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THE EFFECTS OF FEEDING PLANT MATERIALS ON FEED EFFICIENCY AND GAS EMISSIONS IN DAIRY CATTLE

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ABSTRACT

Essential oils, due to their antimicrobial effects, have the potential to alter ruminal fermentation patterns through the inhibition of specific microorganisms. Alterations to ruminal fermentation could increase the efficiency of energy and nitrogen metabolism. In this study, over 60 essential oils native to the United States were analyzed in vitro using a batch culture incubation. From these results, one plant material, oregano, was then selected to be examined in the in vivo portion of the study. Oregano was supplemented at 500 g/cow/day to 8 multiparous Holstein cows, 4 of which were rumen cannulated, in a 4x4 Latin square design. The trial was a switch-over design with periods of 21 days each, for 14 days of adaptation and 7 days of sampling. Effects on milk yield, milk composition, ruminal fermentation, urinary nitrogen losses, urinary and fecal emissions, and total tract digestibility of nutrients were measured. In vitro incubations demonstrated that ruminal methane production was significantly reduced and NDF digestibility was improved for the plant material *Origanum vulgare*, therefore it was selected for in vivo experimentation. In the vivo experiment, *Origanum vulgare* significantly decreased ruminal methane production, illustrating the potential for improved energy efficiency. However, ammonia production within the rumen was significantly increased, suggesting that oregano supplementation may alter the metabolism of some bacteria in the rumen such that their capture of nitrogen is reduced.

TABLE OF CONTENTS

Introduction……………………..……………………………………………………….…...1-24

Secondary Plant Metabolites……………………………...……………………....…5

Essential Oils………………………………………………………………………….....10

Garlic Oil……………………………………………………………………...10

Thymol………………………………………………………………………...13

Eugenol………………………………………………………………………......14

Cinnamaldehyde……………………………………….……………………...15

Anethol……………………………………………………………………...17

Capsaicin………………………………………………………………………....18

Other essential oils…………………………………………………………….19

Supplemental Combinations of Essential Oils……………………………………...21

Materials and Methods…………….……………………………………………….…25-33

In vitro experimental protocol and experimental design……………………………...25

In vivo experimental protocol and experimental design……………………………....29

Results………………………………………………………………………………..34-54

In vitro experimental results…………………………………………………………..34

In vivo experimental results…………………………………………………………...48

Discussion……………...……………………………………………………………….55-65

Essential oil and plant material in vitro screening……………………………………….55

Oregano supplementation in vivo……………………………………………………..60

Overview……………………………………………………………………………….64

Literature Cited……..…………………………………………………………………….66-69

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INTRODUCTION

In the dairy industry, efficiency of the dairy operation is critical to maintain a competitive edge. Since feed costs can make up as much as 60 percent of the total costs on an operation, opportunities exist to enhance feeding strategies for dairy cows. One example has been the use of ionophores to decrease the energy and protein losses of the animals, allowing for better health and production (Calsamiglia et al., 2007). However, in January 2006, the European Union instituted a ban on the usage of ionophores. The rationale was that ionophore use in rations may contribute to antibiotic resistance in ruminal microorganisms and have some health implications for dairy products (Call et al., 2008; Calsamiglia et al., 2007). Therefore even in the United States, the use of alternative feed supplements that are used for human consumption have been the subject of many recent studies. One focus of these studies has been the supplementation of essential oils to the diets of dairy cattle. Since many of these oils have shown properties of interacting with the cell membranes of select gram positive and negative rumen bacteria, they have the ability to reduce losses of energy and protein by the cow by inhibiting methanogenesis and deamination (Calsamiglia et al., 2007). Many essential oils are classified as generally recognized as safe (GRAS) by the Food and Drug Administration, making supplementation a likely possibility (Castillejos et al., 2006).

A large amount of microbial digestion and processing of food occurs in the rumen of dairy cattle. The rumen microbes digest carbohydrates such as cellulose and hemicellulose. The rumen microbes are very important because they can provide up to 50 to 60 percent of the animal's protein and energy needs. The end products of ruminal fermentation of carbohydrates are volatile fatty acids (VFAs); specifically propionate, acetate, and butyrate, which are absorbed by the ruminant for further energy usage in the body. Of all these products, propionate is the

primary VFA used for gluconeogenesis in the liver. As all the cells of the body have glucose requirements for optimum functioning and not VFA requirements, it is essential that gluconeogenesis is supported through the ruminal production of propionate, one of the main glucose precursors. The production of propionate, a product of gram negative bacteria, is correlated with higher glucose availability for cells, thus providing more energy for cell processes and more nutritional efficiency for the cow. Another VFA of great interest, acetate can also serve as an energy source, but is predominantly used in energy storage through fatty acid synthesis. Notably, many gram positive bacteria are involved in the production of acetate as well as butyrate (Busquet et al., 2006). As lipid metabolism does not directly support gluconeogenic pathways, acetate production can be considered less efficient from a production standpoint.

Aside from products of digestion, waste products from ruminal fermentation can greatly affect efficiency. Methane is a common energy waste product of a cow's metabolism. Approximately 8 to 12 percent of the energy ingested in feed is lost to methane production within the rumen (Busquet et al., 2006). To improve metabolic efficiency, the selection against microbes capable of methanogenesis would greatly aid in reducing methane production. Often, such methanogenic microbes attach to or live within protozoa, unicellular eukaryotes found in the rumen (Wallace, 2004). Such an association also makes the protozoa themselves a genus of interest, especially as they also serve in protein and fiber degradation (Wallace, 2004). Through the inhibition of methanogenesis, reducing equivalents such as hydrogen ions are made available that will often be used to produce other compounds such as propionate and butyrate, more energy-conserving products (Van Nevel and Demeyer, 1988). Moreover, methane also

represents a green house gas, so reducing methane production would reduce the overall methane release by the cow, a positive environmental effect.

If degradation of protein results in inefficient capture of nitrogen, ammonia can accumulate and waste nitrogen. Of the nitrogen consumed by a dairy cow, 75 to 85% will be excreted in feces and urine (Busquet et al., 2006). Naturally, cattle have an innate method of nitrogen conservation, through which non-protein nitrogen sources in the body such as urea can be processed in the rumen to produce proteins. With ruminal processing, certain microbes in the rumen can degrade proteins or use components to produce new microbial protein, but some can also produce ammonia waste from the complete breakdown of proteins and peptides. Such proteolytic and peptidolytic actions can occur rapidly, preventing the ruminant from gaining the necessary nutrients from digestion (Wallace and Cotta, 1988). As nitrogen is a valuable nutrient required for the building of many macromolecules such as nucleic acids and proteins, the conservation and use of nitrogen by the body is essential for efficient nutrient use by the animal. To promote nitrogen conservation, primary targets for inhibition are ruminal protozoa and deaminating bacteria that serve as prominent producers of ammonia (Busquet et al., 2006). To decrease nitrogen losses through ammonia production, it would seem that selecting against such microbes could greatly reduce losses, allowing for improvements in feed efficiency and overall production.

Ionophores have been used in agriculture for decades to enhance energy and nitrogen efficiencies (Van Nevel and Demeyer, 1988). With the feeding of streptomycin to chicks, antibiotic use in agriculture first began following World War II with the advent of antibiotic medicine (Gustafson et al., 1997). From this application, research soon showed benefits for livestock that could be had with antibiotic supplementation (Gustafson et al., 1997). Along with

improved feed efficiency and production, antibiotic supplementation was soon shown to also help control endemic disease within large farming operations (Gustafson et al., 1997). In the 1970's, the use of ionophores was officially approved, leading to its wide spread use in the agricultural industry (Calsamiglia et al., 2007). Commonly used in the agricultural industry even today, ionophores improve the overall efficiency of the cow.

One of the most common ionophores used in the dairy industry is Monensin or 2,2-dichloroacetamide. Monensin works by targeting gram positive bacteria within the rumen to produce effects such as increased propionate and decreased acetate production (Busquet et al., 2005a). As gram negative bacteria have a thick outer wall, ionophores are unable to permeate the cell wall (Busquet et al., 2005a). One other effect includes reduced methane production, but Monensin works very differently than typical methane inhibitors (Busquet et al., 2005a). Monensin inhibits methane production through its alteration of the VFA profile, which causes a decrease in hydrogen and formate production, needed substrates for methanogens (Busquet et al., 2005a). Ultimately, Monensin has been a very effective feed additive for enhancing farm productivity; however the possibility of producing antibiotic resistant bacteria has researchers evaluating other feeding strategies and supplements that could be of use.

The current issue with products like Monensin arises with the possibilities of selecting for antibiotic resistant bacteria within the cow gut (Gustafson et al., 1994). In general, consistent antibiotic usage in the livestock population allows affected microbes to adapt. The resistant microbes, unaffected by the antibiotic, will then be able to multiply and spread the resistance gene to other microbial populations. Studies of cattle herds using ionophores have demonstrated increased prevalence of antimicrobial-resistant bacteria (Call et al., 2008). In these ionophore-using herds, strains of *Salmonella* demonstrated resistance to antibiotics such as streptomycin

and sulfonamides, and strains of fecal *E. coli* demonstrated resistance to drugs such as ampicillin, tetracycline, sulfonamides, kanamycin, gentamicin, chloramphenicol and tetracycline (Call et al., 2008). Such effects can become a problem for the human population when antibiotics used in human medicine resemble the antibiotics being supplemented to livestock, as the newly resistant microbial population will no longer be inhibited or killed by traditional antibiotics. Notably, such effects could yield some microbial diseases untreatable (Gustafson et al., 1997). These concerns over the use of ionophores and other antibiotics in agriculture have fueled the efforts to find alternatives to enhance efficiency of nutrient use by the dairy cow. For such reasons, this study aims to examine the effects of essential oils on altering ruminal fermentation, in efforts to improve overall feed efficiency.

Secondary Plant Metabolites

Other potential feed supplements with antimicrobial properties can be found in plant secondary metabolites such as saponins, tannins, and the essential oils. Secondary metabolites are notably compounds produced by plants that do not contribute to development and growth of the plant (Gershenzon and Croteau, 1991). Rather, they can exhibit a variety of effects, including their ability to contribute to plant odor and color, be chemical messengers to the plant's environment, and exhibit antimicrobial properties (Calsamiglia et al., 2007). Saponins have been extensively studied in regards to their possible benefits as feed additives. Saponin molecules possess both hydrophilic and hydrophobic regions, making them a unique class of secondary plant metabolites (Gershenzon and Croteau, 1991). Studies conducted by Busquet et al. (2006) indicated that the effects of saponins fenugreek and yucca were somewhat pH dependent. Although yucca did not alter the ruminal fermentation profile, fenugreek with twice the saponin

content resulted in increased propionate and reduced ammonia nitrogen concentrations (Busquet et al., 2006). As other research supports this reduction in ammonia nitrogen concentration, the proposed mechanism involves an effect on ruminal protozoa, one of the main ammonia producers within the rumen (Busquet et al., 2006). Uniquely, saponins contribute to a decrease in ruminal pH, which likely contributes to decreased protozoa counts through the change in the rumen environment (Busquet et al., 2006). Moreover, saponins have the ability to form irreversible complexes with cholesterol in the protozoa cell membrane, eventually leading to the degradation of the membrane and cellular death (Busquet et al., 2006). Saponins have also been reported to inhibit gram positive bacteria within the rumen, thus contributing to increases in propionate through the selection for gram negative bacteria such as *Selenomonas ruminantium* (Busquet et al., 2006).

Found in high concentrations in birdsfoot trefoil and sulla, tannins, as well, have shown benefits for ruminant supplementation. As shown by Min et al. (2003), these phenolic plant-derived compounds improve protein digestion, through the inhibition of proteolytic bacteria. Along with effects on nitrogen metabolism, overall animal efficiency and production have been shown to improve when feeding either fresh high-tannin forage or concentrated tannin (Min et al., 2003). Moreover, tannins have been examined for use in parasite control. In parasitized sheep, feeding high-tannin forages such as sulla reduced parasite counts and other health issues (Min et al., 2003). However, at high concentrations, tannins may reduce feed intake, digestibility, and rate of growth (Min et al., 2003).

Most commonly known for their fragrant and flavorful properties, essential oils represent another type of plant secondary metabolite. Within the plant, essential oils serve important functions such as protection from bacterial, fungal, and insect attack (Wallace, 2004). To date,

around 3000 different essential oils are known, with approximately 300 of them being used for commercial applications involving fragrance and flavor (Burt, 2004). Specifically, essential oils can be extracted from the plant using expression or fermentation technology (Kung et al., 2008). More commonly, they are collected by the steam distillation of the plant fraction, yielding a combination of volatile active compounds that constitute the oil (Calsamiglia et al., 2007). Terpenoids and phenylpropanoids represent the two most important active compounds of essential oils, which often possess large chains or rings of carbons, contributing to the hydrophobic nature of essential oils (Calsamiglia et al., 2007).

In human medicine, essential oils have been quite beneficial for the treatment of cardiovascular disease, inflammation, tumors, and free radical proliferation (Calsamiglia et al., 2007). These benefits can be attributed to their ability to scavenge free radicals, prevent lipid peroxidation, chelate metals, and stimulate antioxidant enzymes (Calsamiglia et al., 2007). But more importantly, essential oils have been well known for their antimicrobial properties for many years. Their use by the Chinese dates back to around 5000 years ago, where plants were used for various treatments (Calsamiglia et al., 2007). Moreover in 1550 BC, Egyptians used spices such as cinnamon, cumin, and thyme for preserving food as well as mummification (Davidson and Naidu, 2000). Since the 13th century, pharmacies have stocked various essential oils, leading to widespread use and trade of these compounds in 16th century Europe (Burt, 2004).

The scientific proof of the antimicrobial characteristics surfaced in the early 1900's, which eventually led to further research suggesting essential oils as feed additives resulted in increases in daily weight gain in swine and poultry (Calsamiglia et al., 2007). Typically, the antimicrobial action of essential oils is greatest when they are obtained from plants harvested

around flowering (Burt, 2004). Borchers (1965) conducted the first examination of the effects of essential oils on ruminal fermentation, which examined the nitrogen composition when thymol was mixed in vitro with rumen fluid. The results showed this secondary metabolite of oregano and thyme, in fact, increased amino acid nitrogen, while decreasing the amount of ammonia nitrogen, suggesting a decrease in ruminal deamination (Borchers, 1965). Once ionphore usage became common practice in the 1970's, essential oil research for American agriculture fell to the wayside, at least for the next thirty years (Calsamiglia et al., 2007). With the more recent ban of ionophore use in Europe, there has been a resurgence of research in this area, specifically looking for ways to improve production efficiency (Calsamiglia et al., 2007). Various oils have been studied, and some of the studies resulted in varied results due to inconsistent concentrations and active compounds.

Multiple mechanisms have been suggested for the antimicrobial nature of essential oils. Various microbial cellular components have shown sensitivity, including the plasma membrane, cell wall, and cytoplasmic proteins (Burt, 2004). The most commonly proposed mechanism examines the interactions of essential oils with the microbial cell membrane. Somewhat hydrophobic in nature, many essential oils have the ability to interact with the microbial lipid bilayer, thus accumulating between the fatty acid chains, disrupting the stability, and resulting in cell expansion (Calsamiglia et al., 2007). Then, the microbes lose ions through the membrane, upsetting the ionic gradient and causing water to flow out of the cell (Calsamiglia et al., 2007). Even if the bacteria can use ionic pumps to counteract such effects, they may prevent their own death but expend a great deal of energy in the process making them much less competitive (Calsamiglia et al., 2007). Such energy costs prevent the optimum growth of the bacteria, thus decreasing the microbial population (Calsamiglia et al., 2007). As gram positive bacteria do not

possess an outer wall, it would seem as though they would be primarily affected, allowing the essential oil easy access to the cell membrane. However, gram negative bacteria are also affected by most essential oils (Calsamiglia et al., 2007). With a hydrophilic outer wall, the gram negative bacteria are influenced by essential oils because oil functional groups can often form hydrogen bridges that allow for interactions with the outer wall to gain access to the lipid membrane (Griffin et al., 1999). Moreover, some essential oils have been shown to induce the complete disintegration of the cell wall, allowing access to the plasma membrane (Helander et al., 1998).

Another proposed antimicrobial mechanism involves the ability of essential oils to induce the coagulation of some microbial cellular components, especially through protein denaturation (Calsamiglia et al., 2007). Again, side chains are often vital in the formation of hydrogen bridges, ionic bonds, and hydrophobic interactions that result in the structural changes to specific proteins and enzymes within the microbial cell (Juven et al., 1994). Notably, studies have illustrated that amino acid decarboxylase enzymes of *Enterobacter aerogenes* are distrupted by the binding of cinnamon oil and its components (Burt, 2004).

Aside from these possible contributions to positive alterations to ruminal fermentation, essential oils typically do not seem to have a dramatic effect on protozoa numbers (Wallace, 2004). As such an effect is typical of saponins, this clearly sets essential oils apart from other secondary metabolites, making them an important prospective feed additive. From current research, the most promising essential oils for dairy supplementation include garlic oil, thymol, eugenol, cinnamaldehyde, anethol, and capsacium.

Essential Oils

Garlic oil

One of the more well-studied essential oils, garlic oil also has shown potential benefits for cattle production. Garlic oil contains a wide mixture of components including enzymes, sulfur compounds, free amino acids, sterols, steroids, triterpenoid gylcosides, flavonoids, phenols, and organoselenium compounds (Calsamiglia et al., 2007). In addition, to the well-studied antimicrobial properties, garlic oil also exhibits antiparasitic, insecticidal, anti-cancer, antioxidant, immunomodulatory, hypoglycemic, and anti-inflammatory characteristics (Calsamiglia et al., 2007).

A relatively new topic of study, garlic feed additive investigations have illustrated that garlic oil works differently than Monensin (Calsamiglia et al., 2007). While garlic oil reduces acetate and increases propionate production, it also increases butyrate production, making its mechanism resemble the effects of methane inhibitors on ruminal microbes (Busquet et al., 2006). By blocking the production of methane, reducing equivalents such as NADH are produced that are used in butyrate and propionate production (Calsamiglia et al., 2007). Studies by Busquet et al. (2005b) have, in fact, indicated that garlic oil decreased the methane: VFA ratio from 0.20 to 0.05, a more dramatic effect than observed for Monensin. The antimethanogenic properties of garlic oil are quite unique, as they inhibit the growth of ruminal *Archaea* organisms. Importantly, the only *Archaea* found in the rumen are methanogenic, contributing to the methane production and energy loss within the animal (Miller and Wolin, 2001). These *Archaea* possess membranes composed of glycerol linked isoprenoid alcohols, which are synthesized by the enzyme hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase (Busquet et al., 2005b). This enzyme is greatly inhibited by garlic oil and some derived organosulfur

compounds, thus preventing ruminal methane production (Calsamiglia et al., 2007). HMG-CoA reductase also is responsible for the production of cholesterol in the liver, and inhibitors represent the basis of many human cholesterol-lowering drugs (Miller and Wolin, 2001). Studies by Miller and Wolin (2001) demonstrated that known inhibitors lovastatin and mevastatin that reduce this cholesterol production, also can inhibit ruminal *Archaea* in a similar fashions, thus emphasizing similarities with the garlic oil inhibition.

Another study by Yang et al. (2007) indicated that garlic oil at 5g/kg dry matter (DM) could increase dry matter and organic matter digestibilities most likely due to enhanced protein breakdown. Duodenal nitrogen flow decreased for garlic oil, as well as juniper berry, essential oil supplementations (Yang et al., 2007). However, dry matter intake DMI and ruminal pH did not change with the addition of garlic oil (Yange et al., 2007). Milk fat percent did increase with garlic oil supplementation, showing a variation from Monensin, which tends to decrease milk fat percent under certain dietary conditions (Yang et al., 2007). Moreover, this study also examined immune effects, but found no immune system enhancements due to the essential oil supplementation (Yang et al., 2007).

Overall, the effects on nitrogen metabolism seem to be quite skewed, as some studies suggest inhibition of deamination as shown by decrease ammonia nitrogen proportions, while other research suggest only slight changes in ammonia concentrations (Calsamiglia et al., 2007). A possible mechanism for effects on nitrogen metabolism was illustrated by Ferme et al. (2004), as garlic oil modified the microbial populations in a continuous culture study, decreasing the amount of *Prevotella* spp. and especially *P. ruminantium* and *P. bryantii*, bacteria specializing in peptide utilization. Notably, the latter two microbial species represent main contributors to

peptide utilization within the rumen, thus suggesting negative selection within microbial populations could produce effects on nitrogen metabolism (Ferme et al., 2004).

The beneficial effects of garlic oil on ruminal metabolism seem to be pH dependent. As most studies were conducting using a 60:40 alfalfa: concentrate diets at a pH of 5.5, the positive effects included decreased acetate to propionate ratios and ammonia nitrogen production with increases in propionate proportions and total VFA production (Calsamiglia et al., 2007). However, diets consisting of 10:90 forage:concentrate resulted in a rumen pH around 7 and decreased ammonia nitrogen, as well as total VFA production, once again indicating an effect of the oils related to rumen pH (Cardozo et al., 2005).

Multiple chemical components of garlic oil have been attributed to the antimicrobial properties. Busquet et al. (2005b) identified diallyl disulfide, 10.6% of garlic oil, and allyl mercaptan, a garlic oil derivative, to be the main antimicrobial components of garlic. Their comparative studies of garlic oils showed these compounds at doses of 300 mg/L in vitro exhibited the preferred effects of reduced acetate and methane production, and increased butyrate and propionate proportions (Busquet et al., 2005b). Notably, at concentrations of 3000 mg/L, garlic oil and its components can have detrimental effects on digestion, decreasing the total VFA production (Busquet et al., 2005b). Other studies also demonstrated that the organosulfur compounds such as allicin, 0.7% of garlic oil, contribute to antimicrobial properties (Ankri and Mirelman, 1999). Such properties of allicin have been related to its ability to react with thiol groups, thus inactivating important enzymes such as RNA polymerases, alcohol dehydrogenases, thioredoxin reductases, and cysteine proteinases (Ankri and Mirelman, 1999). However, Busquet et al. (2005b) concluded that allicin had little to no effect on ruminal fermentation, possibly due to its volatile, unstable, and immiscible nature at physiological conditions (Busquet

et al., 2005b). Ultimately, the effects of garlic oil are much more pronounced than the individual components, suggesting a synergistic effect among oil compounds (Busquet et al., 2005b).

Thymol

Another essential oil of interest, thymol comes from thyme as well as oregano and is chemically, a monoterpene with a phenolic structure (Calsamiglia et al., 2007). Initial research completed by individuals such as Borchers (1965), using in vitro incubation of rumen fluid, illustrated the inhibition of deamination with the use of thymol, resulting in decreased ruminal ammonia production. Interestingly, in vivo studies by Castillejos et al. (2006) indicated that thymol even at doses of 500 mg/L did not decrease ammonia nitrogen concentrations, but did however increase the amount of peptides and amino acids, improving both proteolysis and peptidolysis. Moreover, recent work by Evans and Martin (2000) examined the effects of thymol on energy metabolism, illustrating that thymol can affect important lactate-producing bacteria, *Streptococcus bovis* and *Selenomonas ruminantium*. Such work has demonstrated that moderate doses of 45 mL is effective in decreasing L-lactate production, and higher doses can actually inhibit microbial processing and overall digestion through reductions in VFA production (Evans and Martin, 2000).

The overall mechanism of antimicrobial activity for thymol seems to involve its action on the cytoplasmic membrane and outer wall of gram negative bacteria. In a study by Helander et al. (1998), the effects of thymol on various bacterial species illustrated that it disintegrated the cell wall, while disrupting the function of the inner membrane. Such effects clearly suggest the ability of thymol to affect both gram negative and gram positive bacteria.

Notably, the effects of thymol on rumen metabolism seem to be greatly pH and diet dependent. At doses of 500 mg/L, thymol actually increased the acetate-to-propionate ratio and decreased total VFA production with a diet of 60:40, alfalfa: concentrate, at a pH of 6.4 (Castillejos et al., 2006). A lower dose of 5 mg/L was more beneficial, without changing the total VFA production, in decreasing the acetate proportion (Castijellos et al., 2006). And with moderate doses of oregano, the acetate-to-propionate ration greatly decreased with 10:90, forage: grain diet, at a pH of 5.5 as shown by Cardozo et al. (2005). These studies suggested that a high concentrate diet, producing a low ruminal pH, may aid in achieving the most desirable effects from essential oil supplementation. Most likely, at low pH values, the hydroxyl groups of this essential oil cannot disassociate, creating a more hydrophobic molecule that can interact with lipids of the cell membrane more easily (Calsamiglia et al., 2007). Therefore, the antimicrobial effects of thymol can be distinctly different at low pH, and the small molecular size of the compound allows it to easily affect both gram positive and negative bacteria with very little selectivity (Calsamiglia et al., 2007). However despite valuable effects of essential oils, low pH can also contribute to poor fiber digestion and even acidosis in cattle, which in turn could detrimentally impact feed intake and milk production. With efforts to reduce the acetate-to-propionate ratio and ammonia production, avoiding the overdose of thymol and enhancing selectivity for efficient ruminal bacteria are of chief importance.

Eugenol

Another prospective essential oil supplement is eugenol, a phenolic compound that accounts for 8% and 85% of the oils found in cinnamon and clove bud, respectively (Calsamiglia et al., 2007). A continuous culture study conducted by Busquet et al. (2005c) demonstrated that

at doses of 2.2 mg/L clove bud oil decreased the proportion of acetate, while increasing proportions of propionate. The main component of clove bud oil, eugenol, however did not exhibit such positive results, suggesting there may be interactions with clove oil components to produce the effects on ruminal fermentation (Busquet et al., 2005c). In addition, this study concluded that peptidolysis was decreased, suggesting postitive effects of clove oil on nitrogen metabolism (Busquet et al., 2005c).

Other studies conducted using eugenol, however, demonstrated improved energy utilization and reduced deamination. In a study by Castillejos et al. (2006), using a 60:40 forage:concentrate diet, reduced ammonia production was observed at the 500 mg/L dose, while acetate/propionate proportions were quite variable. However with the 10:90 straw:concentrate beef-type diet, eugenol decreased overall VFA production, while increasing the acetate-to-propionate ratio, thus suggesting it would not be very useful in beef operations where diets are highly grain based (Calsamiglia et al., 2007). It was suggested that the mechanism of action for eugenol involves its ability to degrade the cell wall and cause cell lysis (Burt, 2004). Also, as seen with *E. aerogenes*, eugenol may also have the ability to coagulate proteins and enzymes, leading to decreased microbial function and eventual death (Burt, 2004).

Cinnamaldehyde

Accounting for 75% of cinnamon oil, cinnamaldehyde is a phenylpropanoid that exhibits antimicrobial properties (Calsamiglia et al., 2007). In a continuous culture, Cardozo et al. (2004) demonstrated that concentrations of cinnamon oil at 0.22 mg/L rumen fluid were able to modify nitrogen metabolism by inhibiting peptidolysis. Notably, cinnamaldehyde seems to have more significant effects when compared to the entire cinnamon oil mixture (Busquet et al., 2006).

However, feeding high amounts of either (greater than 3000 mg/L) can decrease overall VFA production and ammonia nitrogen concentrations (Busquet et al., 2006). Cinnamaldehyde also exhibits unique effects on VFA production, as it increases propionate concentrations, with little changes in butyrate and acetate concentrations (Busquet et al., 2006). Conversely, cinnamon oil only increases proportions of acetate (Busquet et al., 2006). In a similar study by Busquet et al. (2005a), doses of 31.2 and 312 mg cinnamaldehyde/L led to increased molar proportions of butyrate and propionate and decreased proportions of acetate, effects similar to antimethanogenic compounds such as amicloral and carbon monoxide.

Effects of cinnamon oil and cinnamaldehyde appear to be diet and rumen pH dependent. As shown by Cardozo et al. (2005), cinnamaldehyde supplementation to rumen fluid at a pH of 7 (common with highly forage based diets) actually increased acetate-to-propionate ratios and decreased VFA production overall. At a pH of 5.5, overall VFA production increased and the acetate-to-propionate ratio decreased with cinnamaldehyde supplementation, while ammonia nitrogen decreased for both cinnamon oil and cinnamaldehyde compared to the control (Cardozo et al., 2005). Thus, the effects of diet and rumen pH modify the effectiveness of essential oils on metabolic alterations.

Although effects of cinnamon oil on nitrogen metabolism are inconsistent among different studies using different doses, an in vitro study by Ferme et al. (2004) indicated a possible reduction in the bacteria capable of deamination, *P. ruminicola* and *P. bryantii*. It was suggested that the overall effects are most likely due to the presence of a carbonyl group on the cinnamaldehyde structure, allowing for interactions with bacterial componenents (Calsamiglia et al., 2007). By transversing the cell membrane through porin proteins, cinnamaldehyde may actually interact with proteins within the cell or periplasm, rather than disrupting the cell wall

and cytoplasmic membrane as observed with other essential oils (Busquet et al., 2006; Helander et al., 1998).

Anethol

Anethol is another potentially useful essential oil. Anethol is the main component of anise oil contributing to antimicrobial properties through the presence of an ether group attached to an aromatic ring (Busquet et al., 2006). As shown in studies by Busquet et al. (2006), anise oil and anethol both decreased total VFA production and reduced propionate and acetate proportions in vitro, while increasing the proportion of butyrate and having no effect on nitrogen metabolism. A lower anise oil dose of 0.22 mg/L in a continuous culture study, however, showed that anise oil actually stimulated the production of ammonia nitrogen through protein degradation and had little effect on VFA production (Cardozo et al., 2004). Another in vitro study by Cardozo et al. (2005) with anise oil illustrated even more promising effects at a lower ruminal pH, using rumen fluid from beef cattle fed a 10:90, forage: concentrate, diet. With a pH of around 5.5, this study demonstrated that ammonia nitrogen concentrations and acetate proportions decreased, while propionate and butyrate proportions increased, leaving total VFA production unaffected (Cardozo et al., 2005). In addition, another study used growing beef heifers fed a diet of 10:90, straw: concentrate, with a low dose of 2 g/d anise oil (Cardozo et al., 2006). Protozoa counts decreased, as did acetate proportions, while propionate proportions increased (Cardozo et al., 2006).

Capsaicin

Found in hot peppers and composing 10-15% of capsicum oil, capsaicin is a carotenoid that has shown potential for antimicrobial effects (Calsamiglia et al., 2007). With a low number of oxygen molecules within the structure, capsaicin has shown varying effects on fermentation when examined in an in vitro using a 60:40, alfalfa: concentrate, diet (Calsamiglia et al., 2007). Most other antimicrobial terpenes have more oxygen, which is believed to contribute to the antimicrobial properties (Calsamiglia et al., 2007).

Cardozo et al. (2005) used rumen fluid from beef cattle fed 10:90, straw:concentrate, diets adjusted to a pH of 7 to examine the affects of capsicum oil in vitro. They observed a reduction in ammonia nitrogen and total VFA production with an increase in acetate-to-propionate (Cardozo et al., 2005). When rumen pH was adjusted to 5.5, capsicum oil decreased ammonia nitrogen and the acetate-to-propionate ratio, while increasing the total VFA production and propionate proportions (Cardozo et al., 2005). A study conducted by Cardozo et al. (2006), when feeding beef cattle 10:90, forage:concentrate, diets illustrated similar, but less prominent trends. Although there was little change in total VFA production and ammonia nitrogen concentrations, slight decreases in acetate proportions were noted along with higher propionate proportions and amounts of small peptides and amino acids (Cardozo et al., 2006). Such findings suggest that capsicum oil aids in peptidolysis, microbial protein synthesis, and flow of proteins to the small intestine (Calsamiglia et al., 2007). As shown with humans by Calixto et al. (2000) and with cattle by Cardozo et al. (2006), capsicum oil has also been shown to increase dry matter intake and water intake.

Other Essential Oils

Other studies evaluating essential oil components have also illustrated less pronounced effects with compounds such as carvacrol, 9,10-antraquinone, benzyl salicylate, vanillin, and limonene. Carvacrol, constituting 69% of oregano oil, has also been studied to some degree, and as an isomer to thymol, it seems to exhibit similar antimicrobial properties (Busquet et al., 2006). A study conducted by Busquet et al. (2005c) study demonstrated that at an in vitro concentration of 2.2 mg/L, carvacrol decreased large peptide concentrations, potentially stimulating peptidolysis. Busquet et al. (2006) demonstrated that at doses of 300 mg/L, carvacrol increased rumen pH and butyrate proportions, while decreasing the proportions of acetate and propionate, along with total VFA production. However, at higher concentrations of 3000 mg/L, ammonia nitrogen concentrations did decrease, while propionate proportions increased (Busquet et al., 2006).

The proposed mechanism for the antimicrobial activity of carvacol involves the hydroxyl group on the phenolic structure. Through the exchange of the hydroxyl proton with a potassium ion, the carvacrol molecule could potentially serve as a transmembrane carrier, disrupting the proton motive force (Calsamiglia et al., 2007). Such disruption to the cellular stability would eventually lead to cell death due to greater membrane permeability altering the intracellular ionic concentrations and the osmotic balance. In addition, Helander et al. (1998) showed that carvacrol may have the ability to disintegrate the outer membrane of gram negative bacteria while also interfering with cell membrane function, allowing for a wide range of susceptible microbes. Overall, carvacrol seems to elicit somewhat inconsistent and non-specific effects resulting in somewhat varied outcomes on ruminal fermentation.

The main component of aloe and senna oils, antraquinone has exhibited in vitro and in vivo effects that inhibit methane production in ruminants (Garcia-Lopez et al., 1996; Kung et al., 2003). Moreover, antraquinone can alter fermentation patterns of ruminal fluid. As shown by Garcia-Lopez et al. (1996), antraquinone in batch culture was able to increase propionate, while decreasing acetate. Less promising results occurred with increasing doses as total VFA amounts decreased as well as propionate proportions (Garcia-Lopez et al., 1996). A suggested mechanism may be related to antraquinone's characteristic as a redox uncoupler (Kung et al., 2003). Such an effect on methanogenic bacteria would most likely work by inhibiting the electron transfer that reduces methyl coenzyme A to methane (Calsamiglia et al., 2007). However, it was shown that the resulting increase in hydrogen concentrations were not utilized by the rumen to contribute to VFA production as would be expected (Calsamiglia et al., 2007).

With less dramatic effects, benzyl salicylate seems to result in similar fermentation alterations as garlic oil. These effects include an increase in propionate and butyrate proportions with a decrease in acetate proportions (Busquet et al., 2006). It has been proposed that benzyl salicylate, much like garlic oil, acts to inhibit methanogenic *Archaea*, although a mechanism has not been examined (Busquet et al., 2006).

In a study by Castillejos et al. (2006), vanillin and limonene (dill) were tested in vitro batch fermentation using rumen fluid, to also yield limited and less promising results. Vanillin at doses of 5 and 50 mg/L had little effect on ruminal fermentation, but at a 500 mg/L dose resulted in only a slight decrease in acetate (Castillejos et al., 2006). Such results illustrate only limited potential. Along the same lines, the limonene concentrations of 5 and 50 mg/L led to decreases in total VFA production, a highly unfavorable result most likely due to the extreme inhibition of rumen microbes (Castillejos et al., 2006). At the higher dose of 500 mg/L, limonene decreased

ammonia nitrogen concentrations, however, suggesting the inhibition of deamination (Castillejos et al., 2006). Such non-specific and unfavorable microbial inhibitions of vanillin and limonene suggest these essential oil components also are less promising.

Supplemental Combinations of Essential Oils

Various combinations of essential oil components have also been investigated to explore additive, synergistic, and antagonistic effects, with some combinations even being available commercially (Calsamiglia et al., 2007). One blend of essential oils (BEO) containing thymol, eugenol, vanillin, guaiacol, and limonene, known as Crina, seem to inhibit proteolysis to some extent, but effects were quite dependent on diet and the length of adapation (Calsamiglia et al., 2007). A study by McIntosh et al. (2003) examined the effects of this BEO proteolytic, peptidolytic, and deaminase activity when dairy cattle rumen fluid was supplemented with 1g/d over a 4 week period. The results suggested that there was a decrease in ammonia production through the inhibition of deamination and that fiber digestion could actually be hindered through supplementation (McIntosh et al., 2003). This group also investigated specifically which microbes were inhibited by the BEO. *Clostridium sticklandi* and *Peptstreptococcus anaerobius,* both hyper-ammonia producing (HAP) bacteria, were sensitive to the BEO with concentrations of 36-42 ppm reducing bacterial growth by 50% (McIntosh et al., 2003). Such results indicated that the ammonia production was decreased through the inhibition of this unique group of gram positive bacteria (McIntosh et al., 2003). And while other bacterial species such as *P. ruminicola* and *P. bryantii* developed a resistance to the BEO, the HAP species did not, a highly favorable outcome (McIntosh et al., 2003). One HAP species, *Clostridium aminophilum* was not sensitive to the BEO, whereas commercially available Monensin is effective against all HAP

species (McIntosh et al., 2003). In addition *N. frontalis*, a rumen fungus, also was affected by the BEO at 40 ppm, showing decreased hydrogen production, while the methanogen *M. smithii* seemed unaffected by the supplement (McIntosh et al., 2003). As some of the affected bacteria and fungus possess fibrolytic and cellulolytic activity, there could be effects on other digestion processes (McIntosh et al., 2003). Importantly, the sensitive species required higher concentrations of essential oils than what would feasible in the rumen, due to feeding regiments only permitting roughly a maximum ruminal concentration of 17 ppm (McIntosh et al., 2003). However, on the surfaces of plant materials, the essential oil concentration could possibly be higher in vivo, allowing for more of an effect on sensitive species (McIntosh et al., 2003).

Other rumen effects of the Crina BEO seem positive. The effects on VFA production seem to be quite favorable to energy efficiency, but somewhat variable. An in vivo study indicated that butyric and acetic acid were reduced when feeding an essential oil diet, whereas propionate concentrations increased (Kung et al., 2008). However, Benchaar et al. (2007) suggested that Crina actually had little effects on VFA production, protozoa counts, and nitrogen metabolism (Calsamiglia et al., 2007). Moreover, this study showed an increase in rumen pH resulting from the BEO diet, from 6.30 to 6.40, which is typical of essential oil supplements (Benchaar et al., 2007).

Milk composition and production have also been investigated for effects of essential oils. The milk fatty acid profile could also be altered by dietary essential oils, as specific microbes are involved in the biohydrogenation of fatty acids in the rumen (Benchaar et al., 2007). This subject was examined by Benchaar et al. (2007) and Kung et al. (2008) who suggested that the Crina supplement actually did not alter the type of fatty acids in milk. Kung et al. (2008) observed increases in DMI and no change in milk protein concentrations with Crina

supplementation. On the contrary, a study by Tassoul and Shaver (2009) suggested that supplementation during the first 15 weeks of lactation reduced milk protein concentrations and increased milk lactose concentrations. In addition, this BEO contributed to an overall decrease in DMI, and thus an increase in feed efficiency (Tassoul and Shaver, 2009). Kung et al. (2008) and Tassoul and Shaver (2009) both reported that the body condition scores of cows fed BEO were unaffected. Studies also have typically indicated that milk yield is unaffected by the Crina BEO supplementation, with only Kung et al. (2008) indicating a slightly elevation in milk yield (Tassoul and Shaver, 2009; Benchaar et al., 2007). Overall, Crina BEO can contribute to the maintenance of milk quality with improvements in feed efficiency.

Another well studied BEO contains cinnamaldehyde and eugenol. Issues with palatability were suspected in a study by Cardozo et al. (2006) as at intake levels of 180 mg/d cinnamaldehyde plus 90 mg/d eugenol resulted in DMI and water intake reductions. The most apparent effect was a decrease in ammonia nitrogen and an increase in amino acid nitrogen and small peptides, suggesting the inhibition of deamination (Cardozo et al., 2006). Also, microbial changes suggested alterations to the ruminal protozoa counts as a decrease in *Entodinium* spp. and an increase in *Isotricha* spp. 3 hours after eating was noted (Calsamiglia et al., 2007). However, in another study, Cardozo et al. (2006) encapsulated the cinnamaldehyde/eugenol combination, which resulted in no change in DMI, indicating the palatability issue could be minimized. This study used even higher doses of 600 mg/d of cinnamaldehyde and 300 mg/d of eugenol that resulted in greater effects (Cardozo et al., 2006). Importantly, the proportion of propionate increased, while acetate proportions decreased (Cardozo et al., 2006). In addition, the total VFA production remained unchanged, and there was a noted inhibition of deamination

through the reduction of ammonia nitrogen concentrations and increase in small peptides and amino acids (Cardozo et al., 2006).

With previous research showing the possible benefits of essential oils in dairy nutrition, the study reported here aimed to evaluate in vitro effects of 102 essential oils and plant materials on rumen fermentation, and then supplement the oils showing the greatest potential to dairy cattle to investigate their effects in vivo. Native to the United States, the plants used in this experiment are recognized for elevated concentrations of one or more of the following compounds: eugenol, anethole, thymol, carvacrol, saponins, and cinnanaldehyde. This study used a batch culture incubation system conducted in duplicate and in concentrations of 0, 10, 50, and 100 ppm. One essential oil or plant material from this study showing the most potent beneficial effects, such as reduced methane production, high propionate proportions, low acetate proportions, and reduced ammonia production, was then selected for further study. The selected oil was supplemented to 8 multiparous Holstein cows, 4 of which were cannulated, in a 4x4 Latin square design. Effects on milk yield, milk composition, ruminal fermentation, urinary nitrogen losses, urinary and fecal ammonia emissions, and total tract digestibility of nutrients were examined.

MATERIALS AND METHODS

In Vitro Experimental Protocol and Experimental Design

The initial part of this investigation focused on screening 88 essential oils and 14 plant materials in vitro, as listed in Tables 1 and 2, to examine their effects on ruminal fermentation. The ruminal contents were collected from a cannulated cow of the Penn State dairy herd, which was being fed the university diet, 215 DIM, and producing 84 pounds of milk per day. Four liter samples were taken from the ventral sac and feed mat of the rumen at approximately 6:30am. Cows were routinely fed at 8am each day. The collected rumen contents were then strained through two layers of cheese cloth to collect 0.5 liters. The rumen solids were retained in the cheesecloth, and agitated in 0.5 L of warm (39°C) McDougall's buffer with 5 g/L of glucose. This mixture was then strained through two layers of cheesecloth again. Next, 0.5 L of the original strained rumen fluid and 0.5 L of the buffer solute were combined into a warm thermos at the farm. The ruminal contents were then transported quickly to the laboratory and placed into 2 L graduated cylinders. These were then placed into an oven at 39°C and incubated for 30 to 45 minutes to separate particulate matter. The particulate matter was then removed using a vacuum aspirator.

Table 1. List of plant materials tested in vitro

Number	Plant
1	Ambrosia artemisisfolia
2	Artemisia annua (Brazilian)
3	Asiminia sp.
4	Oplopanax horridus (berry pulp)
5	Oplopanax horridus (root logs)
6	Heraclem lanatum
7	Origanum vulgare
8	Artemisia absinthium
9	Artemisia afra
10	Artemisia annua (Chinese)
11	Oplopanax horridus (root bark)
12	Origanum majorum
13	Rhus typhina
14	Spilanthes acmella

Table 2. List of essential oils tested in vitro

Number	Oil Name	Number	Oil Name
1	O. basilicum cv. Mesten	45	O. basilicum #6 accession
2	O. basilicum #14 accession	46	O. basilicum #7 accession
3	O. basilicum #22 accession	47	O. basilicum #10 accession
4	O. basilicum #23 accession	48	O. basilicum #17 accession
5	O. basilicum #24 accession	49	O. basilicum #19 accession
6	O. basilicum #25 accession	50	O. basilicum #20 accession
7	O. basilicum #26 accession	51	O. basilicum #34 accession
8	O. basilicum #28 accession	52	Metha piperita (mitcham)
9	O. basilicum #29 accession	53	Menthaxgracilis sole
10	O. basilicum # 31 accession	54	Mentha spicata L.
11	O. basilicum #32 accession	55	M. avensis cv. Arvensis 2
12	O. basilicum #40 accession	56	Artemisia absinthium
13	O. basilicum #41 accession	57	Melissa officinalis L.
14	O. basilicum #8 accession	58	Satureja montana
15	O. basilicum #9 accession	59	Salvia officinalis
16	Ocimum sanctum	60	Organum Majorana
17	Anethum graveolens (dill weed)	61	Agastache foeniculum L.
18	Artemisia dracunculus	62	Hyssopus Officinalis L.
19	O. basilicum #21 accession	63	Ocimum sanctum
20	Chrysantha parthenium	64	Cymbopogon
21	Lavandula hybrid	65	Rosmarinus officinalis
22	Lavandula latifolia	66	Artemisia annua (Lebermooth)
23	M. arvensis cv. Arvensis 3	67	Artemisia absinthium (Lebermooth)
24	Majorana hortensis	68	Artemisia annua (Brazilian)**
25	Mentha piperita	69	Artemisia annua (Chinese)**
26	Metha arvensis	70	Asiminatriloba
27	Organium majorana	71	Fumeria officindis
28	Salvia sclarea	72	Chrysantha parthenium
29	Satureja hortensis	73	Draccocephalum
30	Satureja hortensis L.	74	Ruta graveolens
31	Tagetes minuta	75	Perilla
32	Valeriana officinalis	76	Chamomilla recutita
33	O. basilicum cv. German	77	Lavandula vera cv. Sevtopolis
34	O. basilicum #12 accession	78	Monarda fistulosa
35	O. basilicum #15 accession	79	Foeniculum vulgare var dulce
36	O. basilicum #16 accession	80	Coriandrum sativum
37	O. basilicum #27 accession	81	Anethum graveolens (dill seed)
38	O. basilicum #33 accession	82	Cymobopogon Winterianus
39	O. basilicum #35 accession	83	Agathophyllum aromatic
40	O. basilicum #36 accession	84	Thuja occidentalis
41	O. basilicum #37 accession	85	Cedrus atlantica
42	O. basilicum #39 accession	86	Juniperus communis
43	O. basilicum #4 accession	87	Achillea millefolium
44	O. basilicum #5 accession	88	Hypericum perforatum

**-70% EtoH Extract

For sample preparation, the rumen inoculum was kept warm and under carbon dioxide, while constantly mixing with a stirring rod on a magnetic plate. The overall process used 13-14 100-mL serum bottles every half hour. In each serum bottle, 0.7 g alfalfa hay, 0.1 g extracted-soybean meal, and 0.2 g corn starch were added to which buffer and 1 mL buffered essential oils were added at three inclusion levels (10, 50, and 100 mg/L) in duplicate. For plant materials, the inclusion levels were 12.5, 50, 100, and 200 mg, replacing equal part of alfalfa hay. At each half hour, one bottle was left as a negative control, without essential oil, and every other half hour, Monensin was added to one additional bottle mixture to serve as a positive control. To these mixtures, 19 mL McDougall's buffer and 20 mL of rumen inoculum were also added. After such preparations, the mixtures, under continuous carbon dioxide flow, were stopped with rubber tops and crimped shut. These bottles were then incubated for 6 hours at 39°C.

After the serum bottles were incubated for 6 hours, gas pressure readings were taken using an electronic meter. Then, 12 mL of gas from the bottle was removed and placed in an evacuated 100-mL serum bottle filled with 108 mL of nitrogen gas. These samples were then placed in a cooler and at a later time were analyzed for methane gas using the Photoacoustic Field Gas Monitor INNOVA 1412.

After gas sampling, the incubated serum bottles were then used for VFA sample collection. Five mL of fluid contents from the bottles was removed and placed in a tube. To this sample, 1 mL of 0.6% 2-ethylbutyric acid and 25% mono-phosphoric acid were added. Then, these tubes were centrifuged for 20 minutes at 4C three times, each time decanting liquid into a new tube. From the final liquid obtained, 1.5 mL was later analyzed for VFAs using a gas chromatograph to separate VFAs (Agilent 7890A Gas Chromatography System, Agilent Technology, Foster City, CA). In addition, a sample was also removed to be tested for ammonia

using a colorimetric procedure in conjunction with a spectrometer (Chancey and Marbach, 1962).

After incubation, pH readings were recorded from each serum bottle, and they were compared to the pH values of the control inoculum. The remaining contents of the serum bottles were then mixed and 10 mL were placed in a tube to be stored if needed later.

Neutral detergent fiber (NDF) samples were prepared using 15 50-mL tubes each half hour. Within each tube, 0.35 g alfalfa haylage, 0.1 g corn starch, and 0.05 g solvent-extracted soybean meal was added. Then, while under continuous carbon dioxide flow, 10 mL of McDougall's buffer was mixed with essential oils (at inclusion levels of 10, 50, and 100 mg/L) or plant extracts (at inclusion levels of 6.25, 25, 50, and 100 mg replacing alfalfa hay). During each round, one tube was left without essential oil/plant material to serve as a blank, while every other round, one extra mixed tube was run with Monensin. To these mixtures, 10 mL of warmed rumen inoculum was added, and the tube was flushed again with carbon dioxide before sealing with a stopper containing a Bunsen valve. These bottles were then incubated for 24 hours at 39°C.

After incubation of the 50-ml tubes for 24 hours, NDF digestibility was then determined using the Ankom A200 filter bag technique (Ankom Technology, Macedon, NY) according to Van Soest et al. (1991). First, these tubes were centrifuged for 10 minutes at 4C, and liquid was then decanted. The remaining solids were then placed in an oven at 50°C for 48 hours, and afterwards, dried solids were placed in a small bag. These particles were then broken up, and 0.25 grams was weighed into an NDF bag. Following NDF protocol, 22 samples and 2 alfalfa standards were placed in the rack. Then, 2 liters of NDF solution were added along with 20 grams of sodium sulfite and 20 mL alpha amylase. After running for 1 hour and 15 minutes, the

solution was drained. Then, the setup was filled with water and 20 mL alpha amylase to be agitated for 5 minutes. The water/alpha amylase procedure was then repeated. The NDF bags were removed drained of water and covered with acetone for 5 minutes. After pouring off the acetone, the NDF bags were then placed in an oven at 102°C for 24 hours. And lastly, the bags were placed in desiccators to cool and were weighed. NDF was calculated using a procedure by Van Soest et al. (1991).

In Vivo Experimental Protocol and Experimental Design

The in vivo portion of the trial used 6 ruminally-cannulated cows and 2 intact lactating cows from the Penn State herd, with 2 additional cows fed the control TMR diet. The 8 experimental cows were divided into 2 groups of 4, each including 3 cannulated and 1 intact cow. Using these groupings based on days in milk, current milk yield, and parity, the trial was a switch-over with 2 periods of 21 days each, including 14 days of adaptation and 7 days of sampling. After the first period, the groups switched diets, which included the control and experimental oregano diets. The control diet was the typical TMR fed to the rest of the Penn State herd without oregano, and the oregano diet was the typical TMR with 500 g/cow/day of oregano (DM basis) replacing 500 g of grass hay. The oregano and grass hay were top-dressed and mixed in the morning feeding daily. Cows had ad libitum access to feed with at least 8 hours between AM and PM feedings and received rBST according to the Penn State Dairy protocol at 63 DIM.

Intake and refusal weights were recorded daily, and TMR and feed waste, known as orts, were composited every other day (MWF), and the oregano plant was analyzed for chemical composition weekly. Forages were mixed together from weekly samples during each period, and

3 samples of concentrate feed were randomly collected and composited. All of these samples were frozen and oven-dried at 65°C to a constant weight. Then these were ground through a 1-mm screen and analyzed for organic matter (OM), nitrogen (N), acid detergent fiber (ADF), NDF, starch (TMR only), and indigestible NDF (INDF; TMR only).

Urine and feces were collected from all 8 cows at various time points during the first half of week 3 of each experimental period. These samples were roughly 6 hours apart, and were shifted one hour later each of the 4 days to provide day and night time samples. Approximately 300 mL of urine was collected at each sampling using vulval stimulation if needed. An unacidified sample was collected and composited for each cow during each period to allow for ammonia emission analysis. The rest of the sample was then acidified (pH<3) using 2 M H_2SO_4. The acidified urine was composited and analyzed for each period. In addition, the acidified urine was diluted 1:10 with distilled water and frozen at -20°C for later analysis including total nitrogen, purine derivatives (allantoin and uric acid), urea, and creatinine. Daily urinary excretion of nitrogen, urea, and purine derivatives was based on creatinine values. Specifically, urinary purine derivatives were used to estimate microbial N flow. Microbial N flow was calculated as absorption of MPB (mmol/d)= $(PD – 0.385 X BW^{0.75}) \div 0.85$, where PD is the urinary PD excretion (allantoin and uric acid; mmol/d), 0.385 mmol/kg of $BW^{0.75}$ is a correction for endogenous PD, and 0.85 is a recovery coefficient.

Approximately 400 g feces was also collected. From this an equal amount on a DM basis was composited, and the rest was oven-dried at 65°C. Apparent nutrient digestibilities of OM, DM, NDF, and starch were estimated using dietary and fecal concentrations of INDF. The chemical composition of the orts was also used to adjust nutrient intake.

Composite urine and fecal samples from each cow for each period were used for the ammonia and methane emissions analysis. Approximately 1 kg feces and 1 kg urine were used. The samples were thawed, and fresh manure was reconstituted based on the 7:1 ratio of feces: urine from other experiments. Ammonia and methane emitted from the manure were measured using the Wheeler's 8-jar system, a steady state chamber system.

For ruminal profiling, rumen sampling also was conducted using the 6 cannulated cows within the 2 experimental groups during 2 consecutive days of the sampling week. Samples were taken every 2 hours, starting at 8 am and ending at 4 pm each day. The sampling procedure included the collection of 2 samples from the whole ruminal contents and 2 samples from the dorsal mat, with each sample constituting about 1 cup each. After thorough mixing, a subsample of 120 mL was taken for microbial profiling which was immediately frozen. The rest of the sample was strained through 2 layers of cheesecloth to obtain about 300 mL of filtrate which was then analyzed for pH. An aliquot was separated for each of the following: protozoa counts, polysaccharide-degrading activities, ammonia, total free amino acids (TFAA), VFA, and bacterial pellet isolation. For the protozoa counts, 5 mL of the filtrate was mixed with 5 mL of protozoal staining solution and was kept at 4°C until counted. These samples were composited and diluted before the microscopic counts were made using a Sedgewick-Rafter chamber. For the polysaccharide-degrading activity, 50 mL of filtrate was frozen immediately at -20°C. In addition 3 45-mL subsamples of filtrate were centrifuged at low speed (500 x g) to remove protozoa and feed particles for 5 minutes at 4°C. The supernatants were then combined for each cow to obtain about 70-80 mL of sample, which was then using for ammonia, TFAA, and VFA analyses, in addition of the isolation of the bacterial pellets.

For ammonia and TFAA, 30 mL of the chilled supernatant was mixed with 2.5 mL of chilled 65% TCA to precipitate the proteins by acidification. After freezing, thawing, and high speed centrifugation (20000 x g) for 15 minutes at 4°C, samples were then analyzed for ammonia and TFAA. For VFA analysis, 5 mL of chilled supernatant was acidified with 25% meta-H_3PO_4. This mixture was stored frozen and analyzed after thawing and high speed centrifugation. The remaining filtrate (35 mL) was mixed with 3.9 mL of 35% formaldehyde solution and refrigerated until bacterial pellet processing within 24 hours. This aliquot was further prepared using 20 minutes of high speed centrifugation at 4°C to sediment the bacteria. The sediment was then combined with distilled water, freeze-dried, and composited per cow to determine the purine: non-ammonia nitrogen (NAN) ratio.

Ruminal methane production was analyzed using the SF_6 tracer technique on the 6 ruminally-cannulated cows (Johnson et al., 1994). A modified rumen cannulae was used, and the SF_6 permeable tubes were inserted into the reticulum 3 days before the first sampling and kept in for the rest of the trial. Gas samples were collected during one day of the sampling week every 2 hours, starting at 10 am and ending at 4 pm. A 60-mL syringe was used for two samplings, and the gas was placed in an evacuated 200-mL serum bottle. After dilution with nitrogen gas (20:1), these samples were then analyzed using a Photoacoustic Field Gas Monitor INNOVA 1412 for methane, carbon dioxide, and SF_6. The production of methane was estimated using the known release rate of SF_6 gas from the SF_6 tube.

Milk yields were recorded at each milking for each cow, and milk sampling was conducted during the sampling week. The sampling was conducted 3 times during each period, and milk was analyzed for milk fat, true protein, lactose, solids-not-fat (SNF), somatic cell count

(SCC) and milk urea nitrogen (MUN) (Pennsylvania DHIA, University Park, PA). Cows were weighed on 3 consecutive days at the beginning of both periods and end of period 2.

Blood sampling was also conducted on 2 consecutive days of week 3 sampling for each period at 10 am, 2 hours after the morning feeding. The samples were placed in heparinized vacuumed tubes and kept on ice. The samples were then centrifuged at 1500 x g for 20 minutes at 4°C and were stored frozen until they were analyzed for blood urea and glucose.

All results were then analyzed using SAS statistical analysis software (2003; SAS Inst. In., Cary, NC), using a cross-over design. Statistically different results were characterized to have a P value < 0.05, with statistical trends between P value > 0.05 and P value < 0.10.

RESULTS

In vitro experimental results

In vitro results of the 88 essential oils and 14 herbs identified various beneficial plant substances. As shown in Tables 1 and 2, the Batch results for herbs labeled 1 through 14 are displayed, along with probability values for effects of treatment, level, and treatment by level interactions. For Batch 1, herbs 1 through 7, there was an effect of level and a treatment by level interaction for NDF digestibility. Treatment effects were observed for methane production, NDF digestibility, ammonia production, and acetic/propionic acid production, but not butyric acid production. For Batch 2, level effects were observed for methane and ammonia production, while treatment by level effect was only seen for NDF digestibility. Treatment effects were observed for methane, NDF digestibility, and ammonia production. For all analyses, the blank was used for comparison, thus serving as the control.

In regards to the effects of herbs on methane production, methane was reduced ($P<0.05$) by the Monensin treatment, with 283.60 µg versus the control production of 350 µg, in Batch 1, however in Batch 2 of Table 4, Monensin caused an increase ($P<0.05$) in methane production with 401 µg versus control production of 285 µg. The seventh herb as shown in Table 3, *Origanum vulgare*, reduced ($P<0.05$) methane by 31.3%, producing 240.46 µg versus the control 350 µg. In Table 4, the ninth herb tested, *Artemisia afra*, increased ($P<0.05$) methane production, 332.28 µg versus the control at 285 µg.

With the plant material NDF digestibility, at the 0.2 g inclusion level, *Ambrosia artemisisfolia, Asiminia spp.,* and *Oplopanax horridus* (berry pulp) all increased ($P<0.05$) NDF digestibility at 37.35%, 35.44%, and 39.92%, respectively, when compared to the control at 24.8% NDF digestibility of Batch 1. As shown in Table 4 with Batch 2, *Artemisia absinthium,*

Artemisia afra, and *Artemisia annua* (Chinese) all increased (P<0.05) NDF digestibility as well, at 34.28, 32.41, and 33.79%, respectively, compared to the control at 27.85%. Decreases (P<0.05) in NDF digestibility at the 0.2 g inclusion level were observed for *Oplopanax horridus* (root logs) and *Heraclem lanatum,* both with 21.66% digestibility compared to 24.8% for the control. In addition, as shown in Table 4, *Oplopanax horridus* (root bark)*,* and *Rhus typhina* also decreased (P<0.05) NDF digestibility with 19.75 and 23.14%, respectively, when compared to the control at 27.85%.

At the 0.1 g inclusion level, when compared to the control at 24.8% digestibility, *Ambrosia artemisisfolia, Artemisia annua* (Brazilian)*, Oplopanax horridus* (berry bulp), and *Origanum vulgare* increased (P<0.05) percent NDF digestibility with 32.92, 28.81, 32.50, and 28.61%, respectively. In this same Batch, *Oplopanax horridus* (root logs) and *heraclem lanatum* decreased (P<0.05) percent NDF digestibility with 22.04% and 16.51%, respectively. For Batch 2 at the 0.1 g inclusion level, *Artemisia absinthium* increased (P<0.05) NDF digestibility, 32%, compared to the control at 27.85%. *Oplopanax horridus* (root bark) decreased (P<0.05) NDF digestibility compared to the blank.

At the 0.05 g inclusion level, when compared to the Batch 1 control of 24.8%, *Asiminia sp., Oplopanax horridus* (berry pulp)*, and Origanum vulgare* increased (P<0.05) the NDF digestibility with percentages of 28.35, 28.79, and 29.96, respectively. On the other hand, *Heraclem lanatum* decreased (P<0.05) NDF digestibility in this batch with a digestibility of 19.65%. In Batch 2 with a control of 27.85%, *Oplopanax horridus* (root bark) decreased (P<0.05) NDF digestibility at 23.87%.

At the 0.0125 g level, effects (P<0.05) were only seen in Batch 1, comparing results to the control at 24.8% digestibility. *Asiminia sp., Oplopanax horridus* (berry pulp), and *Origanum*

vulgare specifically showed increases ($P<0.05$) in NDF digestibility with percentages of 27.33, 28.37, and 29.06, repectively.

Relative to ammonia production, Monensin showed a trend ($P<0.10$) towards increasing ammonia production in Batch 1, producing 0.5 mg/dl versus the control at 0.3 mg/dl, but in Batch 2 demonstrated decreased ammonia production with 0.44 mg/dl versus the control of 0.95 mg/dl. *Ambrosia artemisisfolia* showed an increase ($P<0.05$) in ammonia production in Batch 1, with 0.64 mg/dl. *Oplopanax horridus* (root bark) showed a trend ($P<0.10$) in ammonia reduction, producing 0.47 mg/dl opposed to the control at 0.95 mg/dl. *Rhus typhina* showed a decrease ($P<0.05$) in ammonia production at 0.43 mg/dl when compared to the control at 0.95 mg/dl.

Effects of treatments on VFA production were limited to Batch 1 plant extracts as shown in Table 3. Monensin increased ($P<0.05$) acetic acid production at 53.89 mM compared to the Batch 1 control at 48.13 mM. *Oplopanax horridus* (berry pulp) increased ($P<0.05$) acetic acid production at 54.13 mM, while *Origanum vulgare* showed a trend ($P<0.10$) toward increasing acetic acid production with concentrations of 51.83 mM. For propionic acid, Monensin increased ($P<0.05$) production with 28.38 mM versus the control amount of 24.32 mM. Of the plant extracts, only *Asiminia sp.* and *Oplopanax horridus* (berry pulp) increased ($P<0.05$) propionic acid production at levels of 28.42 and 28.23 mM, respectively. With butyric acid, no effects were detected.

Table 3. Batch 1 Plant Material Results and Significance

Parameter[c]	IL[d]	Con.	Mon.	Treatment[a]							SE	Level of Sig.[b]		
				1	2	3	4	5	6	7		T	L	T x L
Batch 1														
Methane		350.00	283.60[e]	318.61	360.80	362.58	388.87	365.62	363.09	240.46[e]	20.97	***	NS	NS
NDF Dig.	L1	24.80	26.65	37.35[e]	25.19	35.44[e]	39.92[e]	21.66[e]	21.66[e]	27.62	0.58	***	***	***
	L2			32.92[e]	28.81[e]	23.91	32.50[e]	22.04e	16.51[e]	28.61[e]				
	L3			26.30	25.32	28.35[e]	28.79[e]	23.46	19.65[e]	29.96[e]				
	L4			25.63	24.31	27.33[e]	28.37[e]	24.80	23.21	29.06[e]				
Ammonia		0.30	0.50[f]	0.64[e]	0.39	0.17	0.18	0.28	0.42	0.37	0.87	**	NS	NS
VFA														
Acetic		48.13	53.89[e]	48.97	50.17	52.04	54.13[e]	48.79	50.11	51.83[f]	1.35	**	NS	NS
Propionic		24.32	28.38[e]	24.05	25.09	28.42[e]	28.23[e]	24.98	24.71	25.61	1.23	*	NS	NS
Butyric		7.10	6.95	7.34	7.34	7.40	7.87	7.11	7.10	7.33	0.29	NS	NS	NS

NS, not significant ($P > 0.05$).

[a] Treatments defined in table 3-2

[b] Effects of treatment (T), level of inclusion (L) and treatment by level interaction (T × L).

[c] Fermentation parameters tested and labels: Methane= ug; NDF digestibility= %; Ammonia= mg/dl; VFA= volatile fatty acids mM

[d] IL= inclusion level. Levels L1–L4 refer to inclusion of treatments at 0.20, 0.10, and 0.05, and 0.0125g.

[e] Within a row and parameter (L1–L4), marked values differ from their respective control ($P < 0.05$).

[f] Within a row and parameter (L1–L4), marked values tend to differ from their respective control ($P < 0.10$).

* $P < 0.05$

** $P < 0.01$

***$P < 0.00$

Table 4. Batch 2 Plant Material Results and Significance

Parameter[c]	IL[d]	Con.	Mon.	Treatment[a]							SE	Level of Sig.[b]		
				8	9	10	11	12	13	14		T	L	T x L
Batch 2														
Methane		285.00	401.00[e]	264.76	332.28[e]	298.09	272.43	301.20	284.24	261.51	13.68	*	**	NS
NDF Dig.	L1	27.85	26.20	34.28[e]	32.41[e]	33.79[e]	19.75[e]	25.49	23.14[e]	31.24	0.65	***	NS	**
	L2			32.00[e]	24.90	30.79	23.77[e]	26.77	28.33	28.84				
	L3			28.81	29.25	30.85	23.87[e]	25.87	28.60	25.95				
	L4			25.89	27.16	28.61	26.74	26.58	27.53	27.32				
Ammonia		0.95	0.44[e]	0.80	0.74	1.26	0.47[f]	0.62	0.43[e]	0.89	0.17	*	*	NS
VFA														
Acetic		53.40	51.94	57.91	57.76	57.19	56.51	54.87	54.61	58.53	1.64	NS	NS	NS
Propionic		21.43	22.79	22.23	22.97	21.79	23.15	22.68	22.44	23.83	0.85	NS	NS	NS
Butyric		7.56	6.70	8.03	8.03	8.01	8.06	7.91	7.86	8.59	0.29	NS	NS	NS

NS, not significant ($P > 0.05$).

[a] Treatments defined in table 3-2

[b] Effects of treatment (T), level of inclusion (L) and treatment by level interaction (T × L).

[c] Fermentation parameters tested and labels: Methane= ug; NDF digestibility= %; Ammonia= mg/dl; VFA= volatile fatty acids mM

[d] IL= inclusion level. Levels L1–L4 refer to inclusion of treatments at 0.20, 0.10, and 0.05, and 0.0125g.

[e] Within a row and parameter (L1–L4), marked values differ from their respective control ($P < 0.05$).

[f] Within a row and parameter (L1–L4), marked values tend to differ from their respective control ($P < 0.10$).

* $P < 0.05$

** $P < 0.01$

***$P < 0.001$

Results for the 88 essential oil substances screened are presented in Table 5 for all 6 batches. Relative to methane production, multiple essential oils caused alterations in this rumen fermentation characteristic. In Batch 2 of essential oil screening, both *Anethum graveolens* (dill weed) and *Lavandula latifolia* reduced (P<0.05) methane production compared to the control at 369.55 µg, producing 277.53 µg and 252.61 µg respectively. In Batch 3, *O. basilicum #12 accession* demonstrated a trend (P<0.10) towards increased methane production with 274.55 µg in comparison to the control at 236.41 µg. *O. basilicum #35 accession* and *O. basilicum #36 accession* increased (P<0.05) methane production, producing 286.54 µg and 292.55 µg, respectively. Also in Batch 3, *O. basilicum #7 accession* decreased (P<0.05) methane production, producing 190.06 µg once again in comparison to the control at 236.41 µg. All other essential oils did not alter methane production in vitro.

Essential oils had varying effects on NDF digestibility, with the only significant changes resulting in decreases in NDF digestibility. Of Batch 2, *Salvia sclarea* demonstrated a trend (P<0.10) in reduced NDF digestibility at 16.78%, while *M. arvensis cv. Arvensis 3* reduced (P<0.05) NDF digestibility to 15.86% in comparison to the control NDF digestibility at 19.4%. In Batch 5 with a control of 23.81% NDF digestibility, *Draccocephalum* resulted in a trend (P<0.10) for reduced NDF digestibility at 20.6%. In addition, *Rosmarinus officinalis, Artemisia absinthium* (Lebermooth), and *Chrysantha parthenium* all reduced (P<0.05) NDF digestibility with results of 19.13, 18.68, and 19.65%, respectively.

Relative to the VFA production in vitro with essential oils, effects also varied. With acetic acid specifically, the only effects were obtained in Batch 1, for which the control produced 52.08 mM. *O. basilicum #23 accession* and *O. basilicum #32 accession* both increased (P<0.05)

acetic acid production in vitro, resulting in 56.46 mM and 57.68 mM, respectively. The remaining essential oils did not alter rumen acetic acid production.

Propionic acid production however was affected to a much greater extent with the addition of essential oils. From Batch 1, *O. basilicum #31 accession* showed a trend ($P<0.10$) for increased propionic acid production at 19.67 mM when compared to the control at 18.52 mM. Moreover, *O. basilicum #23 accession* and *O. basilicum #32 accession* increased ($P<0.05$) propionic acid, producing respective amounts of 20.52 mM and 21.22 mM. Of Batch 2 for which the control was 21.29 mM propionic acid, *Artemisia drancunculus, O. basilicum #21 accession, Chrysantha parthenium,* and *M. arvensis cv. Arvensis 3* all increased ($P<0.05$) the production of this VFA with results of 26.08 mM, 25.39 mM, 25.18 mM, and 26.07 mM, respectively. From Batch 4, *O. basilicum #34 accession* and *Mentha spicata L.* both increased ($P<0.05$) propionic acid production, resulting in 21.32 mM and 21.28 mM, respectively, in comparison to the control at 19.40 mM. Of Batch 5 for which the control produced 19.40 mM, *Chrysantha parthenium, Draccocephalum,* and *Ruta graveolens* increased ($P<0.05$) propionic acid production, resulting in 24.83 mM, 22.08 mM, and 23.72 mM, respectively. And finally within Batch 6, *Juniperus communis* increased ($P<0.05$) propionic acid production to 19.85 mM with the control producing 18.89 mM.

Relative to effects of essential oils on butyric acid production, some essential oils increased concentrations, while others decreased concentrations. For Batch 1 with a control producing 6.57 mM, *O. basilicum #14 accession, O. basilicum #23 accession,* and *O. basilicum #32 accession* increased ($P<0.05$) butyric acid production at 8.31 mM, 8.47 mM, and 8.15 mM, respectively. For Batch 2, *O. basilicum #21 accession* demonstrated a trend ($P<0.10$) for increased butyric acid, producing 7.30 mM in comparison to the control production of 6.51 mM.

Also, *Artemisia dracunculus, Chrysantha parthenium,* and *M. arvensis cv. Arvensis 3* increased (P<0.05) butyric acid production at 7.78 mM, 7.59 mM, and 8.18 mM, respectively. Within Batch 4, both *O. basilicum #34 accession* and *Mentha spicata L.* increased (P<0.05) butyric acid production yielding 7.92 mM and 7.83 mM butyric acid, respectively, whereas the control for Batch 4 produced 7.02 mM butyric acid. Of Batch 5, *Perilla* produced a trend (P<0.10) towards increased butyric acid at 8.01 mM, while the control yielded 7.02 mM. In addition, *Chrysantha parthenium* and *Ruta graveolens* both increased butyric acid production, producing concentrations of 8.38 mM and 8.68 mM. And only in Batch 6 with a control production of 8.36 mM butyric acid did Monensin reduce (P<0.05) butyric acid to 6.36 mM. Within this batch, *Juniperus communis* also exhibited a trend (P<0.10) for increased butyric acid production, yielding 8.90 mM.

With ammonia nitrogen production, results for the essential oils greatly varied. Within Batch 1, *O. basilicum #9 accession* resulted in a trend (P<0.10) for increased ammonia production at a concentration of 1.38 mg/dl, in comparison to the control at 0.97 mg/dl. Monensin and *O. basilicum #14 accession* both resulted in an increase (P<0.05) in ammonia production at concentrations of 1.55 mg/dl and 1.45 mg/dl, respectively. *O. basilicum #25 accession*, however, reduced (P<0.05) ammonia production to 0.52 mg/dl. From Batch 2 with the control production of 0.97 mg/dl, *Mentha piperita* and *Satureja hortensis L.* increased (P<0.05) ammonia concentration, with results of 1.41 mg/dl and 1.35 mg/dl, respectively. And *O. basilicum #39 accession* of Batch 3 resulted in a decrease (P<0.05) in ammonia production, yielding 0.34 mg/dl in comparison to the control result of 0.75 mg/dl.

Table 5. Effect of essential oil on methane, NDF digestibility, individual VFA and ammonia

Item	Methane	NDF dig.	Acetic, mM	Prop., mM	But., mM	Ammonia, mg/dl
Batch 1						
Blank	325.74	18.45	52.08	18.52	6.57	0.97
Monensin	325.91	18.00	50.74	18.58	6.07	1.55[*]
1	322.28	17.71	53.61	18.45	6.72	0.75
2	382.69	15.83	55.01[t]	18.98	8.31[*]	1.45[*]
3	318.11	17.10	53.70	18.94	6.87	0.96
4	315.73	15.84	56.46[*]	20.52[*]	8.47[*]	0.90
5	368.56	17.14	53.02	18.62	7.32	0.79
6	298.71	18.51	52.93	19.05	6.50	0.52[*]
7	300.68	18.85	53.83	18.60	6.39	0.79
8	325.33	18.02	53.14	19.07	6.85	0.92
9	381.79	18.53	53.14	18.14	7.01	0.93
10	309.77	17.01	54.48	19.76[t]	6.32	0.84
11	318.30	15.57	57.68[*]	21.22[*]	8.15[*]	1.15
12	384.80	16.45	52.92	18.94	6.92	0.85
13	332.56	18.20	53.71	19.68	6.66	0.94
14	349.04	18.39	53.23	18.41	6.51	0.91
15	346.86	17.13	54.32	18.40	7.46	1.38[t]
16	385.45	17.66	53.74	19.14	6.93	0.91
SE[a]	24.42	1.01	1.18	0.58	0.43	0.15
Batch 2						
Blank	369.55	19.40	48.60	21.29	6.51	0.97
Monensin	386.96	19.73	52.88	20.53	5.92	1.14
17	277.53[*]	19.04	52.84	21.08	6.66	0.90
18	394.44	19.92	56.88	26.08[*]	7.78[*]	0.94
19	420.41	17.68	54.16	25.39[*]	7.30[t]	0.66
20	409.69	17.45	55.31	25.18[*]	7.59[*]	1.15
21	355.22	20.95	52.85	21.54	7.00	1.05
22	252.61[*]	21.46	52.47	20.28	6.77	1.03
23	437.18	15.86[*]	55.48	26.07[*]	8.18[*]	0.68
24	367.26	17.05	52.56	23.52	6.97	1.09
25	376.26	17.84	50.68	21.94	6.85	1.41[*]
26	412.90	17.69	50.85	23.03	6.76	1.01
27	411.07	19.72	51.88	22.23	6.68	0.76
28	415.66	16.78[t]	53.84	23.06	7.04	0.71
29	397.73	17.10	50.36	21.29	6.40	1.04
30	390.36	18.18	50.68	20.96	6.58	1.35[*]
21	360.70	19.29	54.00	21.80	6.99	0.91
32	406.44	18.00	52.69	22.17	6.88	0.97
SE[b]	32.14	1.05	1.73	1.33	0.32	0.12

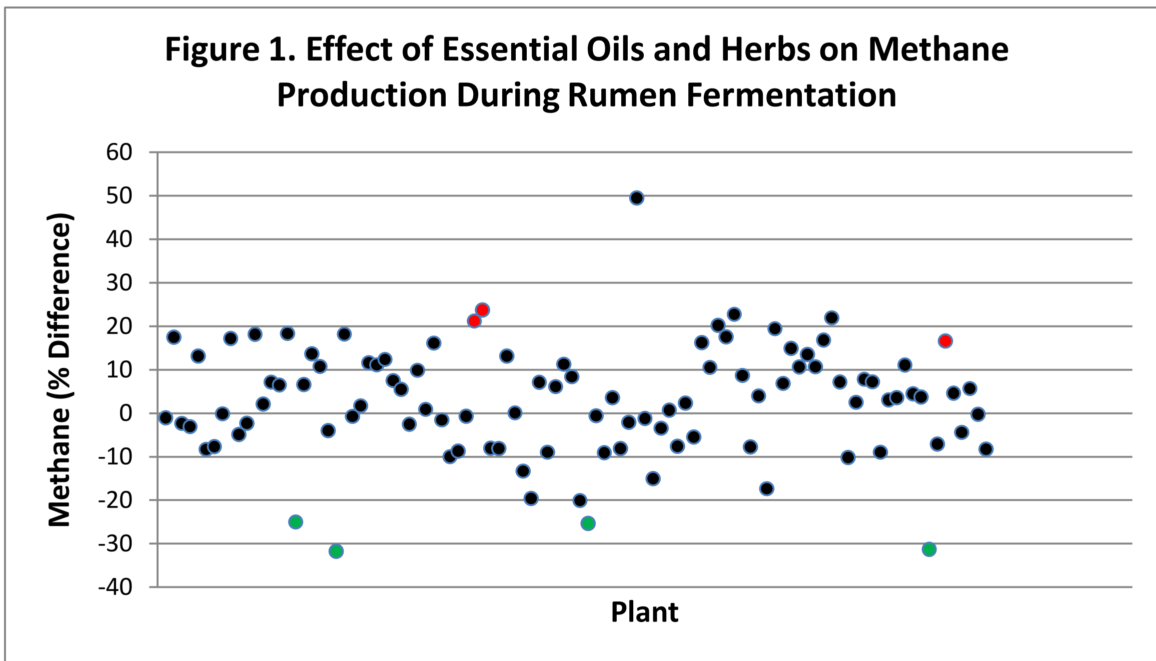
Item	Methane	NDF dig.	Acetic, mM	Prop., mM	But., mM	Ammonia mg/dl
Batch 3						
Blank	236.41	19.15	40.29	16.73	5.62	0.75
Monensin	258.99	19.51	42.46	18.46	5.43	1.05
33	238.54	19.77	46.12	18.13	6.24	0.81
34	274.55[t]	20.43	45.34	17.82	6.31	1.04
35	232.77	17.98	44.83	18.18	6.29	0.52
36	212.78	17.24	45.41	17.75	6.30	0.44
37	215.82	18.87	45.66	18.40	6.14	0.76
38	234.75	18.37	44.66	18.28	6.09	0.66
39	286.54*	18.17	44.48	17.90	6.16	0.59
40	292.55*	18.31	46.13	18.74	6.45	0.48
41	217.42	17.99	44.01	17.53	6.11	0.57
42	217.24	16.93	45.01	17.87	6.16	0.34*
43	267.50	19.50	45.84	18.39	6.38	0.98
44	236.63	19.94	47.29	18.64	6.08	0.67
45	204.98	19.15	43.82	16.25	6.96	0.74
46	190.06*	21.01	44.40	16.87	6.00	1.01
SE[c]	14.81	0.93	1.77	0.54	0.26	0.15
Batch 4						
Blank	178.68	17.35	62.65	19.40	7.02	0.85
Monensin	165.89	13.74	65.40	20.34	7.12	0.87
47	191.38	19.48	63.92	20.02	7.32	0.81
48	162.68	18.43	61.75	19.35	7.07	1.03
49	189.60	16.37	63.74	20.16	7.62	0.76
50	198.81	16.80	62.03	19.88	7.32	0.64
51	193.70	16.58	66.63	21.32*	7.92*	0.76
52	142.82	18.34	63.13	18.87	6.62	0.86
53	133.39	16.42	62.95	19.23	6.67	0.99
54	177.64	18.40	64.45	21.28*	7.83*	0.78
55	162.45	17.16	63.20	20.08	7.13	0.87
56	185.04	16.56	58.64	18.11	6.61	0.64
57	164.20	16.10	59.35	18.13	6.42	0.98
58	174.94	14.45	61.95	19.77	7.30	0.67
59	267.16	14.71	63.13	20.89	7.33	0.74
60	176.56	22.04	60.28	18.63	6.57	1.01
61	151.83	17.88	61.07	18.83	6.83	0.93
62	172.54	18.35	63.35	20.00	6.87	0.82
SE[d]	21.69	1.56	1.65	0.60	0.30	0.15

Item	Methane	NDF dig.	Acetic, mM	Prop., mM	But., mM	Ammonia mg/dl
Batch 5						
Blank	302.30	23.81	62.65	19.40	7.02	1.01
Monensin	293.81	22.60	65.41	20.34	7.12	0.95
63	304.39	21.23	59.30	21.03	7.57	0.91
64	279.37	22.12	59.43	21.54	7.79	1.03
65	309.36	19.13[*]	60.82	21.82	7.77	0.86
66	285.76	23.46	60.52	21.91	7.86	0.69
67	351.36	18.68[*]	60.25	21.65	7.83	0.67
68	334.18	21.26	59.26	21.28	7.71	0.82
69	363.48	23.73	58.57	21.00	7.74	0.94
70	355.47	24.77	58.55	21.00	7.74	0.83
71	371.08	22.14	60.98	21.23	7.81	0.79
72	328.60	19.65[*]	63.36	24.83[*]	8.38[*]	0.94
73	278.91	20.60[t]	58.67	22.08[*]	7.48	0.79
74	314.39	24.07	62.24	23.72[*]	8.68[*]	0.67
75	249.85	23.52	64.15	21.79	8.01[t]	0.62
SE[e]	38.67	1.29	1.94	0.60	0.22	0.15
Batch 6						
Blank	218.93	16.42	54.39	18.89	8.36	0.95
Monensin	191.37	19.98	50.45	19.21	6.36[*]	0.81
76	261.52	18.95	53.48	18.57	8.18	0.70
77	233.94	18.23	53.92	18.77	7.97	1.11
78	251.61	17.34	53.92	18.98	8.42	0.76
79	242.22	16.78	55.55	19.55	8.58	0.90
80	248.56	17.44	54.25	18.88	8.50	0.89
81	242.33	19.34	54.43	19.03	9.36	0.95
82	255.75	18.02	54.95	19.28	8.65	1.05
83	266.96	17.84	53.58	19.02	8.38	0.78
84	234.71	19.88	52.08	18.85	8.12	0.77
85	196.65	20.74	54.40	19.15	8.35	0.90
86	224.42	21.39	54.85	19.85[*]	8.90[t]	0.69
87	236.06	18.40	54.08	19.02	8.27	0.79
88	234.76	17.01	55.13	18.95	9.35	0.93
SE[f]	19.02	1.34	0.78	0.36	0.22	0.15

[a] Effects of treatment, application level, and application level×treatment interaction for Batch 1: P= 0.002, 0.93, and 0.98 (ammonia); P= 0.05, 0.66, and 1.00 (acetic acid); P= 0.03, 0.40, and 1.00 (proprionic acid); P= 0.005, 0.14, and 0.97 (butyric acid).
[b] Effects of treatment, application level, and application level×treatment interaction for Batch 2: P= 0.02, 0.63, and 1.00 (methane); P= 0.02, 0.36, and 0.96 (NDF digestibility); P= 0.002, 0.82, and 1.00 (ammonia); P= 0.03, 0.98, and 1.00 (proprionic acid); P= 0.003, 0.80, and 1.00 (butyric acid).
[c] Effects of treatment, application level, and application level×treatment interaction for Batch 3: P= 0.0001, 0.71, and 1.00 (methane); P= 0.01, 0.66, and 1.00(ammonia); P= 0.08, 0.93, and 1.00 (butyric acid).
[d] Effects of treatment, application level, and application level×treatment interaction for Batch 4: P= 0.07, 0.98, and 1.00 (methane); P= 0.005, 0.54, and 0.72 (proprionic acid); P= 0.02, 0.70, and 1.00 (butyric acid).
[e] Effects of treatment, application level, and application level×treatment interaction for Batch 5: P= 0.03, 0.99, and 0.96 (NDF digestibility); P= 0.03, 0.67, and 1.00 (proprionic acid); P= 0.02, 0.93, and 1.00 (butyric acid).
[f] Effects of treatment, application level, and application level×treatment interaction for Batch 6: = 0.008, 0.51, and 0.76 (acetic acid); P< 0.0001, 0.78, and 0.98(butyric acid).

Summarized results for averaged methane production, NDF digestibility, and ammonia production for both plant extracts and essential oils are presented in Figures 1, 2, and 3. The combined results are displayed graphically and also illustrate the significant alterations by plant substances.

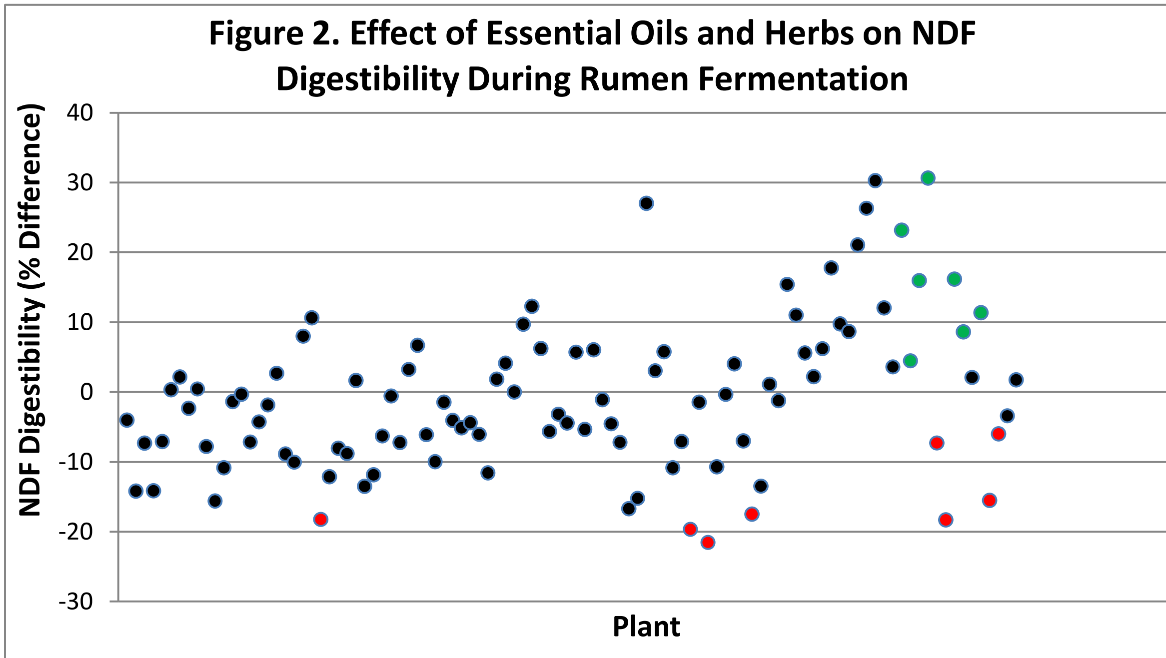
As presented in Figure 1, decreases in methane production were produced by *Anethum graveolens* (dill weed), *Lavandula latifolia, O. basilicum #7 accession,* and *Origanum vulgare.* In comparison to the controls, methane was decreased 25.0% by *Anethum graveolens* (dill weed), 31.8% by *Lavandula latifolia*, 19.6% by *O. basilicum #7 accession*, and 31.3% by *Origanum vulgare*. Increases in methane were observed for *O. basilicum #35 accession* by 21.2%, *O. basilicum #36 accession* by 23.7%, and *Artemisia afra* by 16.6%.



P < 0.05 (Decrease in methane)
In respective order, Anethum graveolens (dill weed), Lavandula latifolia, O. basilicum #7 accession, and Origanum vulgare

P < 0.05 (Increase in methane)
In respective order, O. basilicum #35 accession, O. basilicum #36 accession, and Artemisia afra

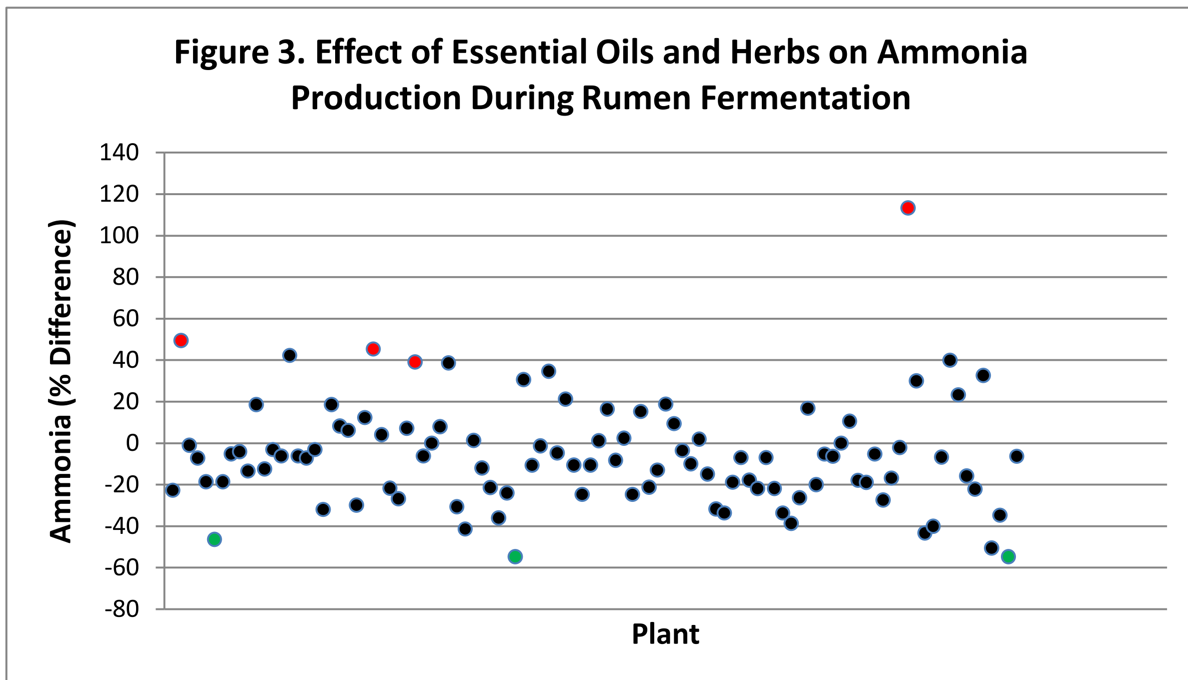
As presented in Figure 2, NDF digestibility was increased (P<0.05) with *Ambrosia artemisifolia* by 23.2%*, Artemisia annua (Brazilian)* by 4.5%*, Asiminia sp.* by 16.0%*, Oplopanax horridus* (berry pulp) by 30.6%, *Origanum vulgare* by 16.2%*, Artemisia absinthium* by 8.6%*,* and *Artemisia annua (Chinese)* by 11.3%. On the contrary, decreases (P<0.05) in NDF digestibility were observed for *M. arvensis cv. Arvensis 3* by 18.2%*, Rosmarinus officinalis* by 19.7%*, Artemisia absinthium* (Lebermooth) by 21.5%*,Chrysantha parthenium* by 17.5%*, Oplopanax horridus* (root logs) by 7.3%*, Heracleum lanatum* by 18.3%*, Oplopanax horridus* (root bark) by 15.5%*,* and *Origanum majorum* by 6.0%.



P < 0.05 (Increase in NDF Digestibility)
In respective order, Ambrosia artemisifolia, Artemisia annua (Brazilian), Asiminia sp., Devil's Club berry pulp, Origanum vulgare, Artemisia absinthium, and Artemisia annua (Chinese).

P < 0.05 (Decrease in NDF Digestibility)
In respective order, M. arvensis cv. Arvensis 3 , Rosmarinus officinalis, Artemisia absinthium ,Chrysantha parthenium , Devil's Club root logs, Heracleum lanatum, Oplopanax horridus, and Origanium majorum.

Figure 3 illustrates decreases in ammonia production by *O. basilicum #25 accession* by 46.4%, *O. basilicum #39 accession* by 54.7%, and *Oplopanax horridus* (root bark) by 54.7%. Contrarily, increases in ruminal ammonia production in comparison to the control were observed for *O. basilicum #14* by 49.5%, *Mentha piperita* by 45.4%, *Satureja hortensis L.* by 39.2%, and *Ambrosia artemisisfolia* by 113.3%.



● **P < 0.05 (Decrease in ammonia)**
In respective order, O. basilicum #25 accession , O. basilicum #39 accession , and Oplopanax horridus (root bark)

● **P < 0.05 (Increase in ammonia)**
In respecti ve order, O. basilicum #14, Mentha piperita, Satureja hortensis L., and Ambrosia artemisisfolia.

Overall, the in vitro portion of this study illustrated that one plant in particular *Organium vulgare* (oregano) could potentially benefit ruminal fermentation in more than one way through reductions in methane production as well as increases in NDF digestibility at the 0.1, 0.05, and 0.0125 g inclusion levels. With such results, oregano was selected to be tested in the in vivo portion of the study, to examine ruminal fermentation changes more effectively.

In vivo experiment results

For the in vivo portion of the study, the composition of the cow diet is presented in Table 6. With the nutrient analysis and components, values as percent of dry matter (DM) were all equivalent among the control and treatment diets. The diet fed consisted of 15.5 percent CP, 20.5 percent ADF, 32.1 percent NDF, and 5 percent fat, with the overall energy of the diet being 1.69 Mcal/kg. Approximately 8 weeks into the experiment, the haylage source was changed, resulting in 5 percent change in dry matter source from ground corn to cracked corn. Dry matter analysis was conducted weekly, and adjustments were made as necessary.

Table 6. Ingredient and chemical composition of diets fed to cows during the *Origanum vulgare* trial.

Item	Diet	SEM
n[1]	8	
Ingredient composition		
Corn Silage[2]	42.34	2.54
Alfalfa Silage[3]	13.30	0.80
Grass/Straw Hay	4.37	0.26
Canola Meal	7.50	0.45
Cookie Meal[4]	6.56	0.39
Ground Corn	7.38	0.44
Cottonseed Hulls	10.81	0.65
Sugar	4.19	0.25
Min/Vit Mix[5]	3.14	0.19
Optigen	0.41	0.02
Chemical composition		
DM, %	51.02	3.06
CP, % of DM	15.50	0.93
Soluble protein, % of CP	5.08	0.30
ADF, % of DM	20.51	1.23
NDF, % of DM	32.07	1.92
NFC[6], % of DM	40.43	2.43
Fat, % of DM	5.00	0.30
NE_L[7], Mcal/kg	1.69	0.10
Ca, % of DM	0.86	0.05
P, % of DM	0.35	0.02
Mg, % of DM	0.34	0.02
K, % of DM	1.24	0.07
Na, % of DM	0.40	0.02
S, % of DM	0.21	0.01
Fe, mg/kg	131.73	7.90
Zn, mg/kg	75.00	4.50
Cu, mg/kg	19.03	1.14
Mn, mg/kg	67.67	4.06

[1]n= number of cows per treatment

[2]Contained 32.2% DM; 6.8% CP, 25.1% ADF, 40.0% NDF, 48.4% NFC, 3.3% fat, 2.8% ash, 0.15% Ca, 0.21% P, and 1.63 Mcal/kg of NE_L (DM basis).

[3]Contained 47.3% DM; 20.4% CP, 30.6% ADF, 39.7% NDF, 31.2% NFC, 2.8% fat, 9.7% ash, 1.22% Ca, 0.30% P, and 1.50 Mcal/kg of NE_L (DM basis).

[4]Dried bakery by-product.

[5]Premix contained 0.296% of sulfur, 7.183 mg/kg of selenium, 4.7 mg/ kg of cobalt, 11.76 mg/kg of iodine, 21.1 mg/kg of vitamin A, 438.56 µg/kg of vitamin D, and 685.887 mg/kg of vitamin E.

[6]NFC = 100 – (%CP + %NDF + %ash + %fat).

[7]Estimated based on NRC (2001).

With the two treatments, milk production and components were also analyzed to identify any oregano effects and are presented in Table 7. Most notably, the fat component was increased ($P<0.05$) with the oregano treatment at 1.44 kg/d, in comparison to no supplemental oregano at 1.36 kg/d. There was also a trend ($P<0.10$) in increased fat percent from oregano-treated cows, with a fat percent of 3.28% and a control of 3.12%. Finally there was an increase ($P<0.05$) in fat corrected milk (FCM) feed efficiency in oregano treated cows, having a feed efficiency of 1.63 compared to the control at 1.53. Other factors including overall milk yield, protein/lactose composition, and somatic cell counts were not different between diets.

Table 7. Milk and milk component production for cows fed either *Origanum vulgare* or control rations.

Item	O[1]	C[2]	SE	*P* Value[3]
n[4]	8	8		
Milk, kg/d	44.14	43.61	3.58	0.61
Feed Eff., kgmilk/kg feed	1.71	1.66	0.07	0.11
Fat, %	3.28	3.12	0.28	0.09
Fat, kg/d	1.44	1.36	0.11	0.04
Protein, %	2.96	2.97	0.10	0.61
Protein, kg/d	1.30	1.29	0.14	0.87
Lactose %	4.75	4.77	0.05	0.43
Lactose, kg/d	2.08	2.07	0.19	0.85
MUN[5], mg/dl	13.34	13.36	0.82	0.93
SCC[6]	61.19	50.06	20.29	0.50
3.5% FCM[7], kg/d	42.20	40.88	0.84	0.10
3.5% FCM[7] Feed Eff.	1.63	1.53	0.01	0.002

[1]O= *Origanum vulgare*
[2]C= control
[3] Probability of significant effect ($P<0.05$) using SAS®
[4]n= number of cows per treatment
[5]MUN= milk urea nitrogen
[6]SCC= somatic cell count
[7]FCM= fat corrected milk

For the nutrient intakes, fecal matter, and apparent digestibilities, the results are displayed in Table 8, with P Values shown from the SAS analysis. In general, there were no effects on

either nutrient intake or fecal matter, from nutrient analysis comparisons. Moreover, the apparent digestibility was thus unaffected by the oregano supplementation.

Table 8. Nutrient intakes and apparent digestibilities of cows fed either *Origanum vulgare* or control rations.

Item	O[1]	C[2]	SE	*P* Value[3]
n[4]	8	8		
Intake, kg/d				
DM	26.04	26.67	0.83	0.24
OM	24.73	25.29	2.79	0.39
CP	4.47	4.57	0.50	0.40
NDF	7.87	8.06	0.90	0.36
ADF	5.01	5.14	0.57	0.36
TNC	9.80	9.80	1.07	0.40
Fecal matter, kg/d				
DM	9.65	9.86	1.05	0.32
OM	9.58	9.45	0.36	0.75
CP	1.80	1.82	0.09	0.86
NDF	5.20	5.33	0.60	0.38
ADF	3.43	3.64	0.29	0.16
TNC	0.82	0.84	0.06	0.57
Apparent digestibility, %				
DM	63.32	63.34	0.57	0.98
OM	64.76	64.71	0.55	0.93
CP	58.02	59.59	3.19	0.53
NDF	34.02	33.77	1.14	0.86
ADF	30.10	28.50	3.34	0.62
TNC	91.16	91.29	0.46	0.80

[1]O= *Origanum vulgare*
[2]C= control
[3] Probability of significant effect ($P<0.05$) using SAS®
[4]n= number of cows per treatment

As shown in Table 9, ruminal fermentation characteristics were relatively unaffected, with the exception of ammonia production. Most notably, ammonia nitrogen was increased ($P<0.05$) with the oregano diet. The control ruminal ammonia nitrogen concentration was 7.78 mg/dl, while the oregano diet resulted in 9.46 mg/dl, illustrating the increase in ammonia

production. Overall, though, the ruminal pH, VFA production, protozoa, and free amino acids were not affected by supplementation.

Table 9. Ruminal fermentation characteristics of cows fed *Origanum vulgare* or control rations.

Item	O[1]	C[2]	SE	*P* Value[3]
n[4]	6	6		
pH	6.13	6.04	0.09	0.47
NH_3N, mg/dl	9.46	7.78	0.78	0.0005
VFA[5], µmole/ml				
Total VFA	128.98	132.21	6.64	0.28
Acetate (A)	79.7	80.8	2.25	0.20
Propionate (P)	28.4	30.5	1.73	0.15
Butyrate	14.5	14.24	0.34	0.37
Isobutyrate	1.15	1.12	0.05	0.45
Valerate	2.77	3.24	0.32	0.14
Isovalerate	2.35	2.32	0.10	0.50
A:P	2.62	2.68	0.27	0.38
Protozoa, count/ml	306,875	297,500	31,414	0.77
Log Protozoa	5.46	5.42	0.06	0.59
TFAA[6], mM	3.01	3.16	0.57	0.81

[1]O= *Origanum vulgare*
[2]C= control
[3] Probability of significant effect (P<0.05) using SAS®
[4]n= number of cows per treatment
[5]VFA= volatile fatty acid
[6]TFAA= total free amino acids

The blood metabolite results are displayed in Table 10. Blood glucose remained relatively unchanged by the oregano diet, and averaged 61.56 mg/dl among cows. However there was a trend (P<0.10) in increased blood urea nitrogen (BUN) production resulting with the oregano diet, producing 9.50 mg/dl while the control diet resulted in 8.81 mg/dl.

Table 10. Blood metabolites of cows fed *Origanum vulgare* supplemented or control rations.

Item	O[1]	C[2]	SE	*P* Value[3]
n[4]	20	20		
Glucose, mg/dl	60.53	62.59	1.87	0.15
BUN[5], mg/dl	9.50	8.81	0.28	0.052

[1]O= *Origanum vulgare*
[2]C= control
[3] Probability of significant effect (P<0.05) using SAS®
[4]n= number of cows per treatment
[5]BUN= blood urea nitrogen

Methane and fecal emissions results are presented in Table 11, demonstrating a notable reduction in ruminal methane production. The control at 1,149,493 mg/m^3 was significantly higher (P<0.05) than the oregano-treated cows rumen production of only 696,208 mg/m^3. In addition, ruminal methane production on a daily basis was reduced (P<0.05), with oregano and control diets averaging 450.77 g/d and 744.66 g/d, respectively. In regards to cumulative emissions from manure, the overall production of ammonia, methane, and carbon dioxide were not altered with oregano supplementation.

Table 11. Rumen methane production and manure gas release for cows fed either *Origanum vulgare* or control rations.

Item	C[1]	OV[2]	SE	*P* Value
n[3]	6	6		
Rumen gas production				
Methane, mg/m3	1,149,493	696,208	286,088	0.02[4]
Methane, g/h	34.21	24.00	10.08	0.13[5]
Methane, g/d	744.66	450.77	196.56	0.004[6]
n[3]	8	8		
Fecal gas release				
Ammonia	0.09	0.06	0.04	0.60
Methane	0.05	0.07	0.01	0.22
Carbon Dioxide	0.07	0.07	0.01	0.93

[1]C= control
[2]OV= *Origanum vulgare*
[3]n= number of cows per treatment
[4]Time, P = 0.09, Treatment x time, P = 0.48
[5]Time, P = 0.86, Treatment x time, P = 0.05
[6] Time, P = 0.02, Treatment x time, P = 0.05

Urine analysis results are shown in Table 12. Oregano had relatively small effects on urine composition. There was however, a trend for decreased urine nitrogen in oregano-fed cows, producing 0.23 kg/d versus the control at 0.24 kg/d, and a trend for decreased urea nitrogen in oregano-fed cows, releasing 59.01 g/d as compared to 66.28 g/d of the control. Milk nitrogen was increased in oregano-fed cows, as they produced 28.51% of nitrogen intake while the control produced 27.76% of nitrogen intake.

Table 12. Nitrogen metabolites for cows fed either the *Origanum vulgare* or control diet.

Item	O[1]	C[2]	SE	*P* Value[3]
n[4]	8	8		
Allantoin, mmol/day	378.47	412.72	60.65	0.47
Uric Acid, mmol/day	77.87	85.70	9.30	0.18
Total PD, mmol/day	456.34	498.41	68.55	0.39
Microbial N flow, g/day	301.69	332.83	49.28	0.39
Microbial CP flow, g/day	1885.57	2080.22	308.02	0.39
Urine N%	1.10	1.10	0.03	0.99
Urine N, kg/day	0.23	0.24	0.02	0.06
Urine N, % of N intake	31.51	32.26	0.66	0.48
Urine output, kg/d	20.76	22.07	2.70	0.30
Fecal N, % of N intake	41.98	40.40	3.19	0.53
Total N Excretion, kg/day	0.51	0.53	0.04	0.42
Total N Excretion, % of N intake	73.48	72.67	3.33	0.78
Urea N, g/day	59.01	66.28	8.31	0.05
Urea N, % of urine N	26.05	27.29	0.98	0.35
Milk protein N, kg/day	0.20	0.20	0.02	0.87
Milk protein N, % of N intake	28.51	27.76	0.33	0.04

[1]EO= *Origanum vulgare*
[2]C= control
[3] Probability of significant effect ($P<0.05$) using SAS®
[4]n= number of cows per treatment

DISCUSSION

In light of recent controversy over the use of ionophores in the dairy industry, the use of plant extracts and essential oils could be a useful alternative. Most notably, essential oil compounds such as thymol from plants alter cell membranes of select microbes to ultimately disrupt ion gradients and osmolarity to induce cell death (Calsamiglia et al., 2007). Through selection against methanogenic or peptidolytic microbes for instance, it is quite possible that ruminal fermentation can be positively affected for more efficient metabolic processes. In this study, the effects of 88 essential oils and 14 plant extracts were examined. Each produced a wide variety of effects on methane, ammonia, and VFA production as well as NDF digestibility, however oregano yielded the greatest effects and was selected for further study in an in vivo trial.

Essential oil and plant material in vitro screening

For the in vitro examination, methane production was reduced ($P<0.05$) by *Anethum graveolens* (dill weed), *Lavandula latifolia, O. basilicum #7 accession,* and *Origanum vulgare* (Tables 3, 4, and 5). Such observations agree with multiple studies, suggesting that essential oils and plant extracts decrease ruminal methane production. Ruminal in vitro screenings of plant substances by García-González et al. (2008) likewise demonstrated the ability of rhubarb root, bark of alder buckthorn, and dried garlic bulbs to reduce methane production. Similarly, a study by Bodas et al. (2008) found that 35 of 450 different plant species were able to decrease ruminal methane production in vitro. Evans and Martin (2000) demonstrated that thymol, an essential oil found in thyme and oregano, decreased methane production when supplemented to rumen fluid in vitro at a level of 400 μg/ml. Moreover, Busquet et al. (2005b) showed that in vitro,

garlic oil along with its components diallyl sulfide, and allicin decreased methane production of rumen fluid. Notably, such reductions in methane production could improve feed efficiency of the cow, preventing hydrogen losses in the form of methane and incorporating the energy into usable products such as propionic acid (Calsamiglia et al., 2007).

On the contrary, multiple essential oils and plant extracts actually increased the production of methane, a highly unfavorable result contributing to energetic losses for the cow. These plant components included *O. basicilcum #35 accession, O. basilicum #36 accession,* and *Artemisia afra* (Table 3; Table 5). Such an increase in methane production was observed in screenings by García-González et al. (2008) as well as Bodas et al. (2008) and ultimately suggests the loss of energetic hydrogen and reduction in efficiency for the cow. After all, this means methanogenic bacteria were allowed to flourish as a result of the plant supplementation, indicating an excessive release of highly energetic methane molecules.

Monensin only produced significant alterations in methane production in the plant material batches, producing an increase in methane production in Batch 1 and a decrease in methane production for Batch 2 (Table 3; Table 4). The decrease in methane production agrees with a Garcia-Lopez et al. (1996) study, illustrating in vitro reduction in ruminal methane production with Monensin supplementation.

Essential oils and plant extracts demonstrated highly variable effects on NDF digestibility, with 7 plant materials increasing NDF digestibility and 8 plant substances decreasing NDF digestibility. NDF digestibility was increased by *Ambrosia artemisifolia, Artemisia annua* (Brazilian), *Asiminia sp., Oplopanax horridus* (berry pulp), *Origanum vulgare, Artemisia absinthium,* and *Aretmisia annua* (Chinese) (Table 3; Table 4). Increases in NDF digestibility indicate that, within the ruminal fluid, beneficial cellulolytic microbes were favored

as a result of the supplementation, and ultimately such changes would allow the cow to gain more energy from a given fibrous feed source.

On the contrary, many studies have reported a decrease in NDF digestibility, as was observed for *M. arvensis cv. Arvensis 3, Rosmarinus officinalis, Artemisia absinthium, Chyrsantha parthenium, Oplopanax horridus* (root logs), *Heracleum lanatum, Oplopanax horridus* (root bark) and *Origanum majorum* (Tables 3, 4, and 5). Castillejos et al. (2006) demonstrated a decrease in NDF digestibility with supplementation of thymol at concentrations of 500 mg/L. Such a reduction in NDF digestibility could be attributed to the non-specificity of the plant materials resulting in the inhibition of cellulolytic microbes. However in the same study, the essential oil eugenol had no effects on NDF digestibility, which agrees with the minimal effects seen for 87 of the 102 plant substances screened in this study (Castillejos et al., 2006). In addition, Monensin did not affect NDF digestibility. Such results are contrary to several studies, including Busquet et al. (2005a) suggesting reduced NDF digestibility as a result of Monensin supplementation.

The effects of essential oils on VFA production were also somewhat variable in vitro. Of the essential oils and plant materials, *O. basilicum #23 accession*, *O. basilicum #32 accession*, and *Oplopanax horridus* (berry pulp) contributed to significant increases in acetic acid production (Table 3; Table 5). Notably, acetic acid more directly contributes to lipid metabolism, thus being a much more indirect and less efficient energy source than the other VFAs including propionic acid. However, acetic acid also serves as a precursor in butter fat synthesis, which could result in higher milk fat production, a profitable trait for the farmer. These results are in contrast to work by Busquet et al. (2005a), which illustrated reductions in ruminal acetate production with in vitro supplementation of cinnamaldehyde as well as garlic oil.

Evans and Martin (2000) and Castillejos et al. (2006) also demonstrated decreases in acetate production with in vitro supplementation of thymol at 400 μg/ml.

The direct precursor for gluconeogenesis, propionic acid amounts were significantly affected by a variety of essential oils and plant materials. Of the herbs, only *Asiminia sp.* and *Oplopanax horridus* (berry pulp) increased propionic acid production (Table 1). Whereas of the essential oils, *O. basilicum #23 accession*, *O. basilicum #32 accession*, *Artemisia drancunculus, O. basilicum #21 accession, Chrysantha parthenium, M. arvensis cv. Arvensis 3*, *O. basilicum #34 accession, Mentha spicata L., Chrysantha parthenium, Draccocephalum, Ruta graveolens*, and *Juniperus communis* all increased production of propionic acid (Table 5). Notably, multiple studies have demonstrated similar increases in propionate proportions with in vitro supplementation of plant materials. Busquet et al. (2005a) demonstrated increases in propionate proportions with cinnamaldehyde and garlic oil supplementation. Cardozo et al. (2005) demonstrated increased ruminal propionate proportions with in vitro supplementations of garlic, cinnamaldehyde, and yucca at a pH of 5.5.

With butyric acid, effects were only observed for the essential oils *O. basilicum #14 accession, O. basilicum #23 accession, O. basilicum #32 accession*, *Artemisia dracunculus, Chrysantha parthenium, M. arvensis cv. Arvensis 3*, *O. basilicum #34 accession, Mentha spicata L., Chrysantha parthenium,* and *Ruta graveolens* (Table 5). Their effects in vitro all resulted in increases in butyric acid production. These results agree with a study by Castillejos et al. (2006) which demonstrated increased butyrate production with thymol supplementation in vitro, and with a study by Busquet et al. (2005a) which illustrated increase in butyrate proportions with cinnamaldehyde and garlic oil in vitro supplementation. Busquet et al. (2006), on the contrary,

showed reduced butyrate production, but supplemented at much higher levels of 300 and 3000 mg/L.

For Monensin, only in the plant material Batch 1 was there a significant increase in both propionic and acetic acid and only in the essential oil Batch 6 was there a significant decrease in the production of butyric acid (Table 3; Table 5). These results are partially supported by a study by Garcia-Lopez et al. (1996), which suggests Monensin contributes to a decrease in acetic acid amounts and an increase in propionic and butyric acid production. Busquet et al. (2005c) also showed an increase in propionate from Monensin supplementation, but unlike the current results, acetate was also significantly decreased in vitro.

In vitro ammonia production was affected by several plant substances. Most favorably, decreases in ammonia were observed for *O. basilicum #25 accession, O. basilicum #39 accession,* and *Oplopanax horridus* (root bark) (Table 4; Table 5). Such results agree with the first essential oil study by Borchers (1965), which showed an in vitro ammonia reduction from thymol supplementation. Moreover, studies by Cardozo et al. (2005) and Castillejos et al. (2006) also demonstrated in vitro reductions in ruminal ammonia concentrations when supplemented with essential oils such as cinnamaldehyde, garlic oil, and eugenol. Although present in small numbers, hyper-ammonia producing (HAP) bacteria are large contributors to ammonia and highly susceptible to essential oils (Wallace, 2005). Thus, through inhibition of HAP bacteria, these plant products could be reducing ruminal ammonia production.

Increases in ammonia production were also noted with treatments of the essential oils *O. basilicum #14 accession, Mentha piperita, Satureja hortensis L.,* and *Ambrosia artemisisfolia* (Table 5). Such increases in ammonia production have not been duplicated in other in vitro studies.

In vitro Monensin increased ammonia production in two of the batches, but decreased ammonia production in another batch (Tables 3, 4, and 5). These results partially agree with a study by Garcia-Lopez et al. (1996) that was unable to detect effects of Monensin on ruminal ammonia production. Mixed results have been observed by a variety of studies however, including a decrease in ammonia observed by Busquet et al. (2005a) and an increase in ammonia concentrations with Busquet et al. (2005c).

Oregano supplementation in vivo

With the beneficial effects of decreasing methane production by 31.3% and increasing NDF digestibility by 6.0%, *Origanum vulgare* was selected for the second portion of the study, involving in vivo experimentation with 8 dairy cows. The essential oil of oregano is known for having antimicrobial properties, and is composed of a wide variety of compounds. Although quite variable with cultivation techniques, this essential oil composition typically includes 79.58 percent carvacrol and 2.45 percent thymol, in addition to compounds such as γ-terpinene and *p*-cimene (Calsamiglia et al., 2007). Carvacrol has been shown to decrease large peptide concentrations, while not affecting ruminal ammonia concentrations, suggesting peptidolytic stimulation (Busquet et al., 2005c). The mechanism behind carvacrol's antimicrobial activity is thought to involve its disruption of ion gradients via diffusion across the microbial plasma membrane, transporting of hydrogen ions in and potassium ions out (Calsamiglia et al., 2007). A highly studied essential oil component, thymol supplementation has been found to decrease ammonia production, increase free amino acids, and reduce methane production (Calsamiglia et al., 2007). Thymol likely is able to interact with cellular membranes, allowing for the disruption of ionic gradients, leading to eventual microbial death (Calsamiglia et al., 2007).

Of the in vivo milk production traits, fat component amounts were significantly increased with the oregano diet, with cows producing 0.08 kg of milk fat more per day (Table 7). In addition, 3.5% fat corrected milk (FCM) feed efficiency was also increased by 0.1% with the oregano treatment, suggesting the cows were able to use nutrients more efficiently (Table 7). Such effects could arise from improved energy efficiency as seen with the reduction in methane production. The oregano treatment could have contributed to methane retention, through a reduction in methanogenic microbes. Therefore, more hydrogen could be incorporated into energetic molecules such as propionate, a glucose precursor, allowing the cow to direct energy towards productive traits and potentially milk fat secretion.

No significant differences were observed for milk yield, FCM yield, feed efficiency, milk protein, milk lactose, milk urea nitrogen, or the somatic cell count (Table 7). Consistent milk yield and FCM yield among the control and treatment groups agrees with a study by Tassoul and Shaver (2009) which demonstrated essential oil supplementation over 15 weeks of lactation had little effect on both characteristics. Tassoul and Shaver (2009), however, also demonstrated improved feed efficiency with essential oil mixture supplementation, over weeks 8 through 14 of lactation. And although there was no difference in milk protein content, the study by Tassoul and Shaver (2009) demonstrated a 0.15% reduction in milk protein with supplementation of an essential oil blend.

There were also no significant effects observed for intake or fecal matter composition, as well as for the apparent nutrient digestibilities (Table 8). The lack of change in DM intake agrees with another study of essential oils that illustrated DM intake was unaffected by garlic and juniper berry oil supplementation (Yang et al., 2007). Such a result may indicate the palatability of the feed was not an issue for the plant supplementation. However, a Tassoul and

Shaver (2009) study detected reduced DM intake over a 15-week lactation when supplementing a mixed essential oil diet.

Most ruminal fermentation characteristics were unaffected by the oregano diet, including protozoa counts, ruminal pH, and total free amino acids (Table 9). Likewise, a study conducted by Wallace (2005) concluded that essential oils have little impact on protozoal populations. However in contrast, Evans and Martin (2000) demonstrated an increase in rumen fluid pH with in vitro supplementation of 400 µg/ml, and Borchers (1965) found an increase in ruminal amino acids in vitro with thymol treatment.

Total VFA, acetate, propionate, and butyrate production were all unaffected by the oregano supplementation, as was the acetate-to-propionate ratio (Table 9). Thus, the VFA producing microbes were likely unaffected by the oregano supplementation and were allowed to maintain similar populations and activity. Such results are supported by Busquet et al. (2005c) which demonstrated that carvacrol supplementation in vitro had no effect on volatile fatty acid production or ratios. In vitro, thymol, another component of oregano, has elsewhere been shown to decrease both acetate and propionate production while increasing the acetate-to-propionate ratio (Evans and Martin, 2000). Although the reduction in propionate and increase in the acetate-to-propionate ratio are unfavorable to ruminal energy metabolism, these results may indicate variances in component and oregano effects.

The most encouraging result was found in the significant decrease in ruminal methane production observed in vivo (Table 11), reinforcing the in vitro result of a 31.3% decrease in methane production (Figure 1). This pronounced reduction indicates that methanogenic microbes were inhibited by the oregano diet, quite possibly because of the cell membrane interactions of carvacrol or thymol. Thus, less methane was produced in the rumen, allowing for

the incorporation of hydrogen ions into other compounds to be used by the cow for maintenance or production. These results agree with an in vitro study of Evans and Martin (2000), which showed reductions in methane production with thymol supplementation. In addition, studies by Busquet et al. (2005b) have also illustrated the ability of compounds such as garlic oil to reduce ruminal methane.

Interestingly, ruminal ammonia nitrogen concentrations were significantly higher with the oregano diet, suggesting ruminal alterations in microbial populations allowed for the accumulation of ammonia. Likewise, a study by Cardozo et al. (2004) illustrated increases in ruminal ammonia production 4 hours after supplementation in vitro with 0.22 mg/L oregano. However, such an increase in ammonia contradicts the results of Borchers (1965) that demonstrated ammonia production to be reduced in vitro with thymol supplementation. Most likely, such effects were seen as thymol inhibits deamination and peptidolysis by ruminal microbes. Again, such differences among oregano and its component thymol may be a result of synergistic effects of the oregano components.

Blood testing throughout the supplementation also indicated no significant changes in blood glucose levels, but did show elevated blood urea nitrogen (BUN) levels (Table 10). As ammonia levels were higher in the rumen, such results suggest that this ammonia non-protein nitrogen waste could be transported in the blood as urea after processing by the liver. A study by Tassoul and Shaver (2009) also illustrated no effect on blood glucose levels with essential oil mixture supplementation, but in contrast showed no effects on blood urea nitrogen.

Gaseous emissions of carbon dioxide, methane, and ammonia from cow feces were unaffected by the oregano diet (Table 11). As methane was reduced within the rumen, these results indicate that the ruminal decrease did not translate to a reduction in emitted fecal

methane. Areas of the digestive tract, aside from the rumen, may have been contributing to the methane release and preventing the carry-over of methane reduction in fecal emissions.

Urine composition had notable differences between the control and oregano diets (Table 12). From the urine analysis, urea nitrogen amounts were decreased (Table 12), which seems to contradict the increase in ruminal ammonia production. For some reason, the increased ammonia production within the rumen did not result in more urea excreted as waste. Possibly, the ammonia was able to be retained within the cow and used for protein production likely through the use of recycled nitrogen. Because the milk protein nitrogen as a percent of nitrogen intake did increase, the nitrogen may have been used elsewhere.

Overview

The essential oils and plant materials of this study showed a wide variety of effects in vitro, both positive and negative. Showing the most potential, *Origanum vulgare,* or oregano, decreased ruminal methane production and improved NDF digestibility in vitro. The in vivo portion of the experiment served to more thoroughly examine these effects in the cow and illustrated a similar reduction in methane production in the rumen. In addition, the oregano diet in vivo increased milk fat amounts, blood urea nitrogen, and ammonia while decreasing urine urea nitrogen. Overall, these effects demonstrate a potential trade-off with oregano supplementation; as methane production is reduced possibly allowing for milk fat improvements, the ammonia production may rise and enter circulation rather than be incorporated into microbial protein.

Ultimately, potential issues with essential oil supplementation could arise as microbial populations could adapt to the given essential oils (Cardozo et al., 2004). After all, if the group

of microbes being selected against gains a resistance gene or adaptive strategy, they could overcome the selection pressures of the essential oil products. Such an effect would eventually lead to decreased effects on ruminal fermentation. In such a way, the supplementation would no longer be of use to that particular cow or herd.

In the end, further research is extremely important in this area of plant supplementation, as the use of ionophores becomes increasingly controversial. More detailed investigations of plants used in the original screening could provide more solid evidence of ruminal effects, and in addition, further in vivo experimentation could provide a more rounded perspective on whole-cow effects. Oregano, itself, needs to be examined more closely to identify active antimicrobial components, quite possibly carvacrol and thymol, and specifically how they induce changes in ruminal fermentation. As synergistic effects of the oregano components could be contributors to the beneficial outcomes, these interactions need to be examined as well.

Studies of essential oils and plant materials, such as this one, have notably identified advantages for ruminant supplementation. If the production and release of methane and ammonia could be reduced through plant supplements, the environmental impact could be reduced and overall feed efficiency of dairy cows could be increased. Moreover, if NDF digestibility can be increased, allowing for improved fiber digestion, more energy can be released from a given fiber source, providing for more efficient and productive animals. Although more work is needed to clarify the advantages and disadvantages of plant and essential oil supplementation, it is clear that these products may provide an alternative to the current practice of feeding antibiotic supplements.

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