	0.17	0.22	0.23
S	0.25	0.05	- -	0.25
K	1.46	0.75	- -	1.82
Ca	0.42	0.25	- -	1.91
Cl	0.12	0.32	- -	0.65
Mn ppm	52.67	29	- -	36.5
Fe ppm	196.33	77.5	- -	421.5
Cu ppm	12	3.5	- -	9
Zn ppm	74.67	61	- -	60.5
pH, 0-14	- -	3.8	4.35	4.5
Lactic	- -	5.08	0.99	7.17
Acetic	- -	2.84	0.22	2.07

[1] RFV = Relative feed value.

[2] TDN = Total digestible nutrients.

[3] ADIP = Acid detergent insoluble protein.

[4] NDIP = Neutral detergent insoluble protein.

Table 3. Effects of a low starch ration on ruminal fermentation and blood characteristics of cows supplemented SS.

	Treatment			*P*
Parameter	CON	LS	SEM[1]	Treatment
Plasma urea nitrogen	13.65	13.84	1.37	0.92
NH_3-N	9.69	5.84	0.54	< 0.01
pH	6.68	7.02	0.13	0.08
VFA				
Acetate, mM	60.88	57.16	3.50	0.44
Propionate, mM	32.34	28.61	2.32	0.25
Isobutyrate, mM	0.81	0.77	0.05	0.58
Butyrate, mM	9.77	8.39	0.71	0.16
Isovalerate, mM	0.78	0.65	0.06	0.09
Valerate, mM	2.48	1.91	0.27	0.14
A: P	1.90	2.10	0.10	0.16
Total VFA, mM	107.07	97.49	6.54	0.29
Acetate, %	56.96	59.04	0.78	0.06
Propionate, %	30.15	29.03	0.88	0.36
Butyrate, %	17.18	14.32	1.28	0.11
Isobutyrate, %	0.78	0.80	0.04	0.42
Isovalerate, %	0.73	0.68	0.04	0.33
Valerate, %	2.28	1.91	0.18	0.13

[1]Largest standard error of the mean

Table 4. Effects of a low starch ration with the supplementation of SS on BCS, BW, pellet intake, and rumination.

	Treatment			*P*		
Parameter	CON	LS	SEM[1]	Treatment	Time	TRT × Time
BCS	2.40	2.40	0.01	0.12	< 0.01	< 0.01
BW, kg	647.95	653.23	2.66	0.16	0.02	0.64
Pellet intake, kg/d	5.08	5.27	0.08	0.07	< 0.01	0.40
Rumination, min/d	472.51	470.25	87.97	0.89	0.93	< 0.01

[1]Largest standard error of the mean

Table 5. Effects of SS supplementation in combination with a LS diet on production parameters.

	Diet			*P*			
Parameter	LS	Control	SEM	Treatment	Lactation	Day	T×D
Milk yield, kg/d	38.29	37.53	0.90	0.51	<0.01	<0.01	0.01
Milk fat, %	3.33	3.48	0.28	0.54	0.77	<0.01	<0.01
Milk protein, %	3.05	3.06	0.01	0.71	0.55	<0.01	<0.01
Milk fat yield, kg/d	1.26	1.27	0.02	0.53	0.26	<0.01	<0.01
Milk protein, kg/d	1.15	1.14	0.02	0.46	0.64	<0.01	0.01
MUN	16.51	16.81	0.23	0.35	0.25	<0.01	0.56

[1]Largest standard error of the mean

Table 6. The effect of a LS diet with SS supplementation on milk production, intake, rumination, BCS and BW of cows in late lactation.

	Treatment			*P*		
Parameter	CON	LS	SEM[1]	Treatment	Time	Trt × Time
Milk yield, kg/d	28.75	31.88	0.96	0.02	0.11	0.40
Protein yield, kg/d	0.91	0.99	0.03	0.04	0.37	0.60
Fat yield, kg/d	1.19	1.21	0.04	0.72	0.06	0.09
Protein, %	3.16	3.13	0.02	0.27	< 0.01	< 0.01
Fat, %	4.22	3.83	0.10	< 0.01	< 0.01	< 0.01
Intake, kg/d	4.27	4.56	0.27	0.42	< 0.01	0.02
Rumination, min/d	427.98	464.74	30.43	0.37	< 0.01	< 0.01
BCS	2.37	2.39	0.19	0.48	< 0.01	0.20
BW, kg	629.71	640.10	4.13	0.07	0.11	0.94

[1]Largest standard error of the mean

Table 7. The effects of SS on early lactation cows fed a LS diet.

	Treatment			*P*
Parameter	CON	LS	SEM[1]	
MUN	16.71	16.47	0.30	0.57
PUN	10.05	14.48	1.90	0.08
NH_3-N	8.49	5.16	0.93	0.01
pH	6.46	6.97	0.21	0.07
Total VFA, mM	119.08	100.38	13.31	0.28
Acetate, mM	66.87	57.44	10.09	0.57
Propionate, mM	37.27	31.87	4.34	0.34
Butyrate, mM	10.37	7.88	1.19	0.12
A : P	1.79	1.82	0.06	0.78
Acetate, %	56.20	57.09	0.87	0.42
Propionate, %	31.34	31.58	0.62	0.76
Butyrate, %	18.47	13.88	2.20	0.12

[1]Largest standard error of the mean

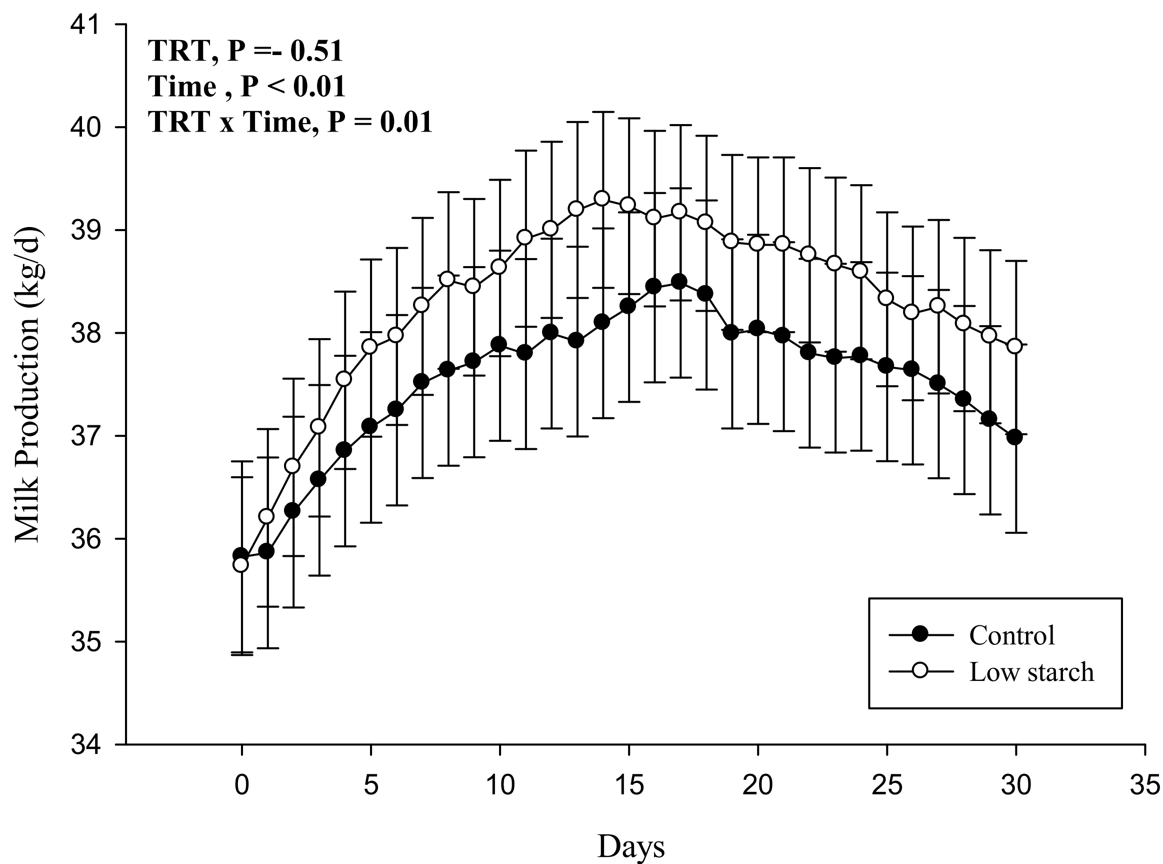

Figure 1. Milk Production. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.
* Significant values.

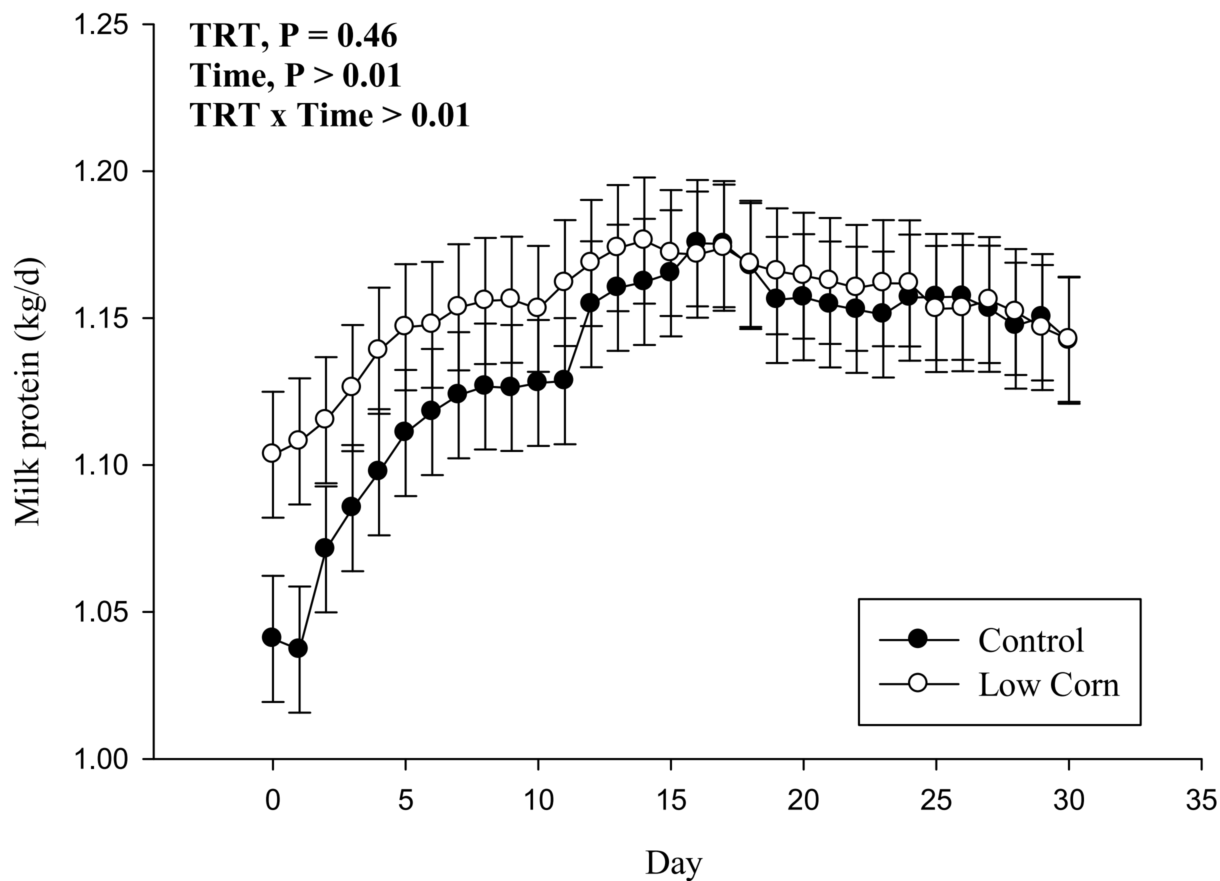

Figure 2. Milk protein yield. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.
* Significant values.

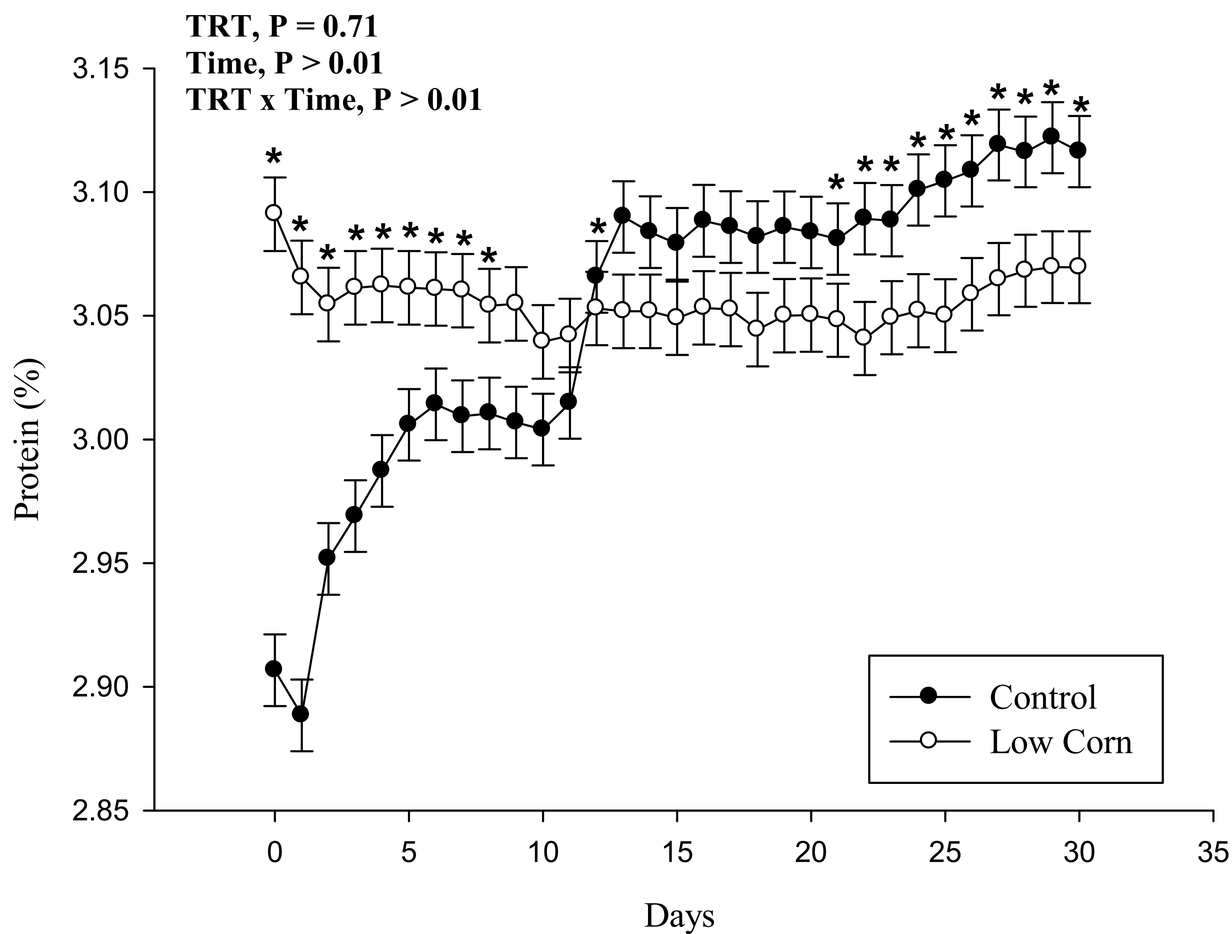

Figure 3. Milk protein %. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.
* Significant values.

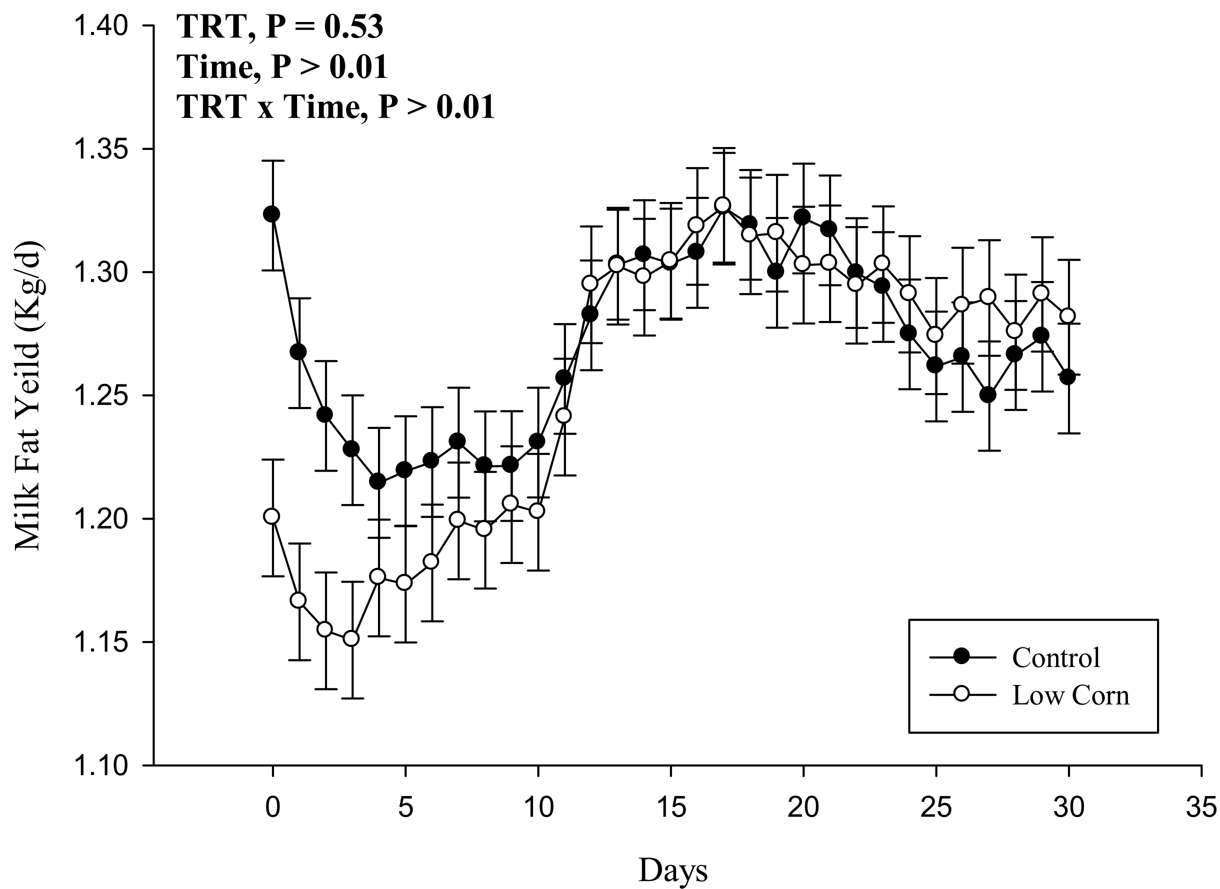

Figure 4. Milk fat yield. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.
* Significant values.

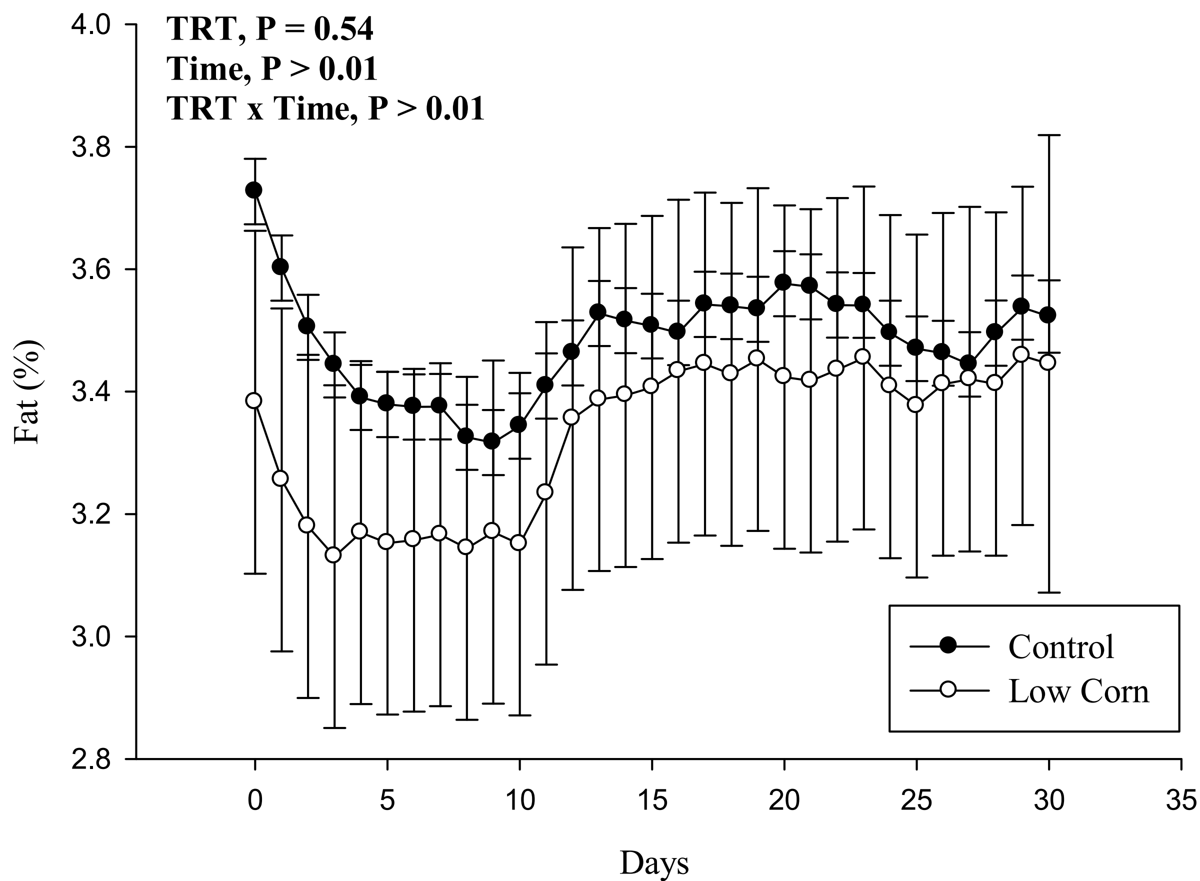

Figure 5. Milk fat %. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.
* Significant values.

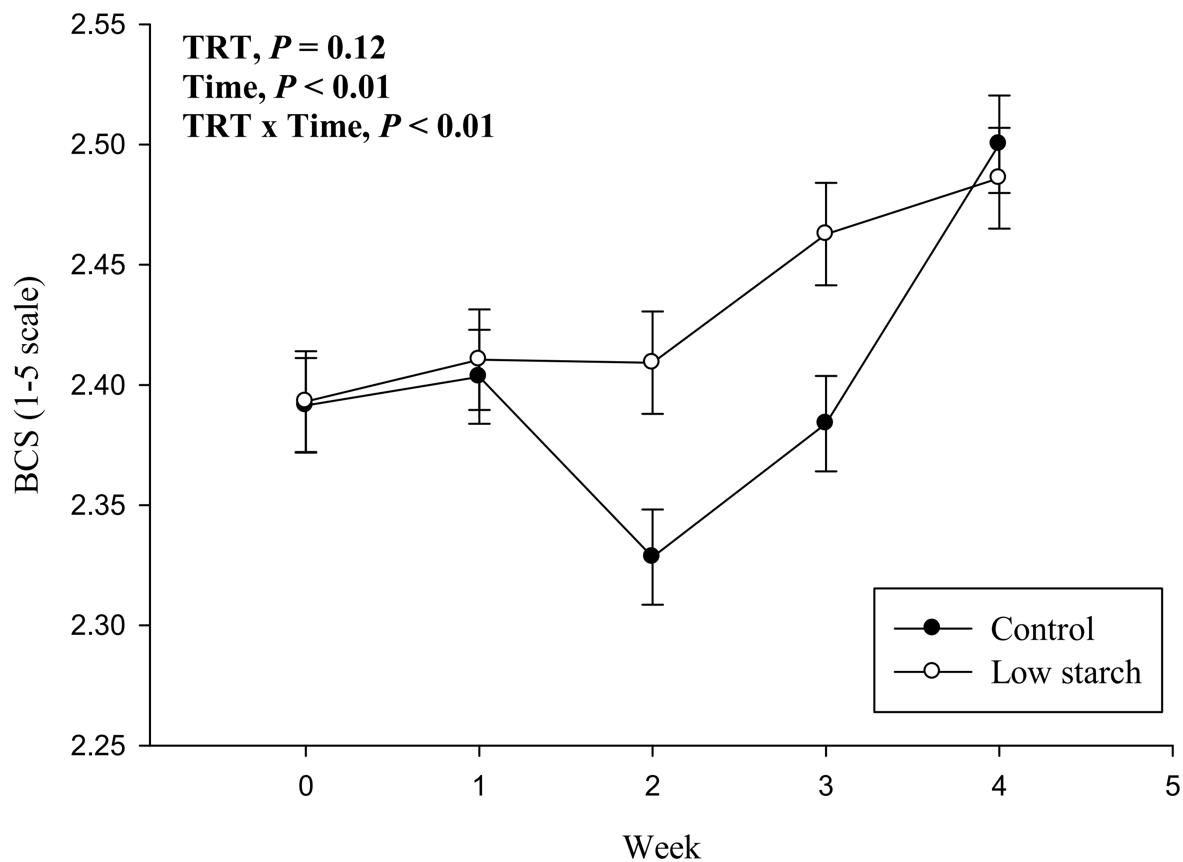

Figure 6. Body condition score. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.
* Significant values.

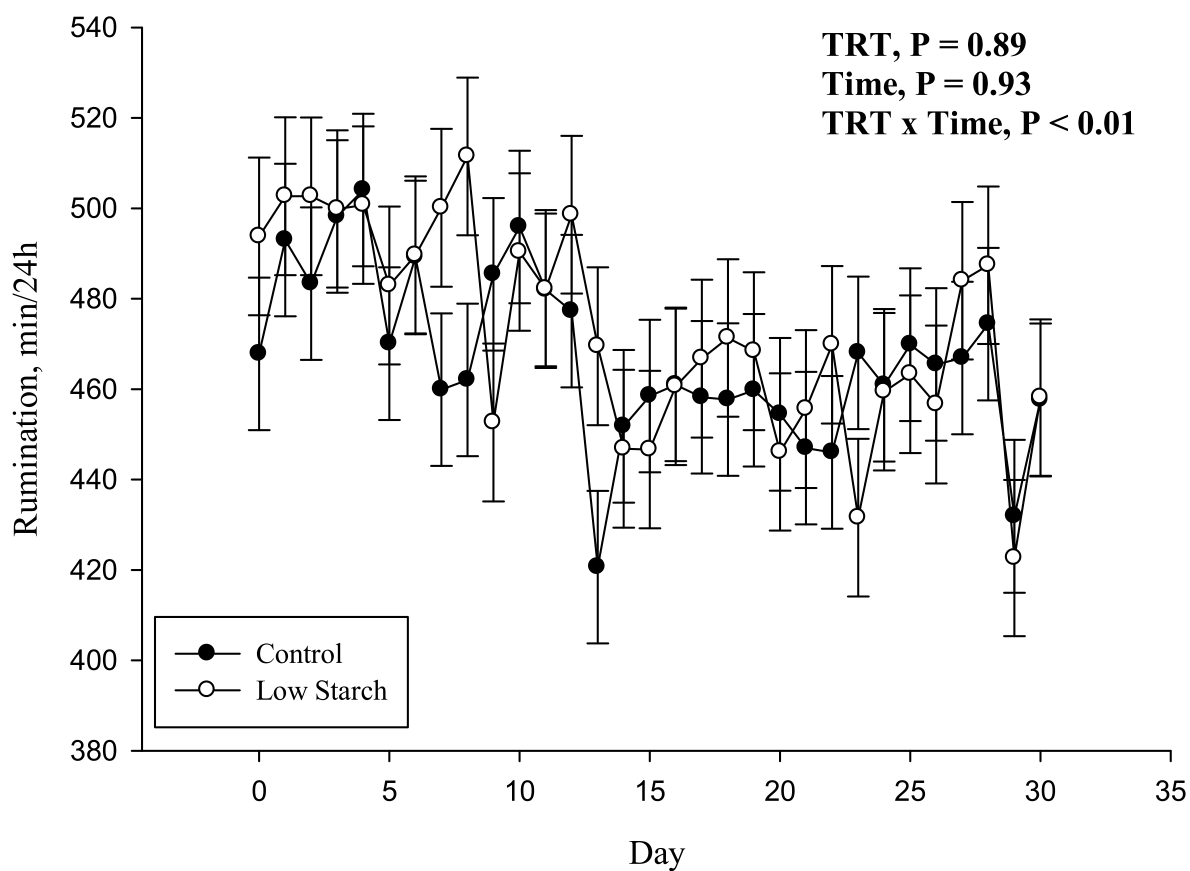

Figure 7. Rumination time. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.
* Significant values.

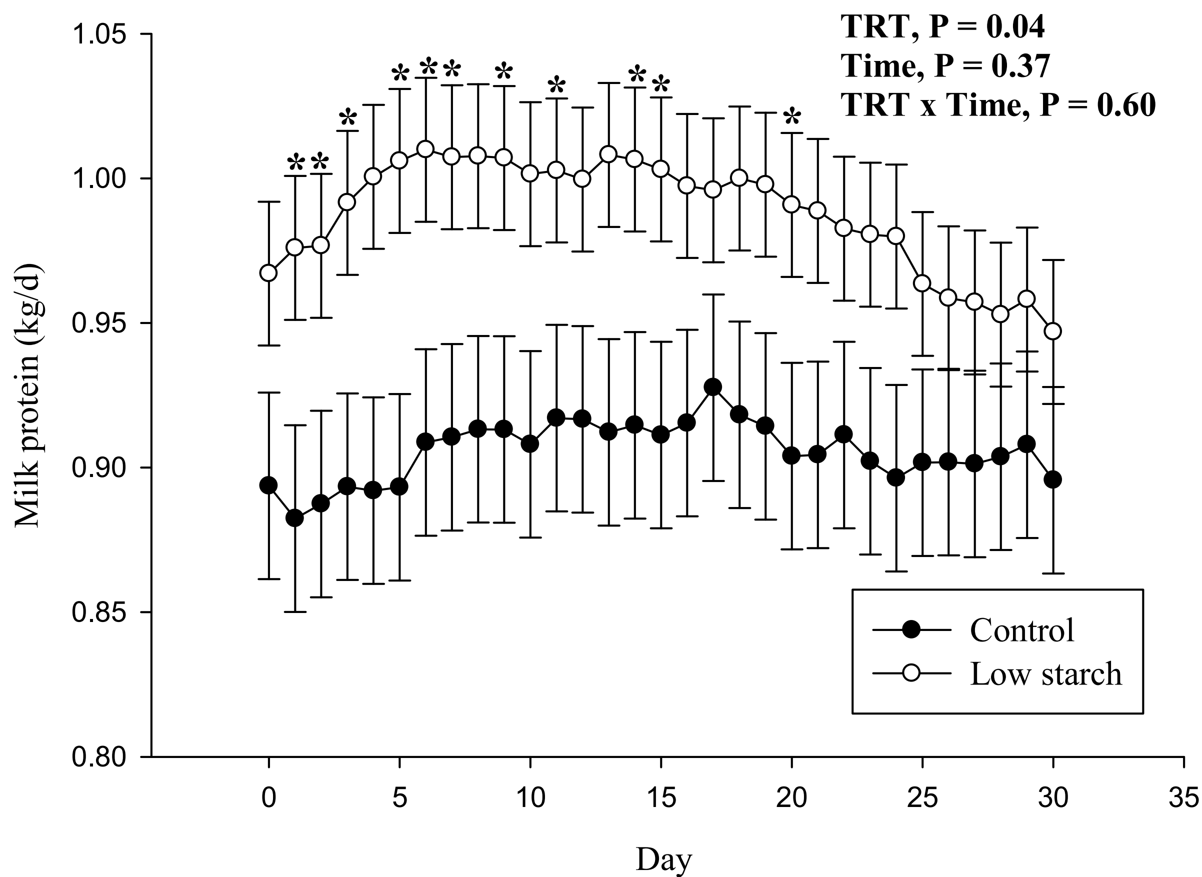

Figure 8. Milk protein yield of cows in late lactation. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.
*Significant values.

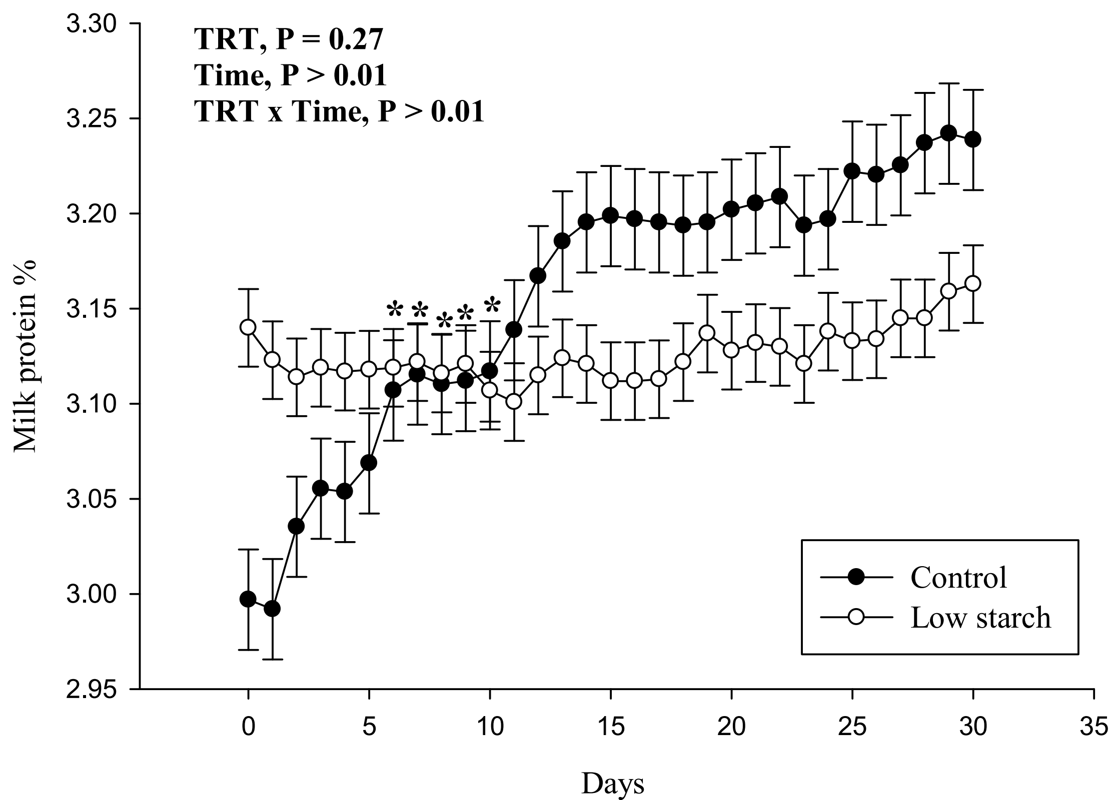

Figure 9. Milk protein % of cows in late lactation. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.
*Non-significant values.

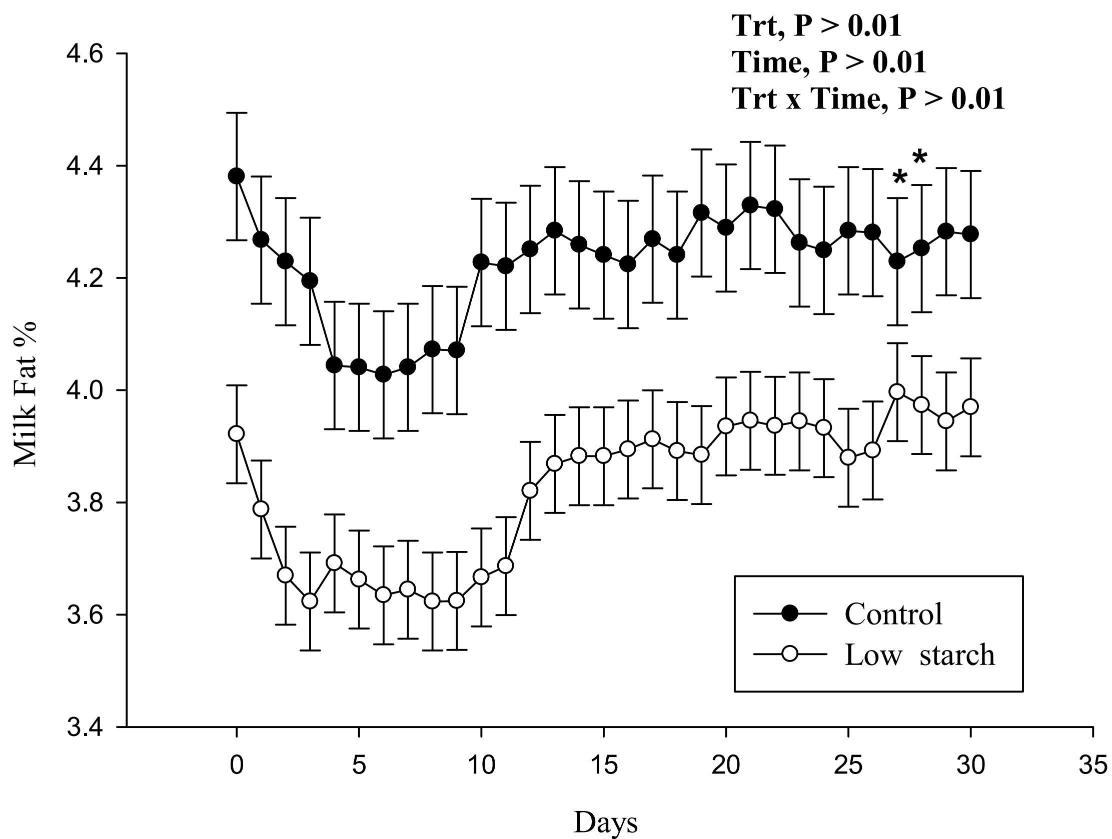

Figure 10. Milk fat % of cows in late lactation. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot. *Nonsignificant values.

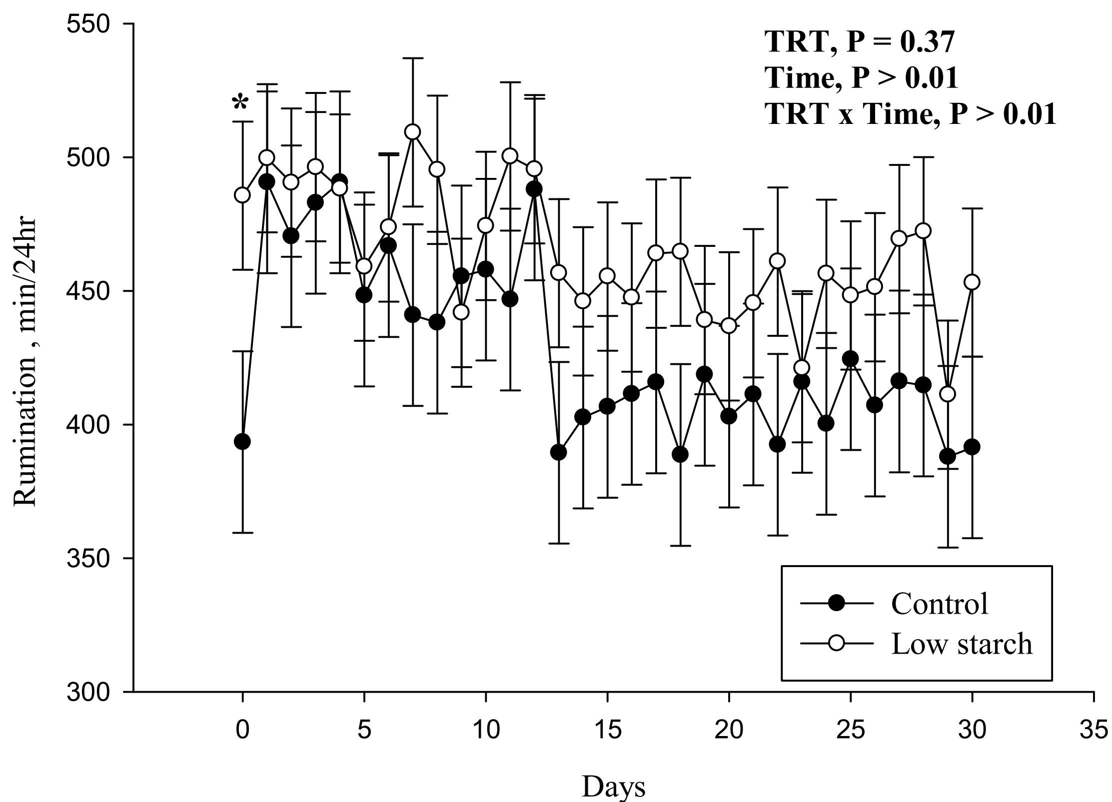

Figure 11. Rumination time of late lactation cows. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.
*Significant values.

CHAPTER 3: The Effect of a blend of EO on the stages of lactation

INTRODUCTION

The use of essential oils has been proposed as a non-antibiotic alternative to rumen modifiers such as Monensin, without the risk of developing antibiotic resistance. Besides dosage, diet, and ruminal pH, the physiological state of the animal may affect the efficacy of EO as a rumen modifier (Calsamiglia et al., 2007, Wall et al., 2014). In fact, Wall et al. (2014) detected a parity by treatment interaction in milk yield, where multiparous cows had greater milk yield than primiparous cows supplemented with 0.35g of EO (17% cinnamaldehyde and 28% eugenol; Pancosma SA, Geneva, Switzerland) and both multiparous and primiparous cows increased DMI. No change in milk production in the primiparous cows may be explained by the change in nutritional requirements due to the physiological state (growth vs. mature) as the cow progresses through several lactation cycles. The metabolizable energy from the increase in DMI in primiparous cows was likely diverted towards growth instead of milk synthesis, in contrast, multiparous cows used the metabolizable energy for milk synthesis. Similar to the growth effect explain above, the stage of lactation may also alter the potential effect of EO as a rumen modifier. As the cow progresses through the lactation cycle, energy requirements and the microbiome will likely change. At the initiation of lactation, the cow has a great demand for energy compared to the late lactation, since energy requirements are mainly driven by milk production, the changes in metabolism, and ruminal microbial populations during a lactation cycle may influence the potential effects of EO. Tassoul and Shaver (2009) reported an increase in milk production during 15 wk of lactation, along with an increase in feed efficiency during 56-98 DIM, in theory the increase in efficiency provided the

cow with an increase in energy allowing her to return to a positive energy balance faster resulting in more energy being diverted towards milk production quicker. The reason for the change in the EO potential to modify the microbial populations may also rely on the change in microbial populations throughout the lactation itself (Jewell et al., 2015).

Given the high diversity of EO types, optimal doses used for supplementation often differ and are less consistent across the literature. It has been demonstrated that the effects of EO may vary with dosage and chemical make-up. Castillejos et al. (2006) observed inconsistent results on rumen microbial fermentation when various EO and doses were evaluated. The inconsistent results on rumen fermentation parameters for various dosages are likely explained by the sensitivity and resilience of certain microbial populations in the rumen. McIntosh et al. (2003), observed various effects on the inhibition of growth on protein and carbohydrate digesting ruminal bacteria in pure culture. The growth of *Prevotella ruminicola*, *Clostridium sticklandii*, and *Peptostreptococcus anaerobius* was inhibited to a great extent with the inclusion of EO, however, *P. ruminicola* and *P. bryantii*, was able to adapt to the new environment and *Streptococcus bovis* even portrayed resistance to EO treatment. This suggests a highly variable range of sensitivity to EO, which only further expand the complexity for an effective manipulation of rumen microbes via EO supplementation.

In Chapter 2, we observed a particular effect of stage of lactation, where late lactation cows supplemented with SS in a low-starch diet produced more milk than the control group. Therefore, the objectives of the present study were to further investigate the effects of SS at different doses over the different stages of lactation (i.e., early, mid, and late). We hypothesize that EO at 2X (i.e., double recommended dose) the dose will

impact ruminal fermentation and kinetics in various stages of lactation differently *in vitro*.

MATERIAL AND METHODS

Experimental Design. The effects of SS on rumen fermentation and kinetics were evaluated in an in vitro batch fermentation system (Ankom Technology Corp., Macedon, NY). The experimental design was conceived as a 2 × 3 factorial arrangement of treatments, with two SS doses (i.e., single and double) and three stages of lactation (i.e., early, mid, and late) for treatment combinations of early-single (ES), mid-single (MS), late-single (L), early-double (ED), mid-double (MD), and late-double (LD). Rumen fluid (RF) was collected from 9 lactating multiparous Holstein cows (n = 3/stage of lactation) at early [days in milk (DIM) 32-36], mid (DIM 144-157), and late (DIM 277-290) lactation via esophageal tubing at approximately 4 h after feeding. The *in vitro* batch fermentation experiment was repeated three times on different days using the same 9 lactating multiparous Holstein cows. After sampling, the pH of each RF sample was measured immediately to ensure the purity of the sample. The RF from the 3 individual cows of each stage of lactation were mixed and strained through 4 layers of cheesecloth. Aliquots of 50 mL of strained RF were added to three 500 mL bottles containing a previously CO_2 gassed 200 mL of Mc Dougalls buffer (McDougall, 1947) and pre-warmed at 39°C. Each *in vitro* batch fermentation was performed in a shaking water bath (Cat#TSSWB27, Waltham, Massachusetts) at 39°C for 24 h. In order to determine experimental treatment effects on NDF and ADF digestibility, sufficient TMR and pellet containing SS, from the in vivo experiment, were ground (Wiley Mill # 3 and 1 micron).

Dacron (57 µm pore size, ANKOM, Macedon, NY) bags containing a total of 1 g of ground TMR and pellets [11% pellets and 89% TMR (i.e., single dose 220 mg/L) or 22% pellets and 78% TMR (i.e., double dose 440 mg/L)] were weighed and sealed using a heat impulse sealer (Cat# MP-8 Intertek). Then, 3 bags containing the same dose were placed in single bottle according to experimental design for later determination of NDF and ADF.

Sample Collection and Analysis. Gas production was measured as pressure per square inch (PSI) in each bottle every 10 minutes using the ANKOM gas production system (ANKOM, Macedon, NY). This system is equipped with gas pressure sensor modules that transmit data via radio frequency, to be recorded by the computer. Gas produced was converted into mL using the equation $V_x = V_j P_{psi} \times 0.068004084$ where V_x is the gas volume at 39° in mL, V_j is the headspace of the bottle in mL (500 mL) and P_{psi} is the pressure recorded by the gas monitor system software, moles of gas produced by $n = Vp / RT$, where n = quantity of gas in moles, P = pressure in kPa, V = volume gas occupied in L, T = temperature in Kelvin (K) and R = gas constant (8.314472 L/kPa·K/mol) and gas produced per 100 mg of substrate by the equation ml per 100mg = ml gas/(mg of substrate/100). Once the 24 h were completed, the Dacron bags containing the TMR and pellet were washed with cold water and dried for 24 h at 102°C, after drying bags were placed in a decanter for 20 minutes and then weighed for dry matter digestibility analysis. The analysis of NDF and ADF was conducted using the filter bag technique method (ANKOM, Macedon, NY). One mL of RF from each bottle was pipetted into a vial containing 0.2 mL of 25% meta-phosphoric acid and frozen at -20°C to be analyzed later

for volatile fatty acid (VFA) concentration. One mL of RF was pipette into a vial containing 20 µl of 50% sulfuric acid (H_2SO_4) and frozen at -20°C until analysis for ammonia N. For the analysis of the rumen fluid, the samples were thawed and centrifuged at 30,000 × g for 20 min at 20°C (Eppendorf 5403, Eppendorf North America, Hauppauge, NY). The NH_3 was analyzed by using Chaney and Marbach (1961) procedures using a colorimetric assay performed on a micro-plate spectrophotometer (Cary 50, Varian Inc., Walnut Creek CA). Rumen fluid samples preserved with metaphosphoric acid were prepared according to Hamada et al. (1968) methods, and acetate, propionate, butyrate, isobutyrate, isovalerate, and valerate concentrations were analyzed by using automated gas-liquid chromatography (model 6850, Agilent, Santa Clara, CA).

Statistical analysis. Data was analyzed using the PROC MIX procedure of SAS 9.4. The model contained treatment, time, and their interaction as a fixed effects and replicate nested within treatment and batch as random. Statistical differences were declared at $P \leq 0.05$ and tendencies at $P \leq 0.15$.

RESULTS

Single dose. Gas production measurements are presented in Table 8. A significant interaction of ($P = 0.02$) TRT × Time was observed for gas production, however; these effects can be associated with transient changes over time ($P < 0.01$) rather than treatment differences over time. Ruminal fermentation parameters and fiber digestibility

determined after the *in vitro* batch fermentation are presented in Table 9. A treatment effect was observed in pH ($P = 0.02$), where L had the greatest pH, followed by ES and MS had the lowest pH. A contrasting decrease ($P < 0.01$) in NH_3-N concentration with an increase ($P < 0.01$) in propionate % was observed for the ES treatment in comparison to MS and LS. The MS treatment had an increase ($P < 0.01$) in butyrate and valerate concentration. The LS treatment had a decrease ($P < 0.01$) in propionate concentrations and the greatest acetate: propionate ration, however, it was only statistically greater than the ES treatment. No treatment differences ($P \geq 0.10$) were observed for acetate and isovalerate concentrations, total VFA production, percentages of acetate, butyrate, isovalerate and valerate, or NDF and ADF digestibility.

Double dose. Gas production measurements are presented in Table 10. Gas production parameters were not affected ($P \geq 0.15$) by any experimental effects. Ruminal fermentation and digestibility parameters are presented in Table 11. The pH ($P = 0.04$) with LD having the highest pH, followed by ED and MD having the lowest pH. There was an evident linear increase ($P < 0.01$) in acetate and isovalerate concentrations from early (ED) to late (LD) stage lactation. Similarly, to acetate, there was a linear increased ($P < 0.01$) over stage of lactation effect on acetate: propionate ratio. Butyrate concentration was greatest ($P < 0.01$) on the MD treatment in comparison to ED and LD. Similarly to butyrate, the MD treatment had the greatest ($P < 0.01$) NH_3-N concentration, followed by LD, and ED treatment had the lowest concentration. There was a treatment effect ($P < 0.01$) on valerate %, where ED had the greatest concentration, followed by LD, and MD had the lowest concentration. No treatment effects ($P > 0.05$) were observed

for propionate, valerate, and total VFA mM concentrations as well as % of acetate, propionate, and butyrate, NDF and ADF digestibility.

Dose comparison. Gas production parameters and ruminal fermentation and digestibility parameters are presented in Table 12 and Table 13, respectively. All gas production parameters presented a dose effect ($P \leq 0.05$), where overall the single dose of EO had greater gas production measured in mL, Moles, and mL/100 mg of substrate than a double dose of EO. Significant dose effect was observed for mL ($P = 0.01$), mol ($P = 0.02$) and mL/100mg ($P = 0.03$) of substrate, acetate ($P > 0.01$), acetate: propionate ratio ($P = 0.03$), and valerate percentage ($P = 0.02$). No dose effects were observed for pH, NH_3-N concertation, propionate, butyrate, isovalerate, and valerate concentrations, acetate, ad butyrate percentages, or NDF and ADF digestibility.

DISCUSSION

Gas production and Fiber digestion. Gas production measured during *in vitro* batch fermentation experiments has been used as an indicator of microbial activity, and in this instance, gas production was not different among treatments for both single and double doses. Similar to gas production, fiber digestibility in terms of NDF and ADF, was not affected by experimental effects, this is consistent with the lack of experimental effects observed in gas production. These findings are in agreement with Benchaar et al. (2007) who reported a decrease in gas production when several types of EO such as eugenol (800 mg/L^{-1}), thymol (200 mg/L^{-1}) and carvacrol (400 mg/L^{-1}) were tested with an *in*

vitro batch fermentation system. In the same study, Benchaar et al. (2007) observed a decrease in NDF digestibility among all of the EO used, which is in disagreement with our results. The decrease in digestibility was attributed to fibrolytic bacteria being more sensitive to EO supplementation especially those rich in phenolic compounds (i.e., eugenol, thymol, and carvacrol). The high concentrations of phenolic compounds exhibited broad spectrum antimicrobial activity resulting in overall inhibition of the fermentation process (Benchaar et al., 2007). The pH of the bottles remained at values (6.64-6.87) that support optimal fiber digestion even though there was a lack of experimental effects on NDF and ADF digestibility, in fact, the latter could be associated with pH differences observed being less biological significant. Benchaar et al. (2007) reported an increase in pH with the addition of carvacrol (400 mg L) eugenol (800 mg L^{-}), thymol (200 mg L) and cinnamon oil (400 mg L) which is in agreement with Busquet et al. (2006) who observed the addition of 300 and 3,000 mg/L of carvacrol and 3,000 mg/L of eugenol the pH increased. While the addition of eugenol and carvacrol at 3, and 30 mg/L had no effect on pH but at the rate of 300 and 3,000 mg/L for carvacrol and 3,000 mg/L of eugenol the pH increased. Patra and Yu (2012) reported a linear increase of the pH when clove oil, eucalyptus oil, garlic oil, origanum oil, and peppermint oil were added in doses of 0.10, 0.25, and 1 g/L.

Volatile fatty acids and NH_3-N. The SS supplementations showed mixed effects on the concentrations and proportions of VFA. In the current study acetate production with a single dose of EO (1,320 mg/L of pellets) was greater than that of the double dose (2,640 mg/L of pellets) while propionate, and butyrate were not affected by the dosage rate. Busquet et al. (2006) observed an increase in acetate production with the addition of

3,000 mg/L of cinnamon oil while lower doses (300 mg/L) of carvacrol and clove bud oil decreased acetate production, propionate production increased with the addition of benzyl salicylate (300, 3,000 mg/L), carvacrol, cinnamaldehyde and eugenol (300 mg/L), the addition of 3,000 mg/L of anise, carvone and tea tree oil decreased propionate production. Butyrate production increased with the addition of anethol and benzyl salicylate (300, 3,000 mg/L) and decreased in the presence of 3,000 mg/L of carvacrol and eugenol in Busquet et al. (2006) study. The acetate: propionate ratio was increased with the addition of a single dose of SS compared to the double dose. Castillejos et al. (2006) reported the addition of thymol (500 mg/L) increased the acetate: propionate ratio while the addition of eugenol (5 mg/L) decreased the acetate: propionate ratio. Busquet et al. (2006), reported no change in total VFA concentrations with the addition of cade, capsicum, dill weed, ginger oils, and yucca and fenugreek extracts at the rate of 3,000mg/L *in vitro*. This is not in agreement with the current study, where the addition of a high rate of SS tended to decrease the total VFA production. The addition of a single dose of SS had no effect on total VFA concentrations which is also in disagreement with Busquet et al. (2006) who reported the addition of anethol, carvacrol, clove bud oil and oregano oil at 300 mg/L decreased the total VFA concentrations.

The single dose of this particular blend of EO had positive effects on rumen fermentation by increasing propionate concentration and percentage and decrease in NH_3-N concentrations when compared to the double dose of SS. The increase in propionate *in vivo* would provide the cow with an increase in energy when she is in a negative energy balance, and the decrease in NH_3-N concentrations suggest lower deamination of AA and

overall lower rumen degraded protein resulting in more metabolizable protein from dietary crude protein going to the host. The TRT × dose interact for acetate with the two doses when compared also indicates the signal dose would be better for cows in mid to late lactation because of the greater amount of acetate even though a significant increase in acetate was observed for the double dose the mean production for the double dose was lower when compared to the single dose. The overall decrease in acetate in the double dose treatment would be conducive to depressed milk fat levels and may also indicate the inhibition of starch-digesting bacteria leading less propionate production ultimately resulting in a decrease in glucose levels and decreased milk yield. The increase in acetate production with the double dose also shows the potential for an increase in growth of fiber-digesting bacteria which could increase feed efficiency. The increase in butyrate levels with both doses used would be beneficial to the cow because butyrate is the main energy source for cells in the gut, this increase could also be beneficial to feed efficiency because the cells would have more energy to use for digestion and absorption. The SS has proven its ability to alter ruminal fermentation, the single dose had the most positive effects when compared to the double dose, but the double dose showed the potential to increase feed efficiency. Further research into an intermediate dose may prove to be beneficial.

CONCLUSION

The findings of this study reveal that overall gas production and rumen kinetics are altered by the stage of lactation at any dosage of SS. However, the TRT × Time effect observed in the single dose of SS on gas production suggests that this dosage level is less

detrimental to microbial activity. This effect is partially supported by the greater total VFA production in the single dose of SS than double. Interestingly, the differential patterns observed in propionate and acetate when rumen microbes were incubated with a single and double dosage of SS, respectively, indicate that this factor can have a fundamental effect on microbial activity and consequently rumen functionality throughout the lactation. Therefore, this effect should be further investigated and doses optimized in order to shift rumen population to account for the changes in rumen population throughout the stages of lactation. Further research should be done to evaluate dosage effects of single or other blends of EO best suited for each stage of lactation. The reason for the changes in microbial populations are still not understood well and thought to be not only due to changes in diet but also other host factors.

Table 8. The effect of a single dose of SS on gas production.

	Treatment				*P*		
Parameter	ES	MS	L	SEM[1]	Treatment	Time	Trt × Time
Ml	201.61	195.69	187.26	9.16	0.54	< 0.01	0.02
Mole	201.72	195.80	187.36	9.16	0.54	< 0.01	0.02
mL/100mg	6.69	6.50	6.39	0.23	0.66	< 0.01	0.02

[1]Largest standard error of the mean.

Table 9. Effects of a single dose of SS on ruminal fermentation and fiber digestibility.

	Treatment				*P*
Parameter	ES	MS	L	SEM[1]	Treatment
Acetate, mM	44.33	44.21	42.37	1.85	0.71
Propionate, mM	28.43[a]	26.43[a]	23.30[b]	0.75	< 0.01
Butyrate, mM	9.24[b]	10.55[a]	9.59[b]	0.26	< 0.01
Isovalerate, mM	1.65	1.92	1.78	0.08	0.10
Valerate, mM	1.79[b]	1.94[a]	1.72[b]	0.03	< 0.01
A : P	1.56[b]	1.70[ab]	1.84[a]	0.05	< 0.01
Total VFA, mM	86.33	97.52	102.15	9.18	0.47
Acetate, %	51.10	47.78	45.92	2.83	0.44
Propionate, %	33.18[a]	28.55[b]	25.37[b]	1.48	< 0.01
Butyrate, %	10.71	10.37	11.40	0.60	0.48
Isovalerate, %	1.92	2.11	2.02	0.17	0.73
Valerate, %	2.09	2.09	1.86	0.11	0.27
NH_3-N, mM	0.91[b]	1.04[a]	0.97[a]	0.01	< 0.01
pH	6.75[b]	6.72[c]	6.77[a]	0.01	0.02
NDF, %	28.64	28.76	27.97	0.37	0.30
ADF, %	18.48	18.50	18.18	0.24	0.53

[1]Largest standard error of the mean.

Table 10. The effect of a double dose of SS on gas production.

	Treatment				*P*		
Parameter	ED	MD	LD	SEM[1]	Treatment	Time	TRT × Time
Ml 1	183.13	173.94	184.17	8.06	0.62	< 0.01	0.22
Mol	183.23	174.04	184.27	8.07	0.62	< 0.01	0.22
ML/100mg	6.08	5.78	6.32	0.20	0.18	< 0.01	0.15

[1]Largest standard error of the mean.

Table 11. Effects of double dose of SS on ruminal fermentation and fiber digestion parameters.

	Treatment				P
Parameter	ED	MD	LD	SEM[1]	
Acetate, mM	34.06[c]	41.81[b]	41.96[a]	1.03	< 0.01
Propionate, mM	25.63	25.45	24.57	0.81	0.62
Butyrate, mM	8.31[b]	9.95[a]	9.74[b]	0.17	< 0.01
Isovalerate, mM	1.54[c]	1.85[b]	2.04[a]	0.06	< 0.01
Valerate, mM	1.80	1.87	1.77	0.04	0.17
A: P	1.33[c]	1.67[b]	1.74[a]	0.04	< 0.01
Total VFA, mM	71.33	93.90	81.70	6.52	0.75
Acetate, %	47.70	47.64	51.46	1.94	0.31
Propionate, %	35.96	29.92	28.90	1.28	0.42
Butyrate, %	11.66	11.42	11.95	0.42	0.67
Isovalerate, %	2.16[c]	2.18[b]	2.51[a]	0.11	0.07
Valerate, %	2.53[a]	2.14[c]	2.17[b]	0.09	< 0.01
NH_3-N, mM	0.92[c]	1.02[a]	0.99[b]	0.01	< 0.01
pH	6.75[b]	6.72[c]	6.76[a]	0.01	0.04
NDF	27.96	28.37	27.74	0.26	0.33
ADF	18.06	18.31	17.82	0.19	0.20

[1]Largest standard error of the mean.

Table 12. The effect of a single and double dose of SS on gas production in an *in vitro* system.

	Treatment and Dose			*P*		
Parameter	Single	Double	SEM[1]	Dose	Time	TRT × Time
Ml	199.00	18502	3.86	0.01	< 0.01	0.50
Mol	199.11	185.12	4.21	0.02	< 0.01	< 0.01
ML/100mg	11.13	10.58	0.19	0.05	< 0.01	< 0.01

[1]Largest standard error of the mean.

Table 13. Comparison of ruminal fermentation characteristic between single and double dose of SS.

Parameter	Treatment		SEM[1]	*P*
	Single	Double		Dose
pH	6.75	6.74	0.01	0.81
NH_3-N	0.97	0.97	0.01	0.97
Acetate, mM	43.64	39.24	0.93	< 0.01
Propionate, mM	26.06	25.21	0.70	0.40
Butyrate, mM	9.79	9.99	0.16	0.40
Isovalerate, mM	1.79	1.81	0.05	0.74
Valerate, mM	1.81	1.81	0.03	0.98
A : P	1.70	1.58	0.04	0.03
Total VFA, mM	95.33	82.31	4.95	0.07
Acetate, %	48.27	48.93	1.49	0.75
Propionate, %	29.04	31.59	1.03	0.09
Butyrate, %	10.83	11.67	0.35	0.10
Isovalerate, %	2.28	2.28	0.09	0.05
Valerate, %	2.01	2.28	0.09	0.02
NDF	28.38	27.84	1.83	0.84
ADF	18.40	17.95	1.17	0.78

[1]Largest standard error of the mean.

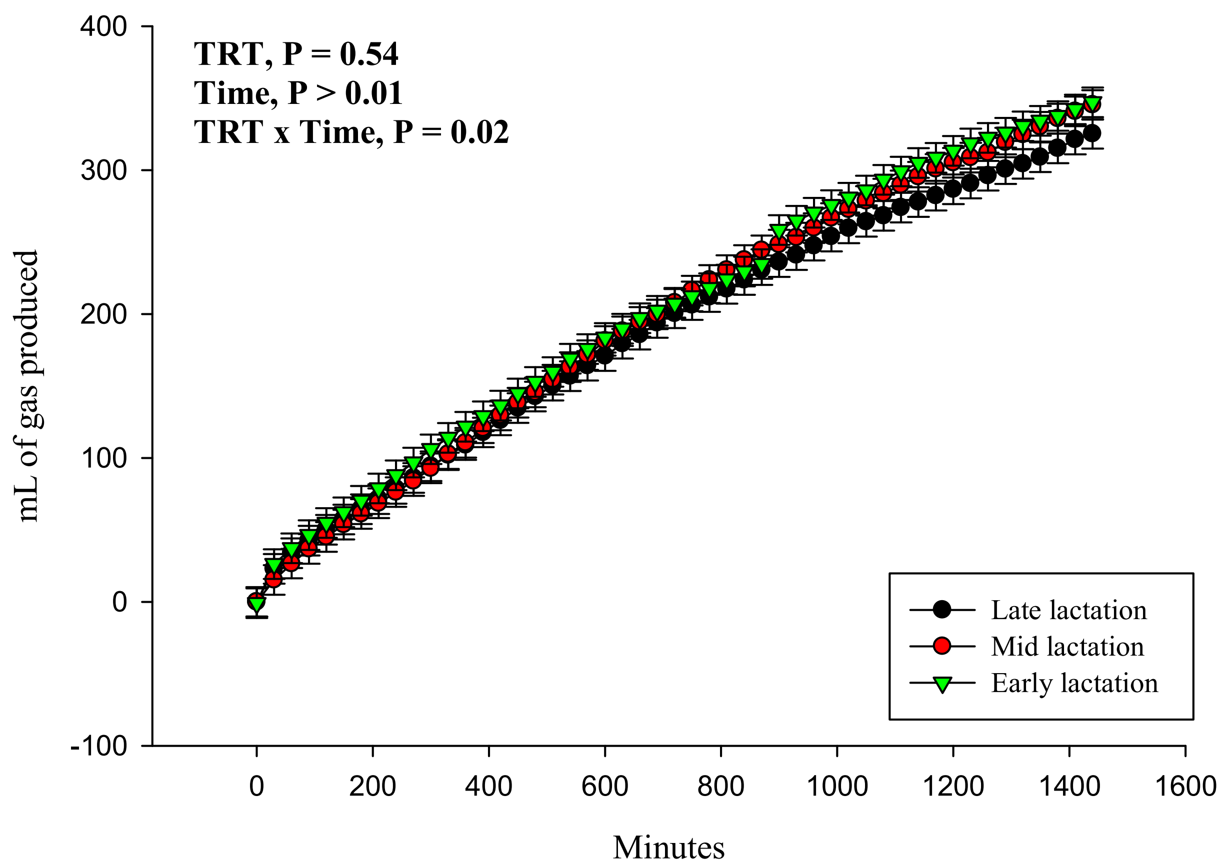

Figure 12. The effect of a single dose of SS on mL of gas produced. Over 24-hour incubation in vitro batch fermentation. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.

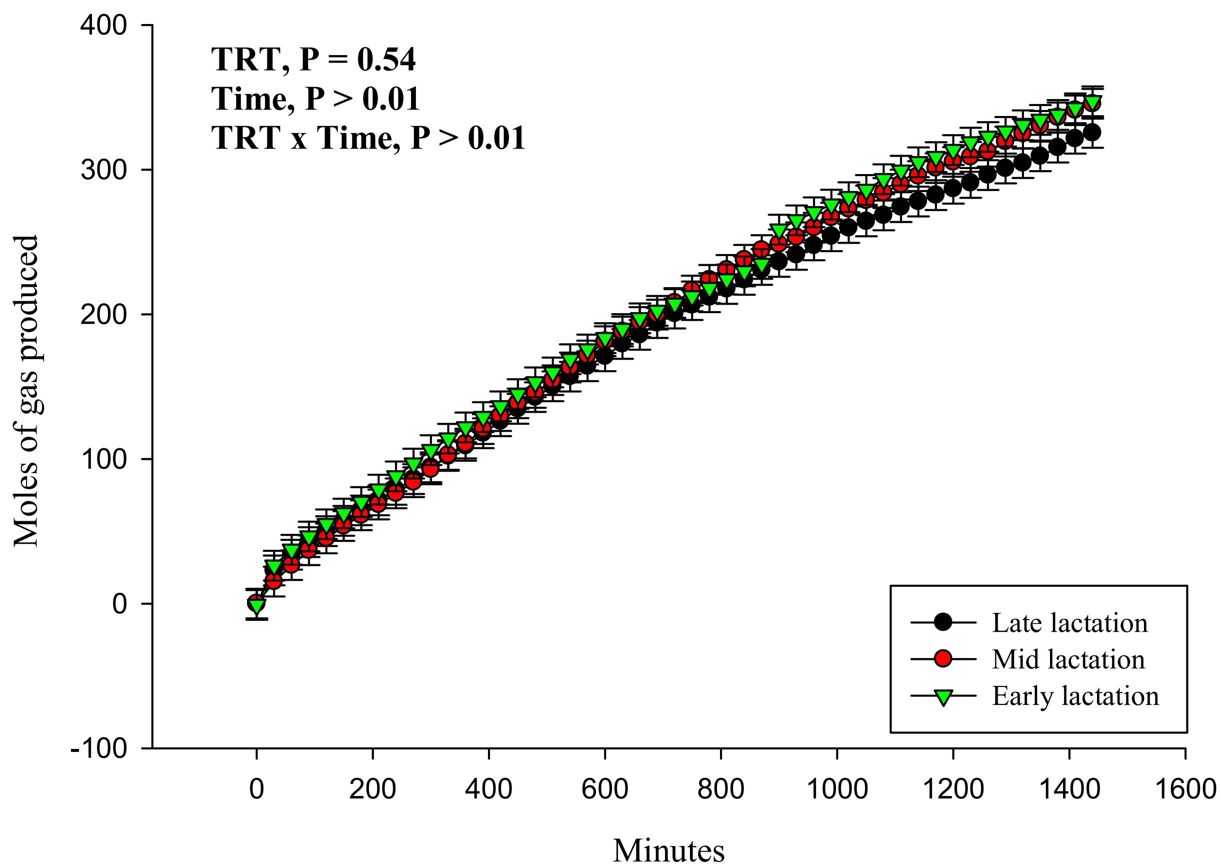

Figure 13. The effect of a single dose of SS on moles of gas produced. Over 24-hour incubation in vitro batch fermentation. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.

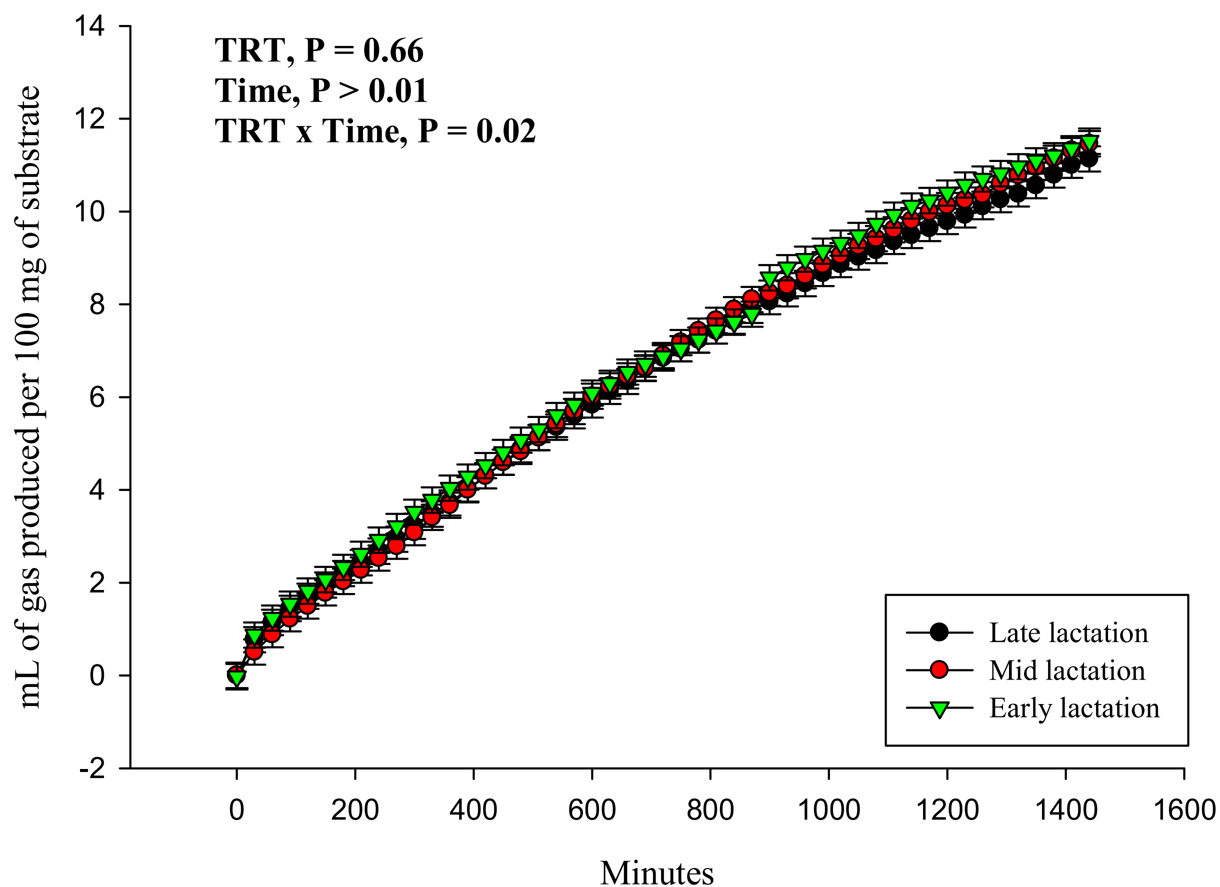

Figure 14. The effect of a single dose of SS on mL of gas produced per 100 mg of substrate. Over 24-hour incubation in vitro batch fermentation. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.

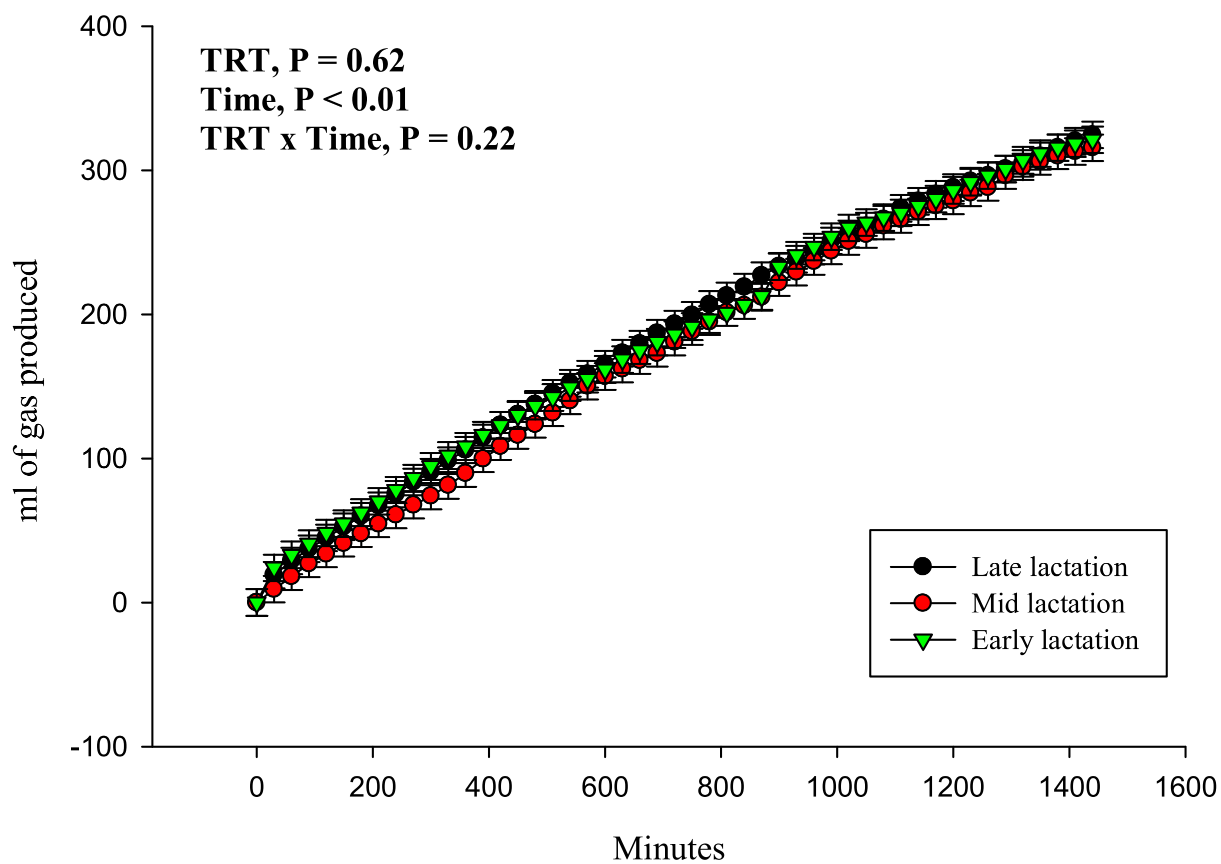

Figure 15. The effect of a double dose of SS on mL of gas produced. Over 24-hour incubation in vitro batch fermentation. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.

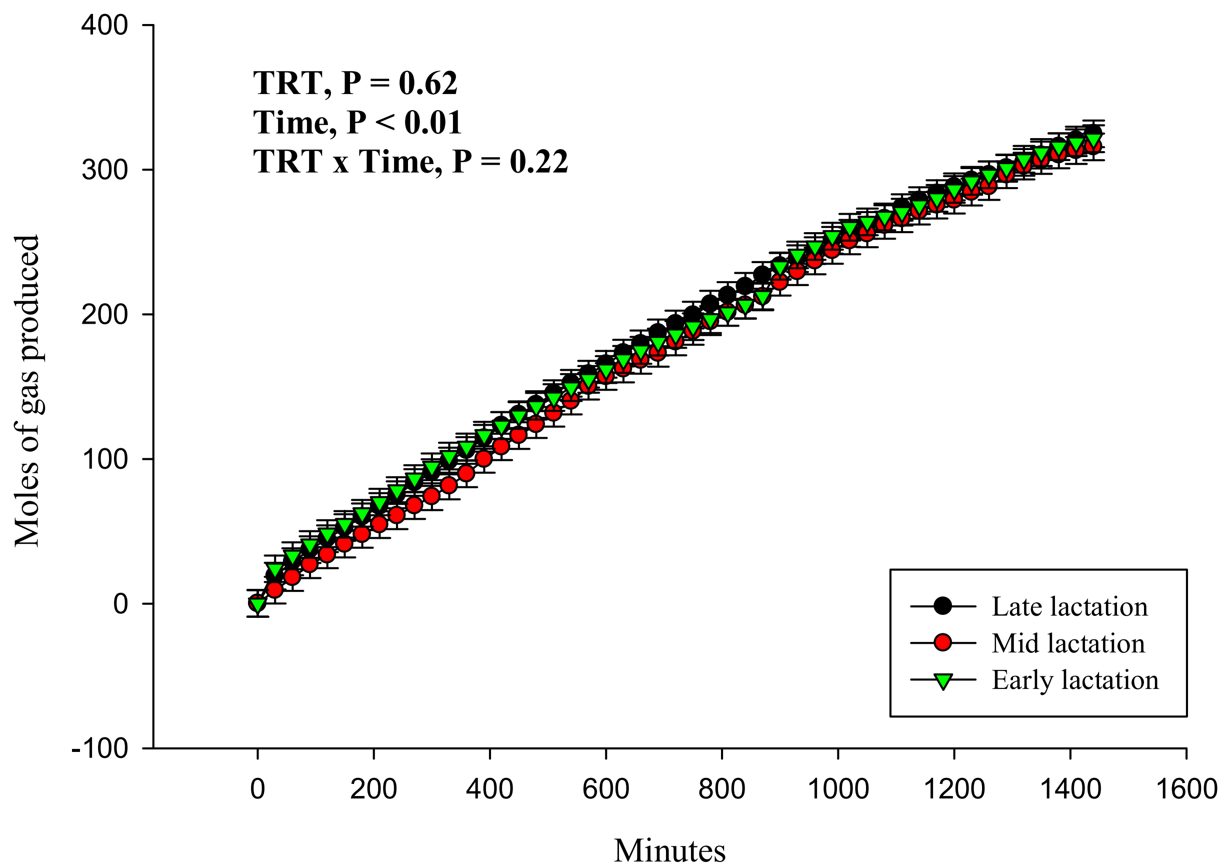

Figure 16. The effect of a double dose of SS on moles of gas produced. Over 24-hour incubation in vitro batch fermentation. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.

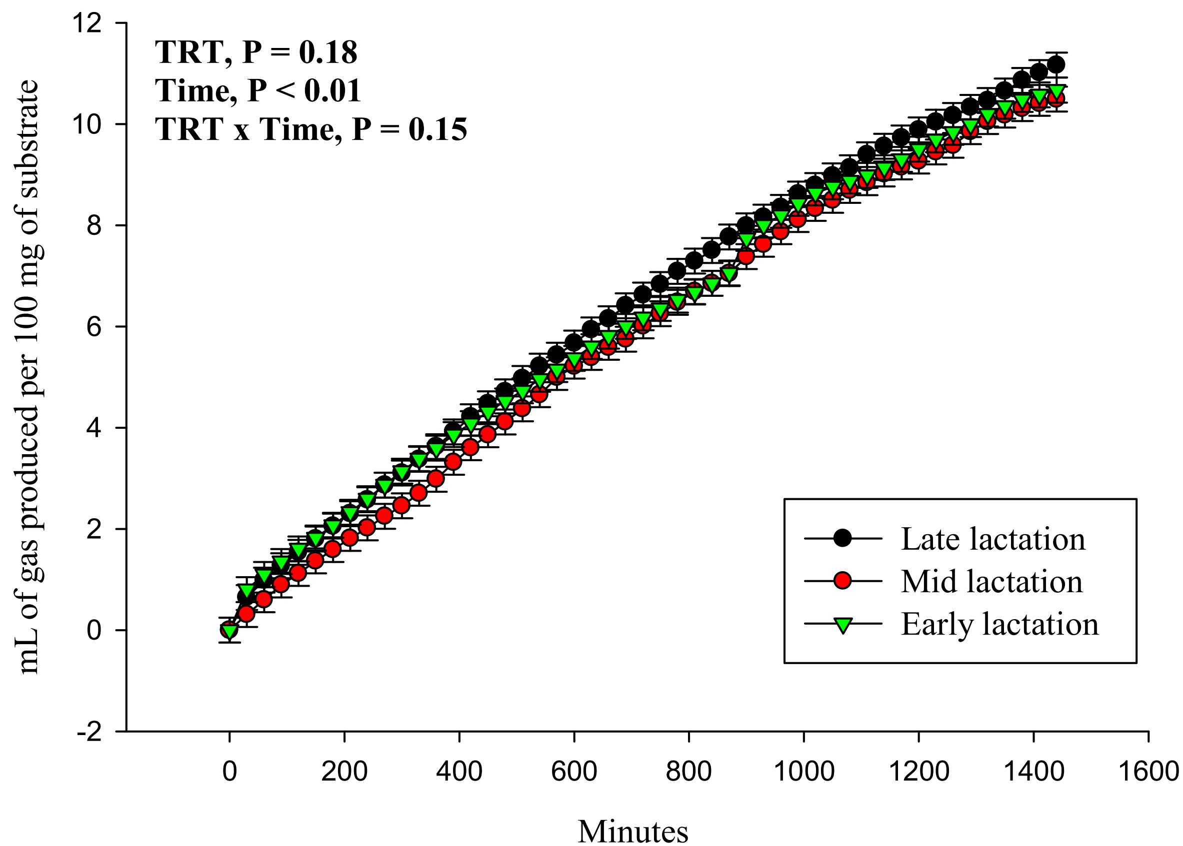

Figure 17. The effect of a double dose of SS on mL of gas produced per 100 mg of substrate. Over 24-hour incubation in vitro batch fermentation. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.

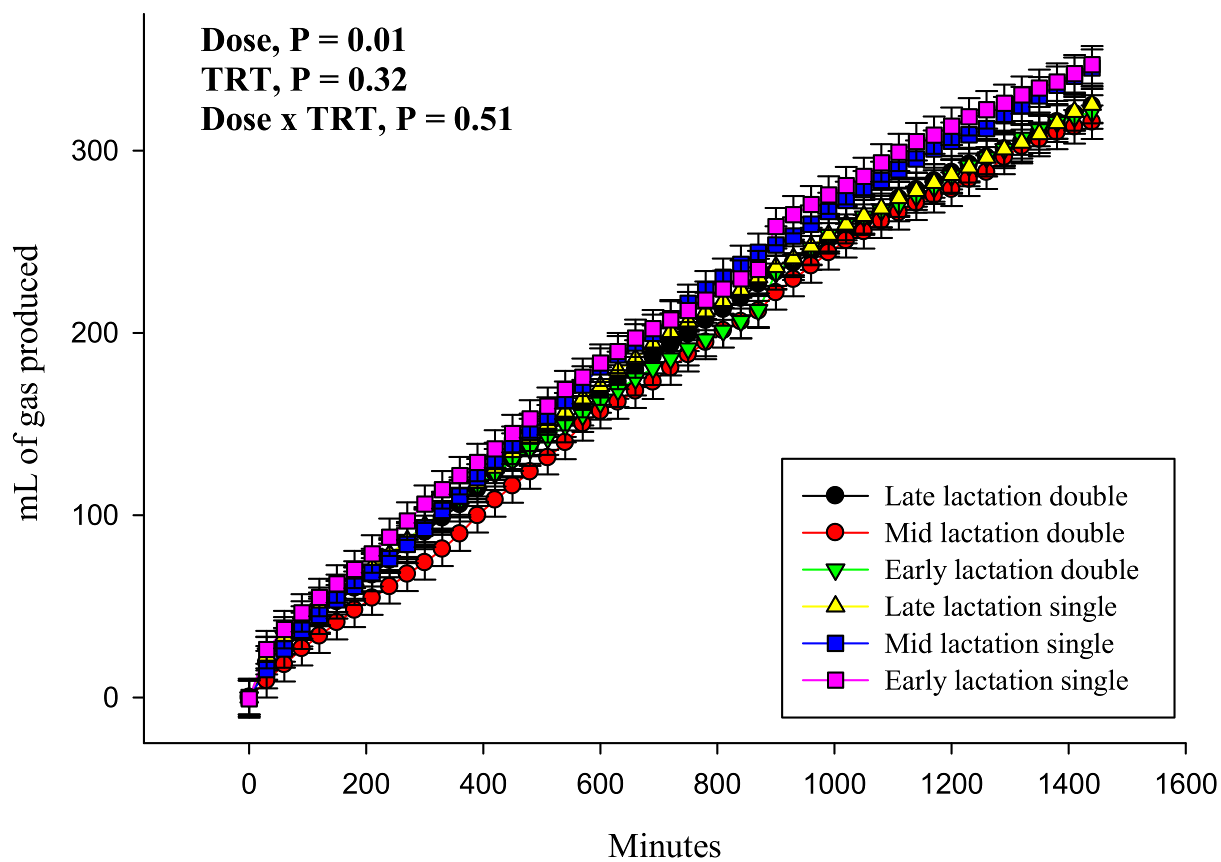

Figure 18. The effect of dose on mL of gas produced. Over 24-hour incubation in vitro batch fermentation. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.

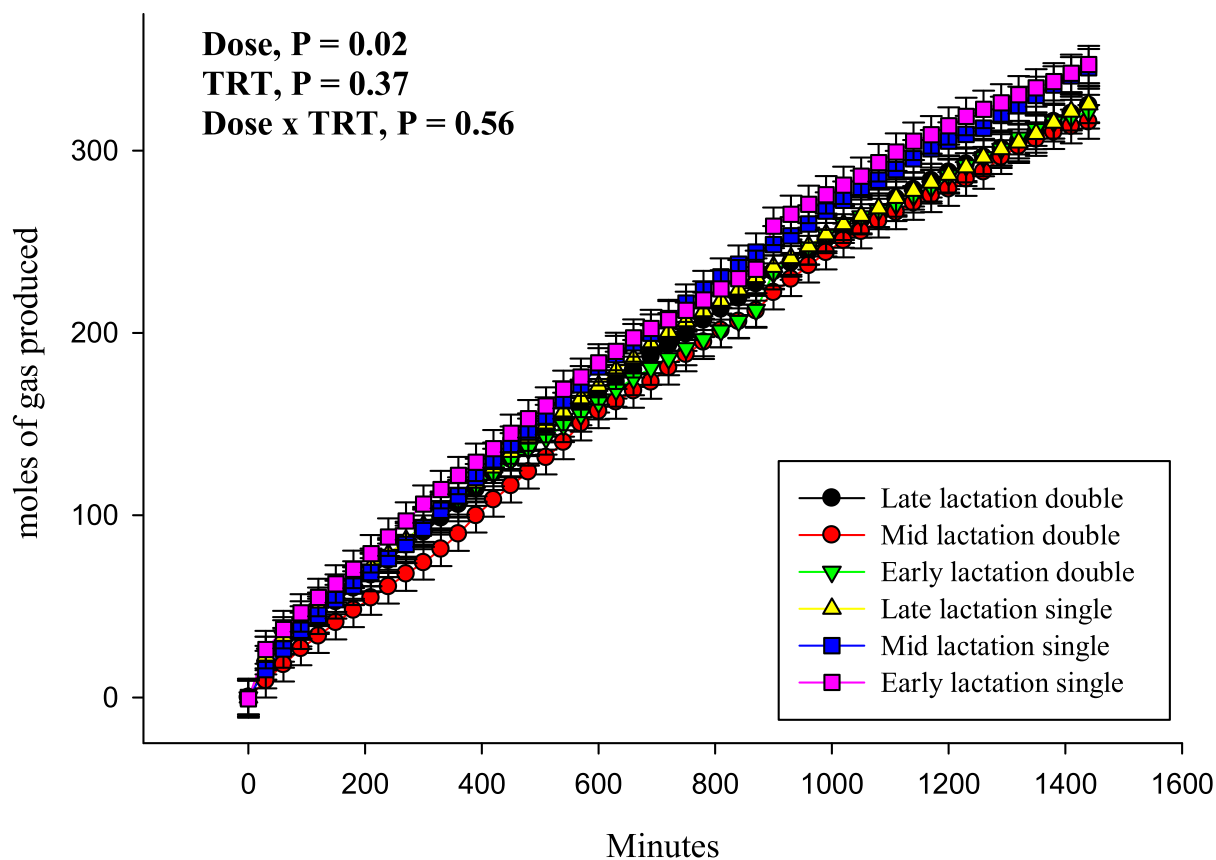

Figure 19. The effect of dose on moles of gas produced. Over 24-hour incubation in vitro batch fermentation. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.

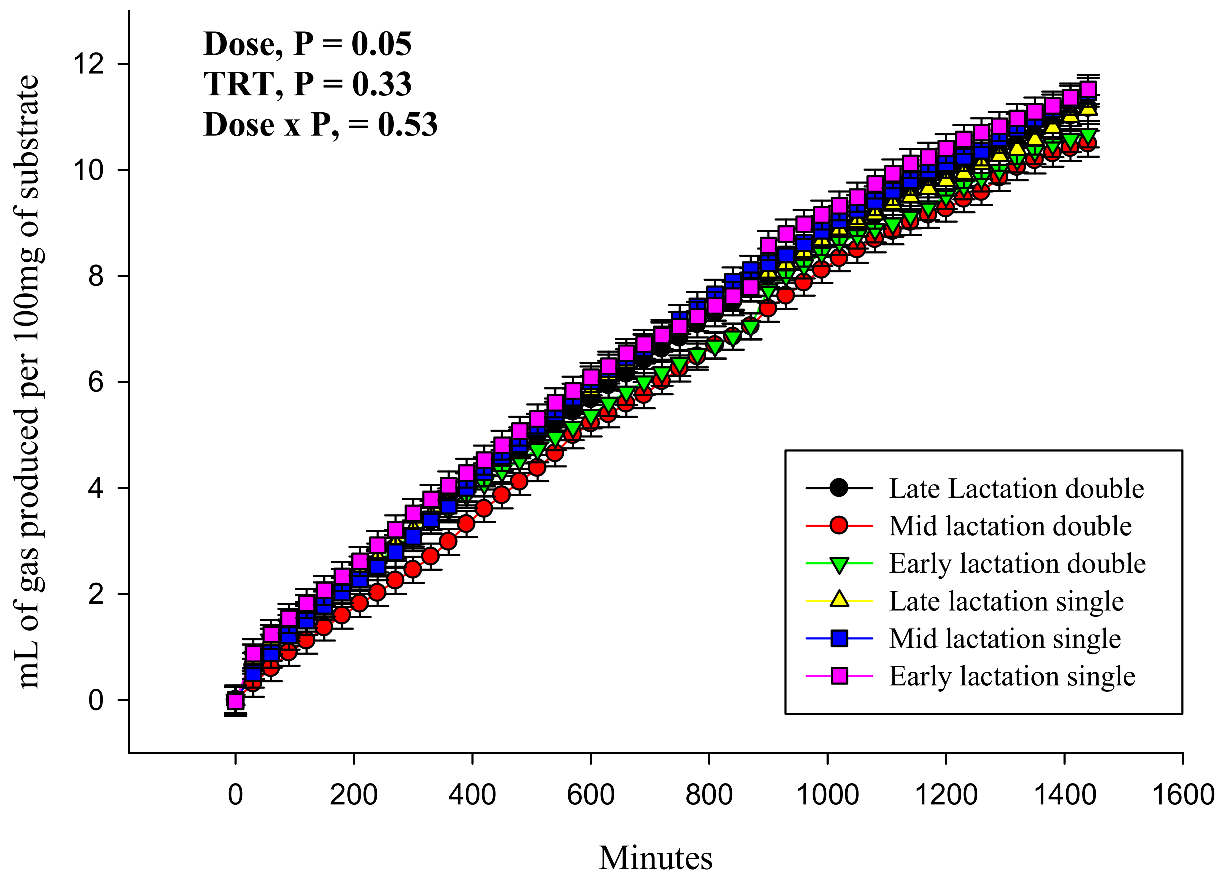

Figure 20. The effect of the dose of SS on mL of gas produced per 100 mg of substrate. Over 24-hour incubation in vitro batch fermentation. The values are means with standard errors represented as vertical bars. The P-values for the main effects are shown in the plot.

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