Effects of essential oils, ionophore and live yeast supplementation on rumen fermentation dynamics in Jersey cows

By

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DECLARATION

I, Phillip Albert Meiring, declare that this dissertation, which I hereby submit for the degree MSc (Agric) Animal Science: Animal Nutrition at the University of Pretoria is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

A.P. Meiring
Pretoria
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SUMMARY

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The prohibition of the use of growth promoting antibiotics in animal feeds within the European Union (EU) and some other countries has led to an increased interest in alternative means of manipulating rumen fermentation. The objectives of our study were to determine the effects of alternative natural feed additives, essential oils (EO, Oleobiotec) and rumen specific live yeast (LY, Levucell), to monensin (MO) and their effects on rumen fermentation and *in sacco* NDF, starch and N disappearance.

The study consisted out of four rumen cannulated lactating Jersey cows that were used in a 4 x 4 Latin square design experiment. The four experimental treatments were: 1) Control (C), a lucerne hay/maize based TMR (10.7 MJ ME/kg, 17% CP, 32.1% NDF), 2) C + MO, 3) C + EO, 4) C + EO + LY. The experimental periods were 25 days with the last four days for rumen sampling and the *in sacco* study.

Mean ruminal pH ranged between 5.80 and 6.02 and tended ($P < 0.10$) to be lower in the control cows compared to the other treatments. Total VFA production, acetate : propionate ratio and rumen ammonia nitrogen concentration did not differ ($P > 0.05$) but lactic acid concentration tended to be lower for the C + EO treatment when compared to MO supplemented cows. *In sacco* TMR starch disappearance after 24 hours were higher for the C + EO treatment when compared to the C and C + EO + LY treatment groups ($P > 0.05$). Milk production and DMI did not differ ($P > 0.05$) but fat % was higher for EO supplemented cows (4.52%) when compared to C cows (4.32%) ($P < 0.05$). Results suggest a negative interaction between the essential oil and live yeast products used in this study. More research is needed on potential complimentary effects and interactions between feed additives.

LIST OF ABBREVIATIONS

AA	Amino acids
A:P	Acetic: propionic acid ratio
ADF	Acid detergent fibre
ADL	Acid detergent lignin
ADIN	Acid-detergent insoluble nitrogen
BCS	Body condition score
BW	Body weight
Ca	Calcium
CF	Crude fibre
CFU	Colony forming units
CP	Crude protein
^{o}C	Degree Celsius
d	Day
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
ECM	Energy corrected milk
EE	Ether extract
EO	Essential oils
EU	European Union
FCM	Fat-corrected milk
g	Gram
GE	Gross energy
GIT	Gastrointestinal tract
HAP	Hyper ammonia producing
H_2O	Water
H_2SO_4	Sulphuric acid
H_3PO_3	Ortho-phoshoric acid
IVOMD	*In vitro* organic matter digestibility
Kg	Kilogram
KJ	Kilojoules
LA	Lactic acid
M	metre
ME	Metabolisable energy
Mg	Milligram
MJ	Megajoules
MUN	Milk urea nitrogen
N	Nitrogen
NEFA	Non-esterified fatty acids
NAN	Non-ammonia nitrogen
NDF	Neutral detergent fibre
NH_3-N	Ammonia-nitrogen

NPN	Non-protein nitrogen
NSC	Non-structural carbohydrates
OM	Organic matter
P	Phosphorus
pH	The negative logarithm to the base ten of the hydrogen ion activity in the solution
r	Ruminal
R	South African Rand
SEM	Standard error of the mean
SCC	Somatic cell count
SD	Standard deviation
Sol CP	Soluble crude protein
Temp	Temperature
TMR	Total mixed ration
USA	United States of America
VFA	Volatile fatty acids
Wt	Weight

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CHAPTER 1

INTRODUCTION

Rumen fermentation processes play a key role in ruminant nutrition (Van Soest, 1994). It is this symbiotic feature between the animal and the rumen micro flora that gives the ruminant animal several advantages in digestive and metabolic processes over non ruminants (DiLorenzo, 2010). The extent of complexity of interactions between diverse groups of microorganisms which inhabit the rumen is such that, even nowadays some pathways and microorganisms are unknown (Van Soest, 1994). The possibility of controlling some of the metabolic processes in the rumen with the objective of achieving more efficient nutrient utilization has been an appealing concept to both nutritionists and rumen microbiologists. Because of several decades of continuous research on rumen fermentation, metabolism and microbiology, the rumen is one of the most extensively studied microbial ecosystems (Van Soest, 1994). The manipulation of rumen fermentation has certain main objectives which include increasing beneficial processes, while at the same time decreasing, changing or removing processes that are inefficient and harmful to the animal (DiLorenzo, 2010).

Ionophore antibiotics such as monensin were approved as a feed additive in ruminant diets in the mid-1970s in the USA (McGuffey *et al.*, 2001). Since then research on ionophores and their effects have multiplied rapidly. This led to several ionophores being discovered and approved as feed additives for dairy cows and this greatly improved the efficiency of the dairy industry (McGuffey *et al.*, 2001). The cost effectiveness of ionophores made it very popular and led to the wide use of this product around the world (McGuffey *et al.*, 2001).

In recent years concern over the use of antibiotics in livestock nutrition has increased due to the emergence of antibiotic resistant bacteria that may represent a risk to human health (Barton, 2000). The use of ionophores as a growth promoter therefore has been banned in the European Union in January 2006 (Regulation 1831/2003/EC) (DiLorenzo, 2010). Consumers demand for safe, high quality nutritious food has stimulated the search for natural alternative additives such as probiotics, yeast products, essential oils and enzymes (Patra, 2011). The contribution of greenhouse gas emissions from enteric fermentation of livestock debate has also redirected research towards natural modifiers of rumen fermentation which has the capacity to reduce methane production (Barton, 2000; Benchaar and Greathead, 2011). Therefore recent research has been mainly focused to exploit plant bio-actives, such as essential oils and yeast products, as natural additives, to improve the efficiency of rumen fermentation, decrease methane production or reduce nutritional stress such as acidosis and bloat and improve overall productivity (Benchaar and Greathead, 2011). Renewed interest has also been shown in the use of essential oils as an inhibitor of feed borne pathogens such as E. coli O157:H7. Ruminant animals were most affected by this food borne pathogen which cause increased foodborne illnesses (DiLorenzo, 2010).

The objectives of this study were to (i) determine the effects of an ionophore (monensin), essential oils (Oleobiotec) and a yeast product (Levucell SC 10 ME-Titan), as well as the potential additive effect of the essential oil and yeast product additives on dairy cow performance by determining their effect on rumen pH and general rumen fermentation and (ii) to determine whether natural alternatives like essential oils and yeast products or a combination of these have the potential to replace ionophore antibiotics as the primary fermentation altering feed additive for lactating Jersey cows.

The following hypotheses were tested in this study:

H_0 = Essential oils, a yeast product or a combination of these additives have the potential to replace ionophores in dairy cattle diets.

H_1 = Essential oils, a yeast product or a combination of these additives do not have the potential to replace ionophores in dairy cattle diets.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Feed additives are typically non-nutritive compounds added to ruminant diets to help animals utilize nutrients better. This leads to better performance, decreased risk of metabolic diseases like rumen acidosis and bloat and reduced impact of the diet on the environment (Adesogan, 2009). Feed additives work in a number of ways: It regulates rumen pH, decrease lactate accumulation, stimulate rumen development in young ruminants, increase rumen organic matter and fibre digestibility and decrease the risk of metabolic diseases (Newbold and Rode, 2006). Feed additives also affect rumen energy and nitrogen utilization (Alexander *et al.*, 2008). Rumen energy usage is improved by decreasing methanogenesis and decreasing the acetate: propionate ratio without lowering milk fat synthesis. Rumen N usage is improved by decreasing proteolysis, peptidolysis and amino acid deamination. This leads to minimal NH_3 losses to the environment (Alexander *et al.*, 2008). Nitrogen usage is also improved by decreasing the activity of rumen protozoa which break down and devour beneficial bacteria, contribute to proteolysis and deamination and act as hosts for methanogens (Adesogan, 2009). Feed additives also increase the synthesis of microbial protein by facilitating coupling of rumen energy and protein supply which also leads to improved N usage (Wang *et al.*, 2001). The main objective of feed additives is to increase the level and efficiency of animal performance but also to be cost effective and approved by the proper legislative authorities (Adesogan, 2009).

The objectives of beef nutrition differ from dairy nutrition because their end products are different. Different volatile fatty acids are needed as end products from fermentation to produce beef and dairy products respectively (Duffield and Bagg, 2000; McGuffey *et al.*, 2001). Beef nutritionists want to increase the molar proportions of propionate while dairy nutritionists want to increase the molar proportions of butyrate and acetate (Ipharraguerre and Clark, 2003). A big increase in propionate at the expense of butyrate and acetate has unfavourable consequences for milk fat content which to a large extent determines the profitability of a dairy enterprise (DiLorenzo, 2010).

There is a substantial variety of feed additives affecting the rumen that is available to dairy farmers. These additives differ in a number of ways. This review will be focussing only on ionophores, essential oils and yeast products.

2.2 Ionophores

2.2.1 Introduction

Ionophores are typically added to ruminant diets for the purpose of increasing the feed utilization efficiency (Dubuc *et al.*, 2009). It is organic compounds mainly from *Streptomyces* species which causes selective transportation of ions across outer cell membranes (Duffield *et al.*, 2008a). The most common ionophores include Rumensin (Monensin Sodium), Bovatec (Lasalocid Sodium), Salinomycin and Cattlyst (Laidlomycin

Propionate Potassium) (Yang *et al.*, 2007). Rumensin is the most widely used ionophore and is generally known as Monensin since monensin sodium is the active ingredient in the commercial product Rumensin (Duffield *et al.*, 2008a).

The dairy industry of South Africa makes widely use of ionophores to enhance milk production. This is done by improving the efficiency of feed utilisation of the animals, which in turn improves profit (AlZahal *et al.*, 2008). Ionophores improve feed conversion by altering rumen fermentation (AlZahal *et al.*, 2008). Rumen fermentation is altered which results in changes in the rumen microbial population (AlZahal *et al.*, 2008). Transmembrane movement and intracellular equilibrium of ions in certain classes of bacteria and protozoa that occur in the GIT is interrupted by ionophores (McGuffey *et al.*, 2001). Ionophores therefore increase the numbers of certain microbes and decrease the numbers of others (Mcguffey *et al.*, 2001). These selected microbes favour the host animal in a number of ways.

Ionophores give very consistent results both *in vivo* and *in vitro* and it is this characteristic that makes it the golden standard against which other feed additives are evaluated (DiLorenzo, 2010).

2.2.2 Mode of action

Ionophores are highly lipophilic and can change the ion exchange gradient over bacterial membranes. The bacteria cells try to correct this which causes an energy spilling cycle. An energy shortage results and cell death soon follows (Russel and Strobel, 1989).

Ionophores affect the rumen mainly by lowering the gram positive bacteria population numbers relative to the gram negative bacteria (McGuffey *et al.*, 2001). Gram negative bacteria have a complex cell wall which gram-positive bacteria don't have. This cell wall has a lipopolysaccharide layer containing protein channels/porins with a size which doesn't allow ionophores to pass through. This leads to ionophores not being able to infiltrate gram-negative bacteria (Ipharraguerre and Clark, 2003). The lack of a cell wall in gram-positive bacteria however allows ionophores to infiltrate the outer cell membrane of these bacteria (McGuffey *et al.*, 2001; Ipharraguerre and Clark, 2003). The Ionophores cause rapid and repeated outflow of intracellular potassium from the cell and inflow of extracellular protons (Sodium and Hydrogen) into the cell (Ipharraguerre and Clark, 2003). This results in acidity and a potassium depletion which repress protein synthesis (Ipharraguerre and Clark, 2003). Protons are transported out of the cells by ATPase pumps which empty the energy reserves used for bacterial growth (Ipharraguerre and Clark, 2003). Cell death results because of the cytoplasmic acidity (McGuffey *et al.*, 2001). Different ionophores might have small differences in their mode of action but the end result is usually the same: a decreased gram-positive bacteria population count in the rumen (Duffield *et al.*, 2008a).

The fermentation end products of ruminal gram positive bacteria include amongst others: acetate, buyrate, lactate, formate, ammonia and hydrogen (Odongo *et al.*, 2007). Methane production is coupled with most of these fermentation processes. Gram negative bacteria on the other hand mostly give propionate and succinate as fermentation end products

(Odongo *et al.*, 2007). Less methane is produced when these bacteria dominate the rumen (McGuffey *et al.*, 2001). This is because of the decreased availability of formate, ammonia and hydrogen. Ionophores therefore indirectly lead to reduced methane production by inhibiting the protozoa that produce these products (Ipharraguerre and Clark, 2003). It has not been observed that methanogenic bacteria are directly inhibited by ionophores (Duffield and Bagg, 2000). The main advantage of including Ionophores in ruminant diets is the shift in acetate to propionate ratio toward increased propionate and decreased acetate and the associated reduction in methanogenesis (Duffield *et al.*, 2008b). Lactic acidosis and certain other metabolic disorders are decreased by ionophores because of the reduction in rumen lactate producing bacteria (Russel and Strobel, 1989). Dietary Protein is used more effectively with the addition of ionophores because it decreases the ammonia production in the rumen (Ipharraguerre and Clark, 2003).

2.2.3 Effects on rumen nitrogen and energy usage

Feed is fermented to less preferred products by Gram-positive bacteria. These include acetate, H^+, CH_4 and NH_3. Ionophores decrease the production of these metabolites by destroying gram-positive bacteria. This leads to improved rumen function and animal performance (Callaway *et al.*, 2003). Decreased acetate: propionate ratio in the rumen also results from ionophores. This increases the effectiveness of rumen energy usage. Rumen methane production is reduced up to 30% by ionophores (Johnson and Johnson, 1995). Ionophores achieve this not by inhibiting methanogens directly but rather by suppressing bacteria producing formate and H_2 which are methane precursors (Dellinger and Ferry, 1984; Johnson and Johnson, 1995).

Peptidolysis and amino acid deamination is decreased substantially by ionophores while proteolysis is decreased only slightly (Newbold *et al.*, 1990). It is caused by the inhibition of gram-positive hyper ammonia producers. This leads to an increase in the postruminal supply of proteins and peptides (Hobson and Stewart, 1997).

2.2.4 Effects on animal performance and health

The ratio of volatile fatty acid (VFA) production is changed by ionophores, propionic acid is increased while acetic- and butyric acid is decreased (Dubuc *et al.*, 2009). Propionic acid is a glucose precursor in ruminants and therefore feeding ionophores to dairy cows has various positive benefits like better energy metabolism and lower incidence of subclinical ketosis, clinical acidosis and abomasal displacement (Dubuc *et al.*, 2009). A decrease in methanogenesis is also associated with this volatile fatty acid ratio change (Russel and Houlihan, 2003). The increased propionic acid production also increased hepatic gluconeogenic flux and the decreased acetic- and butyric acid caused a decrease in milk fat percentage (Duffield *et al.*, 2008a). Ionophores cause decreased milk fat percentages not just by decreasing the ruminal production of acetate and butyrate, but also by the inhibition of ruminal biohydrogenation of long chain fatty acids (Fellner *et al.*, 1997) and by enhancing the trans-10, cis-12 CLA supply to the mammary gland (Griinari *et al.*, 1998; Benchaar *et al.*, 2006), which is a milk fat synthesis inhibitor in dairy cows (Baumgard *et al.*, 2000). Certain nutritional factors like feeding management and diet composition were found to influence the

effect of ionophore supplementation on the milk fat percentage (Duffield and Bagg, 2000). Although various studies showed a decreased milk fat percentage with ionophore supplementation (Phipps *et al.*, 2000; Odongo *et al.*, 2007), others showed ionophore supplementation had no effect on the milk fat percentage (Lean *et al.*, 1994; Duffield *et al.*, 1999). Suggestions have been made that the inconsistency of the fat depressing effect of ionophores may be a result of dietary factors like the ionophore dose level, administration method of the ionophores, lactation stage (Duffield and Bagg, 2000) and feeding system (Duffield *et al.*, 2003).

Ionophores can have positive and negative effects on ruminant digestion depending on the inclusion rate, diet composition and level of feed intake (McGuffey *et al.*, 2001). Other benefits of feeding ionophores to dairy cows include improved milk production, feed efficiency (McGuffey *et al.*, 2001), antiketogenic effects (Duffield and Bagg, 2000), improved body condition scores (Duffield *et al.*, 1999), decreased risk of acidosis by restricting rumen lactic acid production (McGuffey *et al.*, 2001) and control of legume bloat (Maas *et al.*, 2002).

A study by Odongo *et al.* (2007) showed that ionophore supplemented cows had lower milk protein content compared to control cows. These results were in agreement with other studies (Hayes *et al.*, 1996; Green *et al.*, 1999; Phipps *et al.*, 2000). In contrast, some studies showed that ionophores had no effect on milk protein (Ramanzin *et al.*, 1997; Benchaar *et al.*, 2006; Weimer *et al.*, 2008).

Ionophores can also cause a decrease in dry matter intake (Adesogan, 2009). A study by Odongo *et al.*, 2007 however showed no dry matter intake changes as well as no milk yield changes with the addition of ionophores. Various other studies also found no changes in dry matter intake and milk production after supplementing ionophores to the animals (Van der Werf *et al.*, 1998; Duffield *et al.*, 1999; Phipps *et al.*, 2000).

Coccidiosis can be prevented very efficiently with ionophores (Waggoner *et al.*, 1994). Rumen acidosis and bloat can also be prevented to a large extent by ionophores by means of repressing the growth of gram positive lactate producing bacteria such as *Lactobacillus* and *Streptococcus bovis* species (McGuffey *et al.*, 2001). Ionophores stimulate increased rumen propionate production which leads to an increased glucose supply which leads to a decreased risk of ketosis and fatty liver syndrome (Duffield and Bagg, 2000; Adesogan, 2009).

2.2.5 Effect on methane production

Fermentation in the gastrointestinal tract of ruminants produce a colourless, odourless gas named methane (Odongo *et al.*, 2007). One litre of methane equals 39.5 kJ of feed energy and therefor methane production represents a loss of feed energy. Enteric methane emissions are a major source of greenhouse gas produced by agriculture and it is one way in which global methane emissions can be reduced (Guan *et al.*, 2006).

A lot of research and effort is being put into developing strategies to decrease rumen methane production by rumen micro-organisms (Adesogan, 2009). These various strategies

are in different stages of development and only a few inhibitors are commercially available and economically affordable to the producer (Johnson and Johnson, 1995). Ionophores are commonly used in the dairy as well as the beef industry and are therefore feasible as a feed additive used for decreasing methane emissions from ruminants (Guan *et al.*, 2006).

Ipharraguerre and Clark (2003) found that ionophores are highly lipophilic and toxic to various fungi, protozoa and bacteria. Studies showing that ionophore supplementation reduce rumen methane production vary in the extent of the decrease as well as the persistence of the response (Mbansamihigo *et al.*, 1996). In a study by Ushida and Jouany (1996) they found that even though there is no evidence that rumen methanogens are directly affected by ionophores, they can have an indirect effect when protozoa act as a symbiotic host for methanogens.

A study by Guan *et al.* (2006) found that the supplementation of ionophores caused a 27-30% decrease in methane production. These reductions were however short lived and depending on the energy content of the diet, within 2 to 4 weeks the methane production levels returned to the level they were before ionophores were supplemented (Guan *et al.*, 2006).

2.2.6 Summary

Ionophores decrease the gram positive bacteria population of the rumen which leads to a decreased acetate: propionate ratio and decreased methane production. This cause more efficient usage of energy and reduced environmental pollution. Less Gram-positive bacteria also lead to better protein utilization because of decreased peptidolysis and amino acid deamination. Ionophores lower the incidence of acidosis, bloat, coccidiosis and ketosis. It affects milk by increasing the production efficiency and decreasing milk fat concentration. Ionophores are supplemented to lactating cows to increase feed efficiency and to dry cows to decrease metabolic disorders.

2.3 Essential oils

2.3.1 Introduction

In the last few decades, various chemical feed additives like ionophores, antibiotics and methane inhibitors have been evaluated in ruminant diets to regulate rumen fermentation, increase growth and milk yield and also to increase feed intake and efficiency (DiLorenzo, 2010). Microbial adaptation to these additives and toxicity problems to the host animal however led to most of these additives not being used routinely (Barton, 2000). Increased awareness from public health aspects like chemical residues in milk and meat and bacterial resistance to antibiotics resulting from increased use of these additives led to the prohibited use of some of these feed additives. Because of product quality and safety consumers' organisations have criticized these products (Barton, 2000). Consumer demands led to the search for a suitable natural alternative for these chemical feed additives (Barton, 2000). Herbivore diets consist out of plants which allow plants containing bioactive compounds like essential oils with antimicrobial actions to be explored and utilized for the improvement of

feed utilization and health (Cowan, 1999). Research has therefore recently focused on exploiting plant bioactives as natural feed additives to better rumen fermentation by reducing methane production, increasing protein metabolism (Kamra *et al.*, 2008; Patra and Saxena, 2010), bettering animal health and productivity and decreasing nutritional stress like bloat and acidosis (Patra, 2007).

Essential oils (EO) are naturally occurring secondary metabolites and volatile components extracted most commonly from herbs and spices but also from various parts of plants through distillation methods, mainly steam distillation (Patra, 2007). Essential oils have been used for many centuries as folk medicines and food preservatives because of their antimicrobial effect (Burt, 2004). They are complex mixtures of secondary metabolites and volatile compounds (Burt, 2004). Essential oils have antimicrobial activities against both gram-negative and gram-positive bacteria; this is because of the presence of terpenoid and phenolic compounds (Yang *et al.*, 2007). Essential oils are chemically not true oils but rather variable mixtures made up mostly out of two chemical groups (Patra, 2007). These two chemical groups include the most common essential oils: the groups are terpenoids (monoterpenoids and sesquiterpenoids) which are synthesized through the mevalonate metabolic pathway and phenylpropanoids which are synthesized through the shikimic acid metabolic pathway (Calsamiglia *et al.*, 2007; Patra, 2011). Of these two classes the terpenoids are the more diversified group of plant bioactives and are found in a wide variety of spices and herbs (Patra, 2011). They are derived from a basic structure of C5 isoprene units and the number of these units contained in its skeleton determines its classification (Patra, 2011). The most important components of EO of most plants within terpenoids belong to the monoterpenoids and the sesquiterpenoids (Calsamiglia *et al.*, 2007). Phenylpropanoids have a different structure containing an aromatic ring of C6 with a side chain of 3 carbons bound to it (Calsamiglia *et al.*, 2007). Compared with the terpenoids the phenylpropanoids are less abundant (Calsamiglia *et al.*, 2007). Some plants however contain significant proportions of these compounds of EO (Calsamiglia *et al.*, 2007).

The plant species, maturity, botanical fraction and environment determine the composition of the EO and different variations occur as these factors change (Yang *et al.*, 2007). Essential oils are seen as a potential alternative to antibiotic drugs and additives in both animal and human diets because they have antimicrobial properties against a wide variety of microorganisms (Benchaar *et al.*, 2008). Nutritionists and rumen microbiologists have therefore recently shown great interest in exploiting EO as natural feed additives for the purpose of improving rumen fermentation through volatile fatty acid production, inhibition of methanogenesis, improving protein metabolism and increasing the efficiency of feed utilization (Patra, 2011). A wide range of herbs and spices contain EO with the potential to manipulate the rumen and enhance animal productivity. These EO are alternatives that can very successfully replace chemical feed additives (Tager and Krause, 2011). Their effectiveness in ruminant production however has not been proven to be consistent and conclusive. Essential oils have given varying results with regard to rumen microorganisms, rumen fermentation and ruminant performance (Patra, 2011). These results depended upon

the dose, feed composition, animal physiology and chemical structures of the EO (Calsamiglia *et al.*, 2007).

Various parts of plants like the flowers, fruit, bark, leaves, roots, stem, seeds and pulps contain EO (Hart *et al.*, 2008). Various factors such as plant health, stage of growth and environmental factors like temperature, moisture stress and light affect the concentrations of EO (Hart *et al.*, 2008).

Because of the strong antimicrobial properties of various EO, essential oil research has recently been accelerated due to the ban in many developed countries of certain antibiotic growth promoters as feed additives (Benchaar *et al.*, 2006).

2.3.2 Mode of action

Hart *et al.* (2008) found that the main effects of EO in the rumen are due to the lower starch and protein degradation and the inhibition of amino acid degradation because of selective action on certain microorganisms in the rumen, especially bacteria.

Two theories have been proposed to explain the mode of action of EO. The first theory entails EO selectively inhibiting gram-positive bacteria in a similar way as ionophores (Burt, 2004). Some studies however suggest that EO also inhibit gram-negative bacteria (Busquet *et al.*, 2005b). This is possible because of the small molecular weight of EO which allows it to penetrate the walls of these bacteria (Benchaar *et al.*, 2008). Gram negative bacteria have been successfully inhibited by caravacrol and thymol (Helander *et al.*, 1998).

The second theory entails EO changing cells by interacting with certain processes of the cell membrane (Benchaar *et al.*, 2008). These processes include phosphorylation, ion gradients, ATP production, protein translocation etc. Essential oils are lipophilic and hydrophobic and these characteristics contribute to this effect (Benchaar *et al.*, 2008).

Patra (2011) also suggested that EO have 2 different modes of action. His suggestions however differed from the above. He suggested that one mode of action is that EO have an effect on the bacterial colonisation pattern of certain starch rich substrates entering the rumen. The second mode of action he suggested is that EO inhibit hyper ammonia producing bacteria involved in amino acid deamination.

2.3.3 Effect on rumen microbial populations

2.3.3.1 Rumen bacteria

The hyper ammonia producing bacteria in the rumen which cause reduced amino acid deamination is inhibited by certain EO (Wallace, 2004). In a study by McIntosh *et al.* (2003) it was observed that an essential oil mixture inhibited the growth of certain hyper ammonia producing bacteria like *Clostridium sticklandii* and *Peptostreptococcus anaerobius* and had less of an effect on other hyper ammonia producing bacteria like *Clostridium aminophilus*. These inhibitory effects of EO on bacteria can be diet dependant as is shown in a study by Wallace (2004). His study showed that sheep receiving a low protein diet supplemented with

100 mg a day of EO caused the number of hyper ammonia producing bacteria to decrease by 77%, but with sheep receiving a high protein diet the EO had only a small or no effect on hyper ammonia producing bacteria. Ruminal bacteria were also differently affected by individual EO (Patra, 2011). Compared to the corresponding oxygenated compounds the monoterpene hydrocarbons are not as toxic and often stimulatory to microbial activity (Patra, 2011). Wallace *et al.* (2002) found that hyper ammonia producing bacteria have a great capability to produce ammonia from amino acids. Essential oils in low doses can selectively inhibit hyper ammonia producing bacteria whereas higher doses affect all microorganisms (Busquet *et al.*, 2005b). A study by Evans and Martin (2000) showed that the growth of *Selenomonas ruminantium* was selectively inhibited by thymol at a concentration of 90 mg/L whereas *S. bovis* was not inhibited. In comparison a thymol concentration of 400 mg/L inhibited all rumen organisms. The digestion and colonization of readily degradable substrates by amylolytic and proteolytic bacteria can be suppressed by EO without affecting fibre digestion (Wallace *et al.*, 2002). Patra *et al.* (2010) however noted that carboxymethyl-cellulase and xylanase activity were reduced by clove and fennel extracts. This might be the result of the higher concentrations of EO found in the extracts.

2.3.3.2 Rumen protozoa

The effects of EO on rumen protozoa have delivered mixed reports. McIntosh *et al.* (2003) observed that dairy cows supplemented with 1 g/day of mixed EO had no effect on the bacteriolytic activity of rumen ciliate protozoa. Benchaar *et al.* (2007a) and Newbold *et al.* (2004) found similar results showing that dairy cows and sheep fed 750 mg/day and 110 mg/day of a mixture of EO respectively had no effect on their ruminal protozoa counts. Fraser *et al.* (2007) found that the number of ciliate protozoa was not affected when dairy cow diets were supplemented with 0.5 g of cinnamaldehyde per litre of rumen fluid. The protozoa were not affected by the extract of fennel (Patra *et al.*, 2010). The results found in a study by Ando *et al.* (2003) were contradictory to the above in that Holstein steers had a decrease in the total number of protozoa as well as the numbers of Isotrica, Diplodium and Entodinum when they were fed 30 g of peppermint (*Menta piperita L.*) per kg of total dietary dry matter intake. These results were attributed to the presence of EO (Ando *et al.*, 2003). Patra *et al.* (2010) also observed that the total numbers of protozoa, holotrichs and small entodiniomorphs were decreased and the large entodiniomorphs were not affected by clove extracts containing EO. In a study by Cardozo *et al.* (2006) it was found that the numbers of holotrichs increased and the entodiniomorph numbers stayed the same with the addition of a mixture of cinnamaldehyde at 180 mg/day and eugenol at 90 mg/day to beef heifer diets. There was however no effect on these protozoal numbers when the concentrations of both cinnamaldehyde and eugenol were higher at 600 mg/day and 300 mg/day respectively (Cardozo *et al.*, 2006). It was also observed by Yang *et al.* (2010a) that total protozoal as well as Dasytricha, Entodinium and Dasytricha species numbers were not affected by cinnamaldehyde supplementation at 0.4 to 1.6 g/day in steers. In contrast to these findings Cardozo *et al.* (2006) found that the numbers of holotrichs and entodiniomorphs decreased by feeding 2 g/day of anise extract containing 100 g/kg of anethol to beef heifers. The overall conclusion that can be made is that the numbers and activity of ruminal protozoa are not markedly affected by EO and their components.

2.3.4 Effect on digestibility and rumen fermentation

2.3.4.1 Feed digestion

Several studies found that EO didn't affect the digestibility of feeds (Malecky *et al.*, 2009; Meyer *et al.*, 2009; Santos *et al.*, 2010). A study by Yang *et al.* (2007) found that a control diet for Holstein cows containing 60% barley based concentrate and 40% forage had a 13% higher dry matter digestibility when supplemented with 2 g/day juniper berry essential oil. Experimental treatments did however not affect total tract digestibilities of organic matter, dry matter, starch and fibre (Yang *et al.*, 2007). Yang *et al.* (2007) suggested that the increased ruminal digestion of dietary protein of the cows receiving the essential oil supplementation compared to cows receiving only the control diet was the reason for the increased ruminal digestibility. A study by Malecky *et al.* (2009) also showed that different nutrient digestibilities in dairy goats were not affected by a monoterpene blend. Dry matter and fibre digestibility in the rumen is reduced with increased essential oil concentrations (Beauchemin and McGinn, 2006; Yang *et al.*, 2010a).

2.3.4.2 Volatile fatty acids

Most studies showed that the total volatile fatty acid concentrations were not affected by EO (Chaves *et al.*, 2008c; Malecky *et al.*, 2009; Patra *et al.*, 2010). Other studies showed a decrease in the total volatile fatty acid concentrations especially if higher essential oil concentrations were fed (Macheboeuf *et al.*, 2008; Kumar *et al.*, 2009). Some studies however showed an increase in the total volatile fatty acid concentrations with the supplementation of 0.2 g cinnamaldehyde per kg DM intake (Chaves *et al.*, 2008b) and 0.25 g essential oil extract from oregano per kg DM intake (Wang *et al.*, 2009). An *in vitro* study by Castillejos *et al.* (2005) found that the total volatile fatty acid concentrations were increased without the nitrogen metabolism being affected when a blend of EO were added at 1.5 mg/L. The type of substrates fed to the ruminant determines to a large extent what the essential oil responses on the total volatile fatty acid concentrations are going to be (Castillejos *et al.*, 2005). The total volatile fatty acid concentrations of cows fed on an alfalfa silage based diet were not affected, but cows fed a corn silage based diet with an addition of an essential oil mixture of 0.75 g/day had a decrease in volatile fatty acid concentrations (Benchaar *et al.*, 2007a). In some studies the addition of EO increased the acetate to propionate ratios (Benchaar *et al.*, 2007b; Macheboeuf *et al.*, 2008; Agarwal *et al.*, 2009), while in other studies these ratios remained unchanged (Kumar *et al.*, 2009; Wang *et al.*, 2009). A decrease in acetate to propionate ratio is normally associated with a methane production inhibition achieved by specifically targeting the methanogens (Patra, 2011). The essential oil effects may depend on the rumen fluid pH (Cardozo *et al.*, 2005). In a study by Cardozo *et al.* (2005) it was observed that at a low pH the rumen volatile fatty acid profile was affected to a larger extent by some EO. They proposed that the essential oil molecule status of dissociated or undissociated depends on the rumen pH. Spanghero *et al.* (2008) had similar observations of the end products of fermentation being shifted by a blend of EO at a low pH. They observed a decrease in the acetate proportion as well as the acetate to propionate ratio only if the essential oil fluid had a lower pH.

2.3.4.3 Ammonia

Because the rumen hyper ammonia producing bacteria are inhibited by EO it leads to a decrease in ammonia and deaminase activities (Patra, 2011). Only about 1% of the rumen bacteria population consists out of hyper ammonia producing bacteria, but these bacteria have very high deaminase activity (Wallace, 2004). The rate of rumen ammonia production can be decreased by this which in turn may increase the rumen protein efficiency and thereby be nutritionally beneficial to the animal (Wallace *et al.*, 2002). A 25% decrease in bacterial deamination activity *in vitro* has been reported by Newbold *et al.* (2004). Various studies reported an *in vitro* decrease in ammonia concentrations. Cardozo *et al.* (2005) reported an ammonia concentration decrease of 30 to 300 mg/L with oregano oil and a decrease of 0.3 to 300 mg/L with cinnamon oil. Similarly Busquet *et al.* (2006) found a decrease of up to 3000 mg/L with cinnamaldehyde. These effects however don't always apply as was found in certain *in vitro* (Busquet *et al.*, 2006) and *in vivo* (Castillejos *et al.*, 2005; Benchaar *et al.*, 2007a) studies.

Certain EO reduced the ammonia concentrations at low doses compared to other EO in *in vitro* studies. Castillejos *et al.* (2006) found that the rumen ammonia concentrations were reduced at 5 mg/L of Guaiacol while limonene and thymol only caused a reduction at 50 mg/L and eugenol and vanillin tended to reduce the rumen ammonia concentrations at the 50 mg/L and the 500 mg/L concentrations. The importance of the optimum dose for a certain type of essential oil is clearly demonstrated by the above. The type of protein meal in the diet may also affect these effects. Wallace *et al.* (2002) investigated the degradation rate of different protein meals and the colonisation of feedstuffs which were incubated in nylon bags by enzyme activity in the presence of EO. Pea meal which was the most rapidly degraded meal of the protein meals tested was the only meal significantly affected by EO (Wallace *et al.*, 2002). In animals receiving EO the bacterial proteinase and amylase affiliated with the plants protein supplement was likely to be lower, whereas the corresponding fishmeal activities were unaffected (Wallace *et al.*, 2002). Essential oils caused a reduction in the total microbial colonization affiliated with grass hay placed in the rumen, while the colonization of less degradable fibrous substrates like barley straw and grass silage was not affected (Wallace *et al.*, 2002). This served as an indication that the colonization and digestion of readily degradable substrates by proteolytic and amylolytic bacteria can be suppressed by EO without affecting fibre digestion (Wallace *et al.*, 2002). The adaptation of rumen microorganisms and the fast rumen metabolism of EO to a less active form may be the cause of the lack of effect of EO on rumen fermentation (Wallace *et al.*, 2002).

2.3.4.4 Methane production

Certain essential oil components have inhibitory effects on methanogenesis (Benchaar and Greathead, 2011). In a study by Evans and Martin (2000) it was found that the main component namely thymol of the essential oils derived from thymus and origanum plants inhibited *in vitro* methane production strongly and also decreased acetate and propionate concentrations. In another study by Macheoeuf *et al.* (2008) a suppression of methane to the extent of 99% at a concentration of 6 mM of essential oils from origanum vulgare and its component thymol was found. Chaves *et al.* (2008a) found that an inhibition of methane *in vitro* was caused by anethole at a concentration of 20 mg/L.

EO from different spices and plants have shown inhibitory effects on methane production in various studies. Strong inhibitory effects on methanogenesis have been shown by essential oils from cinnamon oil and juniper berry (Chaves *et al.*, 2008a) and peppermint oil (Agarwal *et al.*, 2009). In a study by Macheboeuf *et al.* (2008) it was found that cinnamaldehyde which is the active component of cinnamon oil caused a 94% methane production depression at a concentration of 5 mmol/L rumen fluid. McKay and Blumberg (2006) observed that menthol, menthone and methyl acetate containing antimicrobial properties is found in peppermint oil. Patra *et al.* (2010) noted that *in vitro* methane production was inhibited by methanol and ethanol extracts of clove buds and fennel seeds. Methane production was inhibited by eucalyptus oil by up to 58% at a concentration of 1.66 mg/L (Kumar *et al.*, 2009) and 90.3% at a concentration of 2 mg/L (Sallam *et al.*, 2009). Chaves *et al.* (2008a) found that the eucalyptus oil component p-cymene caused a 29% methane reduction at a concentration of 20 mg/L. Tatsouka *et al.* (2008) however found that the eucalyptus oil component α-cyclodextrin had no effect on methane production up to a concentration of 330 mg/L. Wang *et al.* (2009) performed an *in vivo* study which caused a methane reduction when 0.25 g/day EO derived from oregano plants was included in the diets of sheep for 15 days. In contrast to this the *in vivo* study by Beauchemin and McGinn (2006) showed no effect on methanogenesis when an essential oil mixture was fed to beef cattle at a concentration of 1 g/day for 21 days. The essential oil product was a commercial proprietary blend of EO and plant extracts and was added to the diet at the manufacturer's recommended level (Beauchemin and McGinn, 2006). Similar to the above Soliva *et al.* (2008) found that pinus mugos oil containing various essential oil components had no antimethanogenic activity.

Different responses on methanogenesis were observed for different types of EO. The dose response effects of different EO on volatile fatty acid production and methane inhibition were studied by Macheboeuf *et al.* (2008). They found that methane production was linearly reduced by the essential oil mixture extracted from Anethum graveolens which contained 32% limonene. They observed a negative sigmoidal shape response with a 3 mmol/L rumen fluid threshold dose with cinnamaldehyde extracted from cinnamomum verum (Macheboeuf *et al.*, 2008). A concentration below this threshold dose caused methane and volatile fatty acid production not to be altered. They also found that a negative sigmoidal shape response resulted with the addition of thymol, carvacrol and EO extracted from origanum vulgare and thymus vulgare and contained threshold doses of less than 2 mmol/L rumen fluid (Macheboeuf *et al.*, 2008). Concentrations higher than this caused a rapid decline in fermentation which led to decreased methane production (Macheboeuf *et al.*, 2008).

2.3.5 Effects on rumen nitrogen and energy usage

The effects of EO on rumen fermentation vary because there is a wide range in nature (Calsamiglia *et al.*, 2007). Some EO work similar to ionophores by affecting gram-positive bacteria and rumen volatile fatty acid proportions (Burt, 2004). Other EO decrease the total volatile fatty acid production by inhibiting general rumen bacteria (Busquet *et al.*, 2005a). These differences of their effects imply variations in the chemical structure of EO (Benchaar

et al., 2008). Bacterial activity is inhibited very strongly by oxygenated monoterpenes, and only slightly inhibited or stimulated by monoterpene hydrocarbons (Benchaar *et al.*, 2008).

Amino acid deamination is decreased by certain plant extracts and their active ingredients because of their inhibition of ammonia producing bacteria like Peptostreptococcus anaerobius and Clostridium sticklandii (McIntosh *et al.*, 2003). This results in decreased ammonia nitrogen (McIntosh *et al.*, 2003). Ionophores work in a very similar way as these EO (Calsamiglia *et al.*, 2007; Benchaar *et al.*, 2008). Other EO however have no effect on amino acid deamination (Castillejos *et al.*, 2005). Deamination depends not only on the type of essential oil but also on the diet and dose rate (McIntosh *et al.*, 2003). With the addition of EO a low protein diet resulted in deamination rather than a high protein diet (Benchaar *et al.*, 2008). High dosages of some EO resulted in deamination but also reduced volatile fatty acid production which compromises the energy supply to the animal (Benchaar *et al.*, 2008). Some EO however result in increased volatile fatty acid production (Benchaar *et al.*, 2007b) and some didn't affect volatile fatty acid production (Beauchemin and McGinn, 2006).

The addition of some EO resulted in increased propionate and butyrate proportions and decreased acetate proportion (Calsamiglia *et al.*, 2007). The essential oil eugenol however caused a decrease in propionate concentration (Adesogan, 2009). Microbes have the ability to adapt to EO, which makes short term *in vitro* experiments inaccurate assessment tools (Benchaar *et al.*, 2008).

Certain EO, especially garlic extracts reduce methane production in the rumen (Benchaar and Greathead, 2011). Garlic oil or one of its main components diallyl disulphide caused reductions of up to 70% (Benchaar and Greathead, 2011). Ionophores weren't able to achieve these high reductions (Busquet *et al.*, 2005a). The reason why the EO affected these results was because they suppressed methanogenic bacteria directly rather than suppressing the precursors of methane (Adesogan, 2009). Some EO other than garlic oils also reduce methane production, but can also decrease digestibility and propionate concentration examples include thymol, clove and fennel extracts (Patra *et al.*, 2006)

Some EO are pH and diet dependant only when used under certain conditions and production systems can they be a great advantage (Calsamiglia *et al.*, 2007). Capsaicin for example is advantageous when given in high-concentrate diets and has only small effects in high-forage diets (Santos *et al.*, 2010). Plant extracts act at different levels in the protein and carbohydrate degradation pathways. Carefully selecting and combining them can be a beneficial tool used for rumen microbial fermentation manipulation (Calsamiglia *et al.*, 2007).

2.3.6 Effects on animal performance

EO used as feed additives are still a very new concept and extra research still has to be done on this subject. Various studies done on this have shown that EO have no effect on milk production or composition. A study by Yang *et al.* (2007) showed that garlic and juniper berry essential oils resulted in the same milk production and composition compared to

cows receiving no feed additives. The study done by Benchaar *et al.* (2006) on beef cattle showed an improved feed efficiency with the addition of EO.

2.3.6.1 Feed intake and growth

Mixed observations have been recorded on feed intake depending on essential oil type and dose. Feed intake was not influenced by EO when 250 mg/day of essential oils extracted from oregano plants were fed to sheep (Wang *et al.*, 2009), 2 g of juniper berry essential oils containing 35% α-pinene were fed to cows (Yang *et al.*, 2007), dairy cattle were fed 0.75 to 2 g of an essential oil mixture (Benchaar *et al.*, 2007a) and dairy goats received 0.043 g EO or 0.43 g EO per kg feed intake (Maleckey *et al.*, 2009). Feed intake was however adversely affected in beef cattle by an essential oil mixture of eugenol at 90 mg/day and cinnamaldehyde at 180 mg/day (Cardozo *et al.*, 2006) and in dairy cattle by cinnamaldehyde at high doses of 500 mg/day (Calsamiglia *et al.*, 2007). This feed intake reduction can be a result of palatability problems, implying that this problem can be overcome by encapsulating the product (Calsamiglia *et al.*, 2007; Spanghero *et al.*, 2008). In contrast to this Cardozo *et al.* (2006) found that feed intake and rumen fermentation in beef cattle is stimulated by the addition of capsicum oil to a concentrate based diet. The effect of certain volatile compounds on the alfalfa pellet consumption by sheep was studied by Estell *et al.* (1998). They found that the compounds α-pinene, borneol and camphor inhibited the alfalfa pellet consumption and the compounds limonene, β-caryophyllene and cis-jasmone had no noticeable effect on consumption. Supplementing the proper essential oil dose is very important because certain EO stimulate intake at low doses and may negatively influence intake at higher doses in ruminants (Estell *et al.*, 1998). A study by Yang *et al.* (2010b) showed that a higher feed intake response was linked to a low dose of 0.4 g/day of cinnamaldehyde and a higher dose of 1.6 g/day had no effect on intake in steers.

Limited information is available on the essential oil effects on ruminant performance. A study by Bampidis *et al.* (2005) found that feed efficiency and average daily gain were not affected when diets supplemented with oregano leaves giving 144 mg or 288 mg of oregano oil per kilogram of diet dry matter were fed to growing lambs. Similarly Benchaar *et al.* (2006b) observed no average daily gain change in beef cattle fed silage based diets supplemented with an essential oil mixture of 2 or 4 g/day consisting of eugenol, thymol, limonene and vanillin. The essential oil mixture however had a positive effect on feed conversion with the 2 g/day dose. The 2 g/day dose improved the feed conversion compared to the 4 g/day dose (Benchaar *et al.*, 2006b). In the study by Chaves *et al.* (2008c) it was observed that cinnamaldehyde and carvacrol had no effect on sheep growth when they were fed barley or corn based diets for 11 weeks. The barley based diet did however result in much higher growth rates. Increased average daily gain was however observed in some cases when cinnamaldehyde and juniper berry EO were added to the barley based diet at similar concentrations. The essential oil influence on growth performance thus appears to be diet dependant.

2.3.6.2 Milk production and composition

Milk production is not consistently affected by EO. A study by Santos *et al.* (2010) showed that the total milk fat or fat percentage yield increased but milk production and other

milk components were not affected by an essential oil mixture containing major components of geranyl, eugenol, coriander oil and acetate. Benchaar *et al.* (2007b) found that the supplementation of EO might be the cause of increased acetate production and higher acetate to propionate ratio in the rumen which in turn causes increased fat synthesis. Agarwal *et al.* (2009) found similar results. Santos *et al.* (2010) however found that the increased fat synthesis might be a result of an energetic shift away from body condition gain because essential oil feeding caused a lower dry matter intake without affecting milk yield. This suggested an improved efficiency of nutrient utilization (Santos *et al.*, 2010). In a study by Tassoul and Shaver (2009) the milk and milk fat yields did not change but the milk production efficiency increased with essential oil feeding in dairy cow diets. A study by Kung *et al.* (2008) however showed an increased milk yield with essential oil supplementation.

Adding EO to animal diets could lead to the EO or their metabolic products to be present in the meat and milk products of the animals (Patra, 2011). An example of this is feeding caraway seeds to goats resulted in milk containing limonene and carvone which is the main essential oil component of caraway seed (Bylaite *et al.*, 2001). Feeding camomile to goats however resulted in no essential oil compounds present in camomile being detected in milk (Patra, 2011). Chion *et al.* (2010) found that cows grazing dicotyledons predominated alpine pastures had various monoterpenes present in their milk. Certain nutritional and organoleptic properties of the dairy products can therefore be enriched by the presence of EO and this could provide extra value to the product (Chion *et al.*, 2010)

2.3.7 Combinations between different essential oils

Burt (2004) previously reported about the additive, synergistic and antagonistic effects between different combinations of EO. An *in situ* study by Newbold *et al.* (2004) showed that a blend of EO containing eugenol, thymol, vanillin and limonene inhibited protein degradation. The changes they found were however small and variable depending on the feed being degraded, the type of ration fed to the animals and the adaptation period length (Newbold *et al.*, 2004). One or more EO have been combined in most commercial products, although there is only limited information available on the synergies among them. Research is urgently needed on this subject to ensure proper supplementation of EO.

2.3.8 Summary

Various studies have been conducted in the last decade to exploit EO as feed additives for improving the ruminant production efficiency. Some essential oil components certainly affect rumen production and fermentation positively. The optimum dose, feeding system and physiological status needs to be identified to optimize these effects. Methanogens, Hyper ammonia producing bacteria and other undesirable bacteria can be specifically inhibited by EO which in turn affects rumen fermentation positively by increasing rumen VFA concentrations, inhibiting methane production, increasing CLA production and reducing ammonia concentrations. Most of the recent findings are however based on *in vitro* studies and only a few studies have been conducted *in vivo*. The *in vivo* studies that have been done are inconsistent because the essential oil constituents that have been tested differ in type and dose. Thousands of essential oil active ingredients with different range and mode of actions

in the rumen have been identified. Most studies used an essential oil mixture containing different active ingredients in different proportions. To achieve more accurate results and thereby improve nutrient utilization efficiency and animal performance an optimum dose of essential oil components and their optimum combinations together with their appropriate dietary nutrient composition should be standardized. Micro-organism adaptation to EO has not been studied in detail and this aspect should be regarded for essential oil use in ruminant nutrition.

2.4 Yeast Products

2.4.1 Introduction

Yeasts (Y) are single celled fungi which reproduce by budding and ferment carbohydrates (Chaucheyras-Durand *et al.*, 2008). Yeast cultures based on *Saccharomyces cerevisae* are commonly used in ruminant diets (Adesogan, 2009). The strain of *S. cerevisae* as well as the number and viability of cells present vary widely among available products (Chaucheyras-Durand *et al.*, 2008). A mixture of live and dead *S. cerevisae* cells are found in most commercial products (Adesogan, 2009). Those containing more live cells are classified as live yeasts and those containing more dead cells together with the growth medium are classified as yeast cultures (Adesogan, 2009). Not all strains of yeast are capable of stimulating digestion in the rumen (Newbold and Rode, 2006). This was not related to the amount of live cells, but rather to the metabolic activity of the cells (Newbold and Rode, 2006).

Examples of yeasts include Levucell SC-20 (Lallemand Animal Nutrition) and Yea-sacc (Alltech Inc.) (Adesogan, 2009).

2.4.2 Modes of action

2.4.2.1 Microbial stimulation

Animals fed *S. cerevisae* have a large increase in desirable live rumen bacteria (Fonty and Chaucheyras-Durand, 2006). The rumen fungus *Neocallimastix frontalis* is stimulated by yeast because it supplies thiamine, a vitamin required by rumen fungi for zoosporogenesis. Yeasts also cause enhanced cell wall colonization by the fungi (Chaucheyras-Durand *et al.*, 2008). Fibre digesting bacteria like *Fibrobacter succinogens*, *Butyrivibrio fibrosolvens* and *Ruminococcus species* are stimulated by Yeasts. These factors ultimately lead to increased fiber digestion and higher feed intake (Chaucheyras-Durand *et al.*, 2008).

2.4.2.2 Oxygen scavenging

Although the rumen environment is known to be strictly anaerobic, dissolved oxygen can be found (Fonty and Chaucheyras-Durand, 2006). Oxygen enters the rumen through salivation, rumination and water intake (Fonty and Chaucheyras-Durand, 2006). Most ruminal microorganisms like *Fibrobacter succinogens* are anaerobic and therefor highly sensitive to oxygen. One of the main beneficits of the effects of live yeasts on fibre-degrading bacteria is the ability of yeast cells to scavenge oxygen (Chaucheyras-Durand *et al.*, 2008). The redox potential of the rumen under *in vitro* and *in vivo* conditions was

reduced with the inclusion of live yeasts. This implies that live yeast cells create more favourable conditions for the growth and activities of the anaerobic microorganisms (Chaucheyras-Durand and Fonty, 2002). Because live yeasts could release vitamins or other growth factors to closely associated bacterial cells, their impact on redox potential could also be microorganism mediated and not just a direct effect on oxygen consumption (Jouany, 2006).

2.4.2.3 pH Regulation

Yeasts stabilise rumen pH by stimulating Entodiniomorphid protozoa (Chaucheyras-Durand *et al.*, 2008). This protozoa competes with amylolytic bacteria in the rumen for starch, it engulfs starch granules and thereby avoid it from being fermented to lactate (Mendoza *et al.*, 1993). The protozoa ferments starch at a much slower rate than amylolytic bacteria and the main fermentation end products of the protozoa are volatile fatty acids and not lactate (Mendoza *et al.*, 1993). By delaying fermentation and producing fermentation end products which don't cause such a big drop in pH, the protozoa have a stabilizing effect on rumen pH (Fonty and Chaucheyras-Durand, 2006). Entodiniomorphid protozoa also take up a bit of the lactate and thereby inhibit it from accumulating in the rumen (Adesogan, 2009). Yeasts thereby indirectly decrease the risk of acidosis.

2.4.3 Effect on rumen maturity

Although the newborn rumen is germfree at birth, it is very quickly colonised by a bountiful and complex microbial population (Chaucheyras-Durand *et al.*, 2008). The animal's mother as well as other animals produces saliva and faeces which comes into contact with the newborn (Chaucheyras-Durand *et al.*, 2008). This together with contaminated vegetation provides a continuous supply of rumen microorganisms which colonize in the developing rumen as the conditions in the rumen become more favourable (Hobson and Stewart, 1997; Chaucheyras-Durand *et al.*, 2008). The mother and offspring usually have prolonged contact with each other in smaller farming systems (Chaucheyras-Durand *et al.*, 2008). However in more intensive, larger farming systems the separation of the young from its mother occurs soon after birth. This often causes the transition from liquid milk to solid feed to occur before the complete microbial colonisation of the young rumen (Fonty *et al.*, 1987). An imbalance in the microorganism composition may often be caused by this practice and this can lead to digestive disorders and a higher risk of microbial infections which have been reported to be one of the main causes of mortality and economic losses in livestock (Collado and Sanz, 2007). The development of rumen functions like feed digestion efficiency and absorption ability rely heavily on the establishment of a complex microbial ecosystem. The development of the immune system as well as the gut health also depends to a large extent on the rumen microbe establishment (Hooper *et al.*, 2001).

In a study by Chaucheyras-Durand and Fonty (2001) it was observed that lambs receiving live *Saccharomyces cerevisiae* daily had a higher rate of establishment of the microbial population and this population was also more stable than the lambs receiving no yeast supplementation. Bacterial communities have to be previously colonised in the rumen for ciliate protozoa to be able to be established (Fonty *et al.*, 1988). Chaucheyras-Durand and

Fonty (2002) however found that ciliate protozoa appeared more rapidly in the rumen of lambs receiving active dry yeast products. This implies that maturation of the rumen microbial ecosystem is accelerated by the supplementation of live yeasts (Chaucheyras-Durand and Fonty, 2002). In a study by Galvao *et al.* (2005) it was observed that active dry yeasts was used with a high efficiency on grain intake and growth performance in young calves that did not receive colostrum, especially before weaning. They also found that active dry yeast supplementation decreased the occurrence of diarrhoea in these young animals (Galvao *et al.*, 2005). In the calf study by Lesmeister *et al.* (2004) active dry yeast was supplemented and showed a positive calf performance response in that the dry matter intake and average daily gain was increased. These positive responses could be correlated to improved rumen development of factors like papillae length and width as well as rumen wall thickness (Lesmeister *et al.*, 2004).

2.4.4 Effects on rumen nitrogen and energy usage

In vitro addition of yeast leads to decreased methane production in some studies (Lynch and Martin, 2002), while other studies showed only minor differences (McGinn *et al.*, 2004). This inconsistency might be a result of strain specific effects, stage of lactation or too short duration of *in vitro* studies which inhibit yeasts from stimulating other microbes (Newbold and Rode, 2006).

Yeast increase bacterial numbers which lead to a higher rate of substrate fermentation and microbial protein synthesis (Chaucheyras-Durand *et al.*, 2008). This should lead to increased coupled fermentations which increase NH_3 uptake (Chaucheyras-Durand *et al.*, 2008). Results in practice have actually not been consistent in this regard. In some instances a decrease in rumen NH_3 concentrations were found (Chaucheyras-Durand *et al.*, 2005) and in other instances not (Erasmus *et al.*, 1992). Fungal additives tend to have minor effects on rumen NH_3 and volatile fatty acid concentrations, if they however cause significant effects it has little biological significance (Newbold *et al.*, 1995).

Energy is required for microbial protein synthesis and not all ammonia is incorporated into protein. It is therefore unfavourable to the host animal when amino acids and peptides are converted to ammonia by the rumen microbes. High rumen ammonia levels consequently cause a large amount of N to be excreted in urine and faeces (Chaucheyras-Durand *et al.*, 2008). An example of this is when high amounts of N are fed in an animal production system over half of it is excreted in the urine of the animal mostly in the form of urea (Chaucheyras-Durand *et al.*, 2008). This urea is then rapidly mineralised to ammonia where after it is converted to nitrous oxide which has a global warming potential of 12 times that of methane and 296 times that of carbon dioxide (Chaucheyras-Durand *et al.*, 2005). Nutritional strategies aimed at reducing N losses in the rumen are becoming more important because the role of livestock on global warming is becoming an increasing concern (Moss *et al.*, 2000). The ammonia concentration is the N related parameter which is normally considered when determining the impact of yeasts *in vivo* (Newbold *et al.*, 1995). Ammonia is very variable and depends to a large extent on various factors including the nature of the diet as well as various animal and microbial factors (Newbold *et al.*, 1995). Chaucheyras-Durand and Fonty

(2001) however found that in lambs raised in microbial controlled environments containing a very simplified rumen microbial population the presence of active dry yeast reduced the ammonia concentration. This same occurrence was found in the rumen of newborn lambs (Chaucheyras-Durand and Fonty, 2001). In an adult ruminant study by Kumar *et al.* (1994) daily yeast feeding caused similar effects on the ammonia concentration. The above studies show that the presence of yeasts causes a definite change in the N metabolism of rumen microbes. Various *in vitro* studies have recently indicated that the growth and activity of proteolytic rumen bacteria can be influenced by one yeast strain that inhibits their action on protein and peptides (Chaucheyras-Durand *et al.*, 2008). Chaucheyras-Durand *et al.* (2005) found that competition between the bacteria and live *Saccharomyces cerevisiae* cells for energy supply and through the direct inhibitory effect of yeasts small peptides on bacterial peptidases may be the mechanisms of yeast action. Chaucheyras-Durand *et al.* (2008) suggested that in a production response to probiotic yeast the dietary level of soluble N which included ammonia, amino acids and peptides was a vital parameter. Microbial growth will be enhanced and N loss will be reduced by live yeasts when the correct dietary balance between carbohydrate supply and soluble N are achieved (Chaucheyras-Durand *et al.*, 2008). The risk of acidosis can be decreased by not wasting digested carbohydrates in the form of volatile fatty acids but rather incorporating it into the microbial mass by means of increasing fermentation coupling (Chaucheyras-Durand *et al.*, 2008). Erasmus *et al.* (1992) however found that other yeast products did not give a positive response on the amount and composition of microbial N reaching the duodenum in dairy cows. Putnam *et al.* (1997) found similar results in their study.

2.4.5 Effects on animal performance

Animal performance can be improved by yeast (Adesogan, 2009). The performance enhancement can vary with performance level/ stage of lactation, diet composition and management (Adesogan, 2009). Dry matter intake, milk yield and milk solids are all increased by the addition of yeast to ruminant diets (Robinson and Erasmus, 2008). Yeast products led to a slight decrease in feed efficiency (Robinson and Erasmus, 2008).

A meta-analysis done by Poppy *et al.* (2012) on the effect of a commercially available yeast product on the milk production and other production measures on lactating dairy cows was investigated. Meta analytical methods are used to study the complete breadth of information which relates to a specific treatment. Studying multiple overcomes of all eligible studies can decrease the uncertainty often seen in small individual studies designed without sufficient power to detect differences in treatments (Poppy *et al.*, 2012). The meta-analysis showed that feeding this yeast product resulted in increased production performance of lactating dairy cows. Milk yield, fat yield and protein yield increased which resulted in increased energy corrected milk. The addition of the yeast product also caused increased DMI during early lactation and decreased DMI during late lactation of dairy cows (Poppy *et al.*, 2012).

2.4.6 Summary

Cellulolytic bacteria are stimulated by yeast. This leads to improved feed intake, fiber digestion, microbial protein synthesis and animal performance. Yeast also promotes the growth of anaerobic microorganisms by scavenging rumen oxygen. Rumen pH is stabilized which contribute to a less acidic environment thereby decreasing the risk of acidosis and bloat. Yeasts achieve this by stimulating bacteria which engulf starch and ferment it to less acidic volatile fatty acids.

2.5 Associative effects between feed additives

There is surprisingly very little information available on additive and/or associative effects between feed additives. Wallace and Newbold (1993) suggested that combining yeast cultures with an ionophore might enhance properties of both additives. Erasmus (2000) observed that ionophores are complemented by yeasts through counteracting the decreased DMI caused by ionophores. The depressing effect of ionophores on milk fat is thereby decreased through enhanced fibre digestibility (Erasmus, 2000). The incidence of lactic acidosis can also be decreased by the complimentary effects between ionophores and yeasts (Erasmus, 2000). This can be achieved because yeasts stimulate the growth of lactic acid utilizing rumen bacteria and ionophores depress the growth of lactic acid producing bacteria (Erasmus, 2000). In the study conducted by Frumholtz *et al.* (1992) it was found that bulls had a 12-13% higher weight gain when a combination of ionophores and yeasts were fed compared to bulls receiving only ionophores. They also found that the ability of ionophores to decrease the acetic: propionic ratio in the rumen of sheep was not affected by yeast cultures (Frumholtz *et al.*, 1992). A study by Erasmus *et al.* (2005) found that DMI (as a percentage of BW) was decreased with ionophore supplementation, but yeast cultures tended to alleviate this depression when fed in combination with ionophores. They also found that a combination of ionophores and yeasts had a lower acetic: propionic ratio compared with ionophores or yeast cultures alone (Erasmus *et al.*, 2005).

The additive or complimentary effects between different feed additives is a subject that is often overlooked when feed additives are discussed. More in depth research in this field can lead to major advances in feed additive supplementation. This can lead to higher production efficiency and lower input costs as well as healthier animals.

2.6 Bottom line

In this literature review different feed additives (monensin, yeast products, essential oils), their modes of action and their effects on performance of ruminant animals were discussed. Although positive results were found with all three supplements, results were not always consistent. Furthermore the question still remains whether monensin can be replaced with natural alternatives such as EO or yeast products. Research is also urgently needed on potential additive/synergistic effects between additives. This, however, was not the focal point of this study since the experimental design did not lend itself towards the measuring of such interactions. In the next chapter the materials and methods is described followed by chapters dealing with results and discussion, conclusion and the dissertation is ended off by a critical evaluation.

CHAPTER 3

MATERIALS AND METHODS

3.1 Location

The study was conducted at the Outeniqua Research Farm (Western Cape Department of Agriculture) near George in the Western Cape of South Africa (Altitude: 33° 58'38''S and 22°25'16''E).

3.2 Duration

The project was conducted over a period of 100 days (14 weeks and two days). The 100 days consisted of four experimental periods of 25 days each. The study commenced on 28 March 2012 and lasted until 1 July 2012. Experimental period 1 ranged from 28 March to 20 April 2012, period 2 ranged from 21 April to 14 May 2012, period 3 ranged from 15 May to 8 June 2012 and period 4 ranged from 9 June to 1 July 2012.

3.3 Animals

Four rumen fistulated Jersey cows in mid-lactation were used. They were all disease free and healthy and were selected based on days-in-milk, milk production and body weight.

Table 3.1 Cows used for the study, their body weight, body condition, lactation number, days-in-milk, milk production per day and somatic cell count as it was at the start of the trial on 28 March 2012

Cow nr.	Cow	Bodyweight	Body condition	Lactation nr.	Days in milk	Production per day (kg)
1	Bella 137	421	2.25	6	173	12.0
2	Mart 178	322	2.00	8	177	13.9
3	Mart 169	378	2.00	5	191	13.1
4	Firefly 52	399	2.25	4	228	11.0

3.4 Pens

Each cow was housed in an individual pen measuring 6.10m x 6.10m. Each cow stayed in its own pen throughout the study. The floor was covered with wooden chips to supply adequate bedding for the cow and also to keep unwanted bacteria to a minimum, thereby decreasing the probability for mastitis and other diseases. Fresh water was available *ad lib*.

Figure 3.1 Illustrates a pen in which the individual cows were kept during the trial

3.5 Experimental design and treatments

The experimental design was a 4 x 4 latin-square with 4 dietary treatments, 4 cows and 4 experimental periods. Each experimental period was 25 days with the first 21 days for adaptation to the new diet and the last four days for sampling. According to Dr Paul Weimer, a rumen microbiologist at the University of Wisconsin (pjweimer@wisc.edu) a three week adaptation period is sufficient to prevent any carry over effects from the previous diet. Each cow was subject to a different diet/treatment during each experimental period. With the diet/treatment changing with each period, at the end of the study each cow had been on each diet/treatment in one of the experimental periods. The treatments differed in the sense that it contained different feed additives. Each cow received one of the following treatments during the four experimental periods:

1) Total mixed ration (Control)
2) Control supplemented with ionophore (monensin) (0.3 g/cow/day)
3) Control supplemented with essential oil (EO) (1 g/cow/day)
4) Control supplemented with essential oil (1 g/cow/day) combined with yeast product (EO + Y)(1 g/cow/day)

The different feed additives will be described in more detail later in this chapter.

In the original protocol there was a yeast treatment, however the sponsor of the study requested that the yeast treatment be removed. It is the opinion of the researchers that a yeast treatment would have added much value to the study, especially with interpretation of results.

Table 3.2 Assignment of the different treatments to each cow during each experimental period

Period	Control	Control+ Ionophore	Control + EO[1]	Control + EO + Y
1	Cow 1	Cow 2	Cow 3	Cow 4
2	Cow 4	Cow 1	Cow 2	Cow 3
3	Cow 3	Cow 4	Cow 1	Cow 2
4	Cow 2	Cow 3	Cow 4	Cow 1

[1]EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.

The cows received a total mixed ration as control diet consisting of 50% lucerne hay and 50% concentrate on an as is basis. Sixteen 400 kg lucerne (*Medicago sativa*) bales were put aside at commencement of the project and core samples were taken of each bale to ensure all the lucerne used in the study was of similar nutritional content. The different treatments had the same nutritional quality except for the feed additives. The feed additives were mixed into the vitamin-mineral premix and this was mixed into the concentrate part of the diet by NOVA feeds (NOVA Feeds George, Industrial area, George, Western Cape, South Africa).

The cows were fed *ad lib* and received feed twice a day at 7:00 and at 16:00 directly after milking. Each cow's feed residues of the previous day were cleaned from the feed trough and weighed back. The daily feed allocation per cow was adjusted by taking the weight of the previous day's residues into consideration. Feed allocated was increased or decreased with feed intake until a 10% residue was achieved.

Table 3.3 presents the ingredient composition of the control diet fed to the cows and Table 3.4 present the composition of the different vitamin-mineral premixes of the different treatments.

Table 3.3 Ingredient composition of the control diet fed to the Jersey cows (DM basis)

Ingredient	g/kg DM
Lucerne hay	500
Maize	270
Hominy Chop	80
Wheat Bran	80
Soya Oilcake	50
Urea	3
Mono calcium phosphate	3
$CaCO_3$	2
Salt	5
Magnesium oxide	2
Premix	5

For each ton concentrate a 2.5 kg vitamin-mineral premix unit was added that contained the ingredient composition given in Table 3.4.

Table 3.4 Composition of the different vitamin-mineral premixes based on one ton concentrate

		Treatments[2]			
Item	**Unit[1]**	**Control**	**Ionophore**	**EO**	**EO + Y**
Vitamin E	G	10	10	10	10
Vitamin A	G	6	6	6	6
Vitamin B12	g	0.02	0.02	0.02	0.02
Manganese	g	86	86	86	86
Zinc	g	120	120	120	120
Copper	g	30	30	30	30
Iodine	g	2	2	2	2
Selenium	g	0.3	0.3	0.3	0.3
Ferrous	g	80	80	80	80
Cobalt	g	1.5	1.5	1.5	1.5
Magnesium	g	250	250	250	250
Rumensin[2]	g		150		
EO[3]	g			100	100
Yeast[4]	g				100

[1]g = Gram
[2]Rumensin: contains 20% monensin, 30 g monensin/t concentrate
[3]EO = Essential oil: Oleobiotec was the essential oil used, 100 g oleobiotec/t concentrate
[4]Yeast = Levucell SC 10 ME-Titan, 100 g Levucell/t concentrate

Table 3.5 The chemical composition of the control diet fed to Jersey cows (g/kg DM)

Nutrient[1]	**Control**
DM	890.0
ME[2] (MJ/kg DM)	10.6
Starch (g/kg DM)	245.0
CP	175.0
NPN (g/kg DM)	11.6
Sol CP (g/kg DM)	75.0
NDF (g/kg DM)	330.0
ADL (g/kg DM)	60.0
Invitro (% digestibility)	75.0
EE(g/kg DM)	32.0
Ash	80.0
Ca	8.0
P	4.0
Ca: P	2.1

[1]DM=Dry Matter; GE = Gross Energy; ME = Metabolisable Energy; CP = Crude Protein; NPN = Non Protein Nitrogen; Sol CP = Soluble Crude Protein, NDF = Neutral Detergent Fibre; ADL = Acid Detergent Lignin; EE = Ether Extract; Ca = Calcium; P = Phosphor
[2]Calculated ME MJ/kg DM ME = 0.82 x GE x IVOMD (Robinson *et al.*, 2004)

The diets in this study were formulated according to the NOVA database formulation to contain the chemical composition in Table 3.5. The analysed feed values of the different treatments are given later in the results and discussion chapter.

3.6 Ionophores

Elanco Animal Health, A division of Eli Lilly and Company (Greenfield, Indianopolis, IN 46285, USA) supplied the ionophore product used in the trial. The ionophore product used was Rumensin which contains the active ingredient monensin sodium. Elanco specifies that for optimum efficiency monensin must be fed continuously to dry and lactating dairy cows in a total mixed ration containing 11 to 22 g/ton monensin on a 100% dry matter basis. By mixing 150 g Rumensin that contained 20% monensin sodium, 30 g monensin sodium was added per ton of concentrate. The concentrate comprised 50% of the total diet, therefore the level of monensin was 15 g/ton of total diet (15 mg/kg).

3.7 Essential oils

Phode Laboratoires (Z.I. Albipole, La Martelle, 81150 Terssac, France) supplied the essential oil product used in the trial. The product used was Oleobiotec for ruminants (NS LX 185 P12). It consists of 37.5% sunflower oil; 45% precipitated and dried silicic acid; 17% of a mix of flavouring substances including 0.1-1% D-limonen, 1-4% Anethol and 8-16% Carvacrol; and 0.5% BHT. The product is a yellow powder which smells like aniseed spices and has an apparent density of about 0.6. Oleobiotec is legally declared and labelled as a sensory additive for animal nutrition and is in accordance with European and U.S. Feed laws. Phode specifies that the intake requirement is 1 g of Oleobiotec per cow per day. The EO group as well as the EO + Y group had the Oleobiotec pelleted together with the concentrate portion of the diet at a concentration of 100 g of Oleobiotec per ton of concentrate. The concentrate comprised 50% of the total diet therefore total diet contained 50 g Oleobiotec per ton. At an estimated intake of 20 kg of total diet per cow day^{-1} cows consumed 1 g Oleobiotec per cow per day.

3.8 Yeast

Lallemand S.A.S (19 rue des Briquetiers, 31702 Blagnac cedex, France) supplied the yeast product evaluated in the trial. The yeast strain *Saccharomyces cerevisiae* CNCM I-1077 was registered at the Pasteur Institute collection (CNCM) in Paris under the number I-1077. It is manufactured as a product known as Levucell SC 10 ME-Titan. This product is a micro encapsulated formulation for premix and pelleted feeds. Lallemand specifies that the intake requirement is 1 g yeast per cow per day. The EO + Y group had the yeast pelleted together with the concentrate portion of the diet at a concentration of 100 g of yeast per ton of concentrate i.e. 0.1 g/kg. This concentration resulted in 1 g yeast per cow per day if the cow consumed an average of 20 kg of TMR per day. The yeast concentration of this product is $1x10^{10}$ CFU/g. The EO + Y treatment cows therefore would ingest an average of $1x10^{10}$ CFU yeast per day.

3.9 Mixing of feed

Mixing of the feed was done before the onset of each experimental period. The lucerne and concentrate were mixed together using a Seko Samurai 5 mixing wagon (90 Via Gorizia, 35010 Curtarolo, Italy) with electronic mixing capacity. At the beginning of the weighing process one of the lucerne bales was put into the wagon and the bales weight was recorded. The bale was allowed to be broken up and mixed alone for 5 minutes. The concentrate portion was then added in the exact same weight as the bale weight. This mixture was mixed for 10 minutes. The feed was then poured out into big feeding troughs and from here it was put into feed bags by hand. This process was repeated for each of the four diets.

Figure 3.2 The mixor wagon used for mixing of the experimental TMR's

Figure 3.3 Mixing of the lucerne hay with the concentrate

3.10 Cow and feed identification

Each treatment was assigned a colour to identify the different feeds apart.

Control	= White
Ionophores	= Red
Essential oils	= Orange
Essential Oils + Yeasts	= Green

The control treatment feed was put into white bags, the ionophore treatment feed into red bags, the essential oil treatment into orange bags and the essential oils + yeast treatment into green bags.

Each cow had a coloured tag around its neck which matched the colour of the treatment it was receiving during that period. Each cow's pen also had a colour tag which matched the tag around the cow's neck which was kept in that pen. It also made it possible to allocate the right feed to the right cow by simply providing the feed of which the bag colour matched the cow's tag colour.

3.11 Body weight & body condition

The cows were weighed at the beginning of the study which was 28 March 2012. Thereafter they were weighed on two consecutive days at the first and last day of each experimental period. They were weighed on 19 and 20 April 2012, on 14 and 15 May 2012, on 7 and 8 June 2012 and also on 30 June and 1 July 2012. Possible variations in BW may occur depending on when the cow last urinated, defecated and drank water. This is the reason why the cows were weighed twice. The average BW between these two days was used for analysis.

A body condition score was also performed on the last day of each experimental period. A score of 1 to 5 was given to each cow, one indicating severe under nutrition and five indicates severe obesity (Wildman *et al.*, 1982; Ferguson *et al.*, 1994). Body condition scoring and weighing were done after the afternoon milking.

3.12 Sampling

3.12.1 Sampling period

Samples were taken during the 4 day sampling period at the end of the experimental period.

3.12.2 Milking and milk samples

The cows were milked twice daily at 07:00 and 16:00 in a 20 point dairy swing over milking parlour with weigh all electronic milk meters. Milk samples were taken twice daily on 4 consecutive days during the sampling period. The milking machine in the milking parlour was a twenty point Dairy Master (Total Pipeline Industries, 33 Van Riebeeck St.,

P.O. Box 252, Heidelberg 6665, Republic of South Africa). A 16 ml milk sample was taken during morning milking and 8 ml during the afternoon milking. Each ml of milk represented an hour since the last milking. The two samples from the same cow on the same day were pooled resulting in a representative milk sample. The milk samples were collected in bottles and preserved with sodium dichromate and sent to Lactolab (ARC, Irene) at the end of each sampling period. Milk was analysed for butterfat %, protein %, lactose %, somatic cell count and milk urea nitrogen (MUN) by means of infrared technology using a Milkoscan 6000 (Foss Integrated Milk Testing FT 6000, Foss Electric, Hillerod, Denmark). Flow cytometry using a Fossomatic 5000 (Foss Electric, Hillerod, Denmark) was used to determine the somatic cell count (SCC).

3.12.3 Feed samples

3.12.3.1 Lucerne core samples

Four core samples were taken from each lucerne bale. The 4 samples were pooled to give a 150 g representative sample of each bale. The device used to collect the core samples was a pipe with a length of 525 mm and diameter of 32 mm. The pipe was connected to a winch which was used to drill the pipe into the side of the bale. The samples were then sent to Bloemfontein where it was analysed by Dr G.D.J. Scholtz (Animal-, Wildlife- and Grassland Sciences, University of Free State, P.O. Box 399, Bloemfontein 9300). The samples were analysed for ADF, NDF, CP and Moisture. According to this information the bales were graded. All the bales except 1 were graded as prime lucerne. The one bale with the different grading was not used in the study. The analyses made it possible to use bales with similar nutrient content for the different treatments.

Figure 3.4 Core samples being taken from a lucerne bale

3.12.3.2 Lucerne samples

Each bale was randomly allocated to one of the treatments and before being mixed with the concentrate portion of that treatment, core samples were taken. At the end of the study there were 16 different lucerne samples from the different bales (four of these bales were

assigned to each treatment). The lucerne samples were frozen for later analyses. The different analysis done will be discussed later in this chapter.

3.12.3.3 Concentrate samples

Before mixing of the total mixed rations, 500 g samples were taken of the concentrate portion of each treatment alone. At the end of the study there were 16 concentrate samples in total. These samples were sealed in an airtight plastic bag and frozen for later analyses.

3.12.3.4 Total mixed ration samples

Full TMR samples were taken during the four day sampling period. During this time a grab sample was taken of every treatment with every feeding. All the grab samples of a treatment of a particular experimental period were pooled to give one representative TMR sample of that treatment for each period. There was one sample for every treatment in each period, giving a total of 16 TMR samples. These samples were milled through a 1 mm sieve, frozen and sent to the University of Pretoria (Nutrilab) for analyses together with the lucerne and concentrate samples.

3.12.3.5 Penn State Forage Particle Separator

After each feed mixing a representative sample was taken from each treatment. These samples contained 50% lucerne and 50% concentrate. Each of these samples was individually evaluated using the pen state particle size separator (PSFPS). The PSFPS consisted out of 3 trays which fitted on top of each other. Each tray had different size holes but the bottom pan was without holes. The top tray had the biggest holes and allowed only particles with a size bigger than 1.9cm to remain in this tray. The middle tray had smaller holes and allowed only particles with a size of 0.78 – 1.9cm to remain in this tray. The smallest particles with a size of smaller than 0.78cm remained on the bottom pan. The feed samples were put onto the top tray and by shaking the PSFPS rhythmically back and forth the different feed particles were separated into their respective trays. This was done on a flat horizontal surface with no vertical motion during shaking. The trays were shaken five times in one direction where after it was rotated a quarter turn and shaken another five times. This process was repeated seven times for a total of 40 shakes. The weights of the particles in each tray were then recorded (Heinrichs, 1996).

Figure 3.5 The pen state particle size separator with its three trays fitted on top of each other

Figure 3.6 The pen state particle size separator with its three trays containing the different size particles

3.12.3.6 TMR refusal samples (TMR orts)

TMR refusal samples were taken during the 4 day sampling period. During this time a grab sample was taken each morning out of the feed trough still containing the feed residues of the previous day. Each cow's grab samples were pooled at the end of each sampling period to give a representative sample of the TMR refusals for that cow during the sampling period. There was one sample for every treatment in each period, giving a total of 16 TMR refusal samples. These samples were milled through a 1 mm sieve and sent to the University of Pretoria for analyses.

3.12.4 Ruminal pH

Automatic pH/temperature loggers (Trutrack Data Logger, Model pH-HR mark 4, Intech Instruments LTD, New Zealand) were used to measure the pH in the rumen of the cows during the sampling period. Each cow had its own logger. The pH probe was inserted through the plug of the cannula into the rumen of the cow. Before being inserted into the rumen the logger was calibrated using pH buffer solutions of four and nine. Prior to insertion and in between each calibration with the two buffer solutions, the logger electrode was rinsed with distilled water to remove the former buffer solution or anything else that might affect the accuracy of readings. The computer program Omnilog was used to calibrate systems, adjust settings and to download data. The loggers were set to measure the pH in 10minute intervals and store the measurements in its memory. The logger was left in the cow for 72 hours after which it was taken out and the data was downloaded onto a computer. The loggers were washed and a cap containing Potassium Chloride (KCl) was put on the logger's electrode. The loggers were stored this way until the next sampling period.

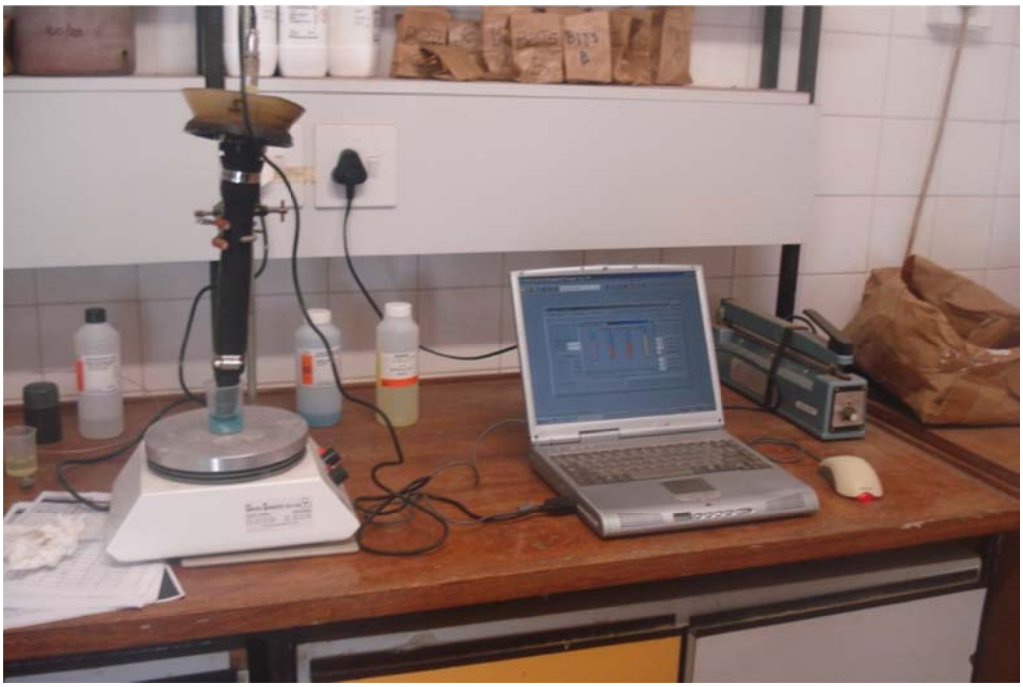

Figure 3.7 The pH logger being calibrated by the computer

3.12.5 Rumen fluid samples

Rumen fluid samples were taken on day 22 of each experimental period which was the first day of the 4 day sampling period. There were four rumen fluid sampling periods during each day namely 02:00, 08:00(after milking), 14:00 and 20:00. Rumen fluid samples were taken while the pH loggers were in the cows. The pH logger plugs had a small hole through which a pipe could be passed. In this way access was gained to the rumen contents. A pump was used to create a vacuum and pump rumen fluid into a bottle which was labelled with the cow's name. Each of the four cows samples were kept apart in separate bottles. Directly after the sample was taken from the rumen, the pH of the rumen fluid was determined with a calibrated handheld pH meter (WTW pH 340i pH meter and data loggers with a WTW Sentix 41 pH electrode; Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). This was done directly after the sample was taken to minimize the change in pH which can be caused by exposing the rumen digesta to oxygen. The rumen samples were filtered through four layers of cheesecloth to remove any solid particles. Three laboratory sample bottles per cow per sampling time were used to store the samples. Each sample bottle was labelled with a number which was linked to a certain cow, sample period, sample time, the date and the purpose of the analyses. The sample from the first of the three bottles was used for NH_3-N analysis, 15ml of the filtered rumen fluid was added to this bottle and the sample was preserved with 2.5ml of a 50% Sulphuric acid solution (H_2SO_4) (Broderick and Kang, 1980). For VFA analysis 18ml of the filtered rumen fluid was added to the second bottle and 2ml of a 25% ortho-phosphoric acid solution (H_3PO_3) was added to preserve the sample (Broderick and Kang, 1980). The third bottle was filled with 20ml of the filtered rumen fluid to analyse lactic acid and no preservatives were added (User Manual, YSI 2300 STAT PLUS Glucose and L-Lactate analyser). Each of the 4 cows had 3 sample bottles for every sampling time. These 12 bottles were frozen and later sent to the laboratory for analysis.

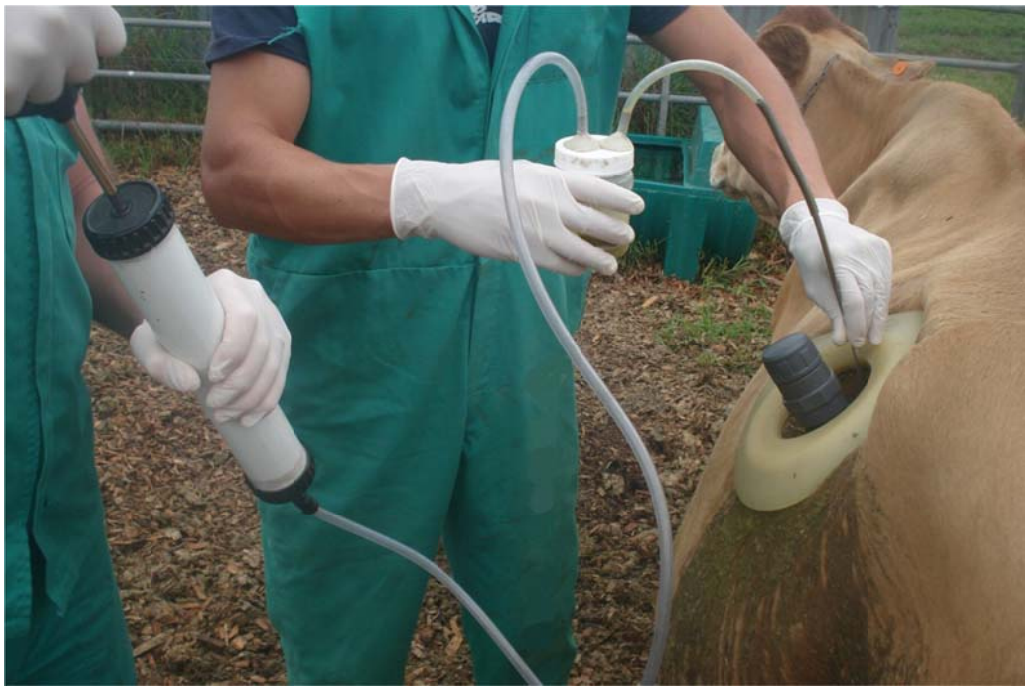

Figure 3.8 Rumen fluid samples being taken from a cow through the pH logger plug

3.12.6 *In sacco* study

Approximately 700 g of lucerne and 700 g of control TMR was milled through a 2mm sieve before the start of the study. The milled lucerne and TMR was put into two separate plastic bags and kept in a dry place at room temperature. Dacron bags were used for the *in sacco* incubations. The empty dacron bags had dimensions of 10cm by 20cm and a pore size of 53 microns (Vanzant et al, 1998). These bags were each given a number which was linked to a certain cow and experimental period. Before each experimental period the bags were labelled and thereafter dried in a forced airoven for 48 hours at 50°C. The bags were then taken out of the oven and weighed on a three decimal Sartorius L420P scale and this weight was recorded. Approximately 5 g of either the milled lucerne or TMR was weighed to three decimals and put into each bag. The bag with its contents was weighed, a cable tie was put around the bag to close the bag and the bag with its contents and the cable tie was weighed again. Bags were placed in stockings according to the *in sacco* method described by Cruywagen (2006). This method made bag retrieval easy and kept exposure to oxygen low. Eight Dacron bags were put into each of the four cows. These eight bags were put into two different stockings. Each stocking thus contained four bags of which two contained lucerne and the other two TMR. The stockings were tied to the inside of the cannula plug of the cow and placed in the rumen at approximately 08:00 on the third day of each sampling period. Twelve hours after incubation one of the stockings containing four Dacron bags were taken out and 24 hours later the other stocking containing the other 4 Dacron bags were taken out. The bags were then washed five times for three minutes each or until the water ran clear in a Defy Twinmaid washing machine. The bags were then spinned in a tumble drier for three minutes to get rid of most of the water after which it was oven dried at 55°C for 72 hours. The bags were then weighed on a three decimal Sartorius L420P scale and this weight was recorded. Four bags (two containing lucerne and two containing control TMR) that were not put into any cow were also washed and dried in order to obtain zero hour values. This process was repeated with each experimental period. At the end of the experimental trial there were 144 bags in total which have undergone this process. Of these bags 72 contained lucerne residues and 72 contained TMR residues. These bags were frozen and sent to the University of Pretoria for analysis. The bags with the same content taken out of the same cow at the same time were pooled together. With every collection time there were two bags containing lucerne and two bags containing TMR collected from each cow. The two lucerne bags were pooled together and the two TMR bags were pooled together. This was done to obtain sufficient dry matter for the required analyses.

Figure 3.9 The canulla plugs with the stockings containing the dacron bags

3.13 Laboratory analyses

The samples taken during the trial were milled (SWC Hammer mill, 1 mm sieve) and sent to UP-Nutrilab (Department of Animal and Wildlife Sciences, University of Pretoria, Pretoria) to be analysed. The following analyses were performed: DM (AOAC, 2000, procedure 934.01), Ash (AOAC, 2000, procedure 942.05), CP (Leco TruMac N determinator, model FP-428, Leco Corporation, St Joseph, MI, USA was used to determine N (CP calculated from N x 6.25(AOAC, 2000, procedure 968.06))), NDF (Ankom 2000 fibre analyser, Ankom Technologies, Macedon, NY, USA; Robertson and Van Soest, 1981), GE (MC – 1000 Modular Calorimeter, Operators Manual), IVOMD (Tiley and Terry, 1963; Engels and van der Merwe, 1967), EE (crude fat; AOAC, 2000, procedure 920.39), Ca (AOAC, 2000, procedure 965.09) and P (AOAC, 2000, procedure 965.17), Starch (Macrae and Armstrong, 1968; Faichney and White, 1983; AOAC, 1984), ADL (Goering and Van Soest, 1970) and Sol CP.

3.13.1 Lucerne samples

Sixteen lucerne samples were taken during the study. Four of these samples were assigned to each treatment. The samples of each treatment were pooled together to give one representative sample of the lucerne used for that treatment. Pooling of these samples resulted in four samples which were analysed for DM, ash, CP, NDF, GE and IVOMD.

3.13.2 Concentrate samples

Sixteen concentrate samples were taken during the study. All 16 of these samples were pooled together to give one representative sample of the concentrate portion fed to the cows. The concentrate portions of each treatment were exactly the same except for the feed additives it contained and were therefore pooled to be analysed for DM, ash, CP, EE, NDF, Ca, P, starch, GE and IVOMD.

3.13.3 TMR samples

Sixteen TMR Samples were taken during the study (Four from each treatment). The samples from each treatment were pooled together to give one representative sample of the TMR of that treatment. After pooling these samples together there were four samples which were analysed for DM, ash, CP, EE, NDF, ADL, GE, Ca, P, starch, soluble CP and IVOMD.

3.13.4 Feed refusal samples (TMR orts)

TMR refusal samples were taken during the study (four from each treatment) and were analysed for DM, NDF and CP.

3.13.5 *In sacco* residues

Hundred fourty four bags in total were collected during the experimental study. Seventy two of these contained lucerne residues and 72 contained control TMR residues. Bags with the same contents, taken out of the same cow at the same time were pooled together. This brought the total number of bags down to 72 (36 containing lucerne and 36 containing control TMR). A sample of the lucerne and a sample of the control TMR used in the bags were also analysed. These two samples were not incubated or washed.

The 36 bags containing lucerne samples as well as the extra unwashed lucerne sample were analysed for NDF and the 36 bags containing the control TMR samples as well as the extra control TMR sample were analysed for N and Starch.

3.13.6 Rumen ammonia N

Sixty four rumen samples consisting of 15ml rumen fluid preserved with 2.5ml Sulphuric acid solution were analysed for NH_3-N according to the procedure described by Broderick and Kang (1980). Catalyzed phenol-hypochlorite and ninhydrin colorimetric procedures were adapted to the technicon autoanalyzer for simultaneous determination of total amino acids and ammonia in rumen fluid or rumen *in vitro* media.

3.13.7 Rumen volatile fatty acids

Sixty four rumen samples consisting of 18ml rumen fluid preserved with 2ml Orthophosphoric acid were analysed for volatile fatty Acid composition (Broderick and Kang, 1980). The rumen VFA composition was determined with the HPLC method. Clean samples with only fermentation products present for analysis were obtained by first subjecting the samples to a clean-up procedure where sugars were removed and rumen fluid deproteinised (Siegfried *et al.*, 1984). A 717 auto sampler fitted with a RI Detector and a Biorad Aminex HPX 87H column was used in this method.

3.13.8 Rumen lactic acid

The YSI 2300 STAT PLUS Glucose and L-Lactate analyser were used to analyse sixty four rumen samples consisting of 20ml rumen fluid without any preservatives for lactic acid according to the User Manual (YSI 2300 STAT PLUS Glucose and L-Lactate analyser).

Each probe is fitted with a three-layer membrane containing immobilized enzyme in the middle layer. The face of the probe, covered by the membrane is situated in a buffer-filled sample chamber into which a sample is injected. Some of the substrate diffuses through the membrane. When it contacts the immobilized oxidase enzyme, it is rapidly oxidized and produces hydrogen peroxide. The hydrogen peroxide is in turn oxidized at the platinum anode which produces electrons. A dynamic equilibrium is achieved when the rate of hydrogen peroxidase production and the rate at which hydrogen peroxidase leaves the immobilized enzyme layer are constant and is indicated by a steady state response. The electron flow is linearly proportional to the steady state hydrogen peroxide concentration and therefor to the concentration of the substrate. The platinum electrode is held at an anodic potential and is capable of oxidizing many substances other than hydrogen peroxide. To prevent these reducing agents from contributing to sensor currents, the membrane contains an inner layer of a very thin film of cellulose acetate. This film readily passes hydrogen peroxide but excludes chemical compounds with molecular weights above approximately 200. The cellulose acetate film also protects the platinum surface from proteins, detergents and other substances that could foul it.

3.14 Statistical analyses

Data were analysed statistically as a latin square design using the GLM model (Statistical Analysis System, 2012). Repeated analysis of variance measures were used for repeated period measures. Means and standard errors were calculated and significance of difference between means was determined by Fischers test (Samuels, 1989). Significance was declared at $P < 0.05$ and tendencies at $P < 0.10$

The linear model used is described by the following equation:

$$Y_{ijk} = U + T_i + P_j + C_k + e_{ijk}$$

Y_{ijk} = variable studied during the period, it's the observation for the i^{th} treatment, the j^{th} period and the k^{th} cow.
U = the overall mean of the population.
T_i = the effect of the i^{th} treatment.
P_j = the effect of the j^{th} period.
C_k = effect of the k^{th} cow.
e_{ijk} = the random error associated with each Y.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Feed analyses

The chemical composition of the total mixed rations containing the different feed additives is represented in Table 4.1. All four rations were identical except for the feed additives it contained. The chemical analysis of the TMR's represents these similarities.

Table 4.1 Chemical analysis of the total mixed rations containing different feed additives

	Treatment[2]			
Nutrient[1]	**Control**	**Ionophore**	**EO**	**EO + Y**
DM (g/kg)	887.5	884.4	880.4	885.2
ME[3] (MJ/kg DM)	10.6	10.8	10.9	10.8
Starch (g/kg DM)	244.8	297.8	216.8	254.1
CP (g/kg DM)	170.6	173.9	179.9	172.9
NPN (g/kg DM)	11.7	11.1	13.5	11.2
Sol CP (g/kg DM)	73.2	69.3	84.5	69.9
NDF (g/kg DM)	327.1	340.7	327.4	330.4
ADL (g/kg DM)	58.6	54.2	58.5	57.1
Invitro (% digestibility)	74.2	76.2	76.3	74.6
EE(g/kg DM)	30.7	26.2	27.9	38.7
Ash (g/kg DM)	78.7	81.4	84.1	71.8
Ca (g/kg DM)	8.0	7.8	8.2	7.6
P (g/kg DM)	3.8	4.1	4.4	4.0
Ca: P	2.1	1.9	1.9	1.9

[1]DM=Dry Matter; ME = Metabolisable Energy; CP = Crude Protein; NPN = Non Protein Nitrogen; Sol CP = Soluble Crude Protein, NDF = Neutral Detergent Fibre; ADL = Acid Detergent Lignin; EE = Ether Extract; Ca = Calcium; P = Phosphorous

[2] EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.

[3]Calculated ME MJ/kg DM ME = 0.82 x (GE x IVOMD) (Robinson *et al.*, 2004)

4.1.1 Total mixed ration

The TMR complies with the nutritional requirements of Jersey cows producing 20L milk/day (NRC, 2001).

4.1.2 Lucerne

Only prime grade lucerne graded by Dr G.D.J. Scholtz (Animal-, Wildlife- and Grassland Sciences, University of Free State, P.O. Box 399, Bloemfontein 9300) was used in the study. The lucerne used for the different treatments had similar chemical compositions, which implies that any differences between treatments are not a result of lucerne used, but rather because of the additive in the diet. Results are therefore not shown.

4.1.3 Feed refusal samples (TMR orts)

The aim of analysing the TMR orts was to check for sorting by the cows, therefore only a few analyses were done. There were no differences in chemical composition of the orts from the four treatments and the TMR fed, therefore sorting did not take place and results are therefore not shown.

4.2 Milk production and cow performance data

In this section performance data of cows are compared with other published data including both 4x4 Latin square design studies with limited cow numbers and long term production studies with high cow numbers. Such comparisons should be interpreted with caution.

The mean DMI, milk production, milk composition, MUN and SCC data is shown in Table 4.2.

Table 4.2 The effect of different feed additive treatments on average dry matter intake, milk production, milk composition, somatic cell count and milk urea nitrogen on cows fed a total mixed ration (n = 16)

	Treatments[1]				
Parameters	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
DMI (kg/day)	22.0^{cd}	21.3^{d}	22.7^{c}	22.3^{cd}	0.5
4% FCM[3] (kg/day)	24.0	23.8	25.1	25.0	0.5
Milk production (kg/day)	22.9^{cd}	22.4^{d}	23.2^{c}	23.4^{c}	-0.37
Feed efficiency	1.04	1.04	1.03	1.05	0.02
Fat %	4.32^{b}	4.45^{ab}	4.52^{a}	4.46^{ab}	0.05
Protein %	3.93	3.93	3.91	3.91	0.25
Lactose %	4.91	4.91	4.88	4.89	0.25
MUN (mg/dL)	14.6	16.0	15.7	16.6	0.89
SCC (x1000/ml)	94.1	70.1	78.0	82.1	11.8

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean, DMI= average dry matter intake of the cows fed that certain diet; MUN = Milk Urea Nitrogen; SCC = Somatic cell count.
[3]4% FCM (kg) = (0.4 x kg of milk) + (15 x kg of milk fat) (National Research Counsil, 2001)
[ab] Means in the same row without a common superscript differs (P<0.05)
[cd]Means in the same row without a common superscript tend to differ (P<0.1)

4.2.1 Dry matter intake (DMI)

The feed intake, milk production, milk composition data, MUN and feed efficiency data is shown in Table 4.2. There were no differences in DMI between cows receiving the different treatments ($P < 0.05$); however cows fed the EO treatment tended to have a higher DMI when compared to the non-supplemented cows ($P < 0.10$). The DMI's were around 5.3% of BW, which is exceptional but is comparable to other studies where Jersey cows were fed lucerne based TMR's (Van Ackeren *et al.*, 2009).

Ipharaguerre and Clark (2003) analysed 12 studies where cows were supplemented with ionophores. There were no significant differences found for DMI in 8 out of the 12 studies when compared to control cow. The remaining four studies however showed a decrease in DMI with the addition of ionophores. These decreases in DMI ranged from 0.4 kg (2%) to 1.7 kg (10%) per day and averaged 1.2 kg per day (7%). The largest decrease in DMI was found by Johnson *et al.* (1988) and was associated with ionophore doses that largely exceeded the ionophore concentration which is effective in dairy diets. Wilkinson *et al.* (1997) determined this effective dose to be 240 mg monensin per cow per day. A study by Ramanzin *et al.* (1997) found that feeding high grain diets, in which the concentrate portion provided 50% of the DM of the diet, caused a larger DMI depression with the administration of ionophores compared to high forage diets in which the forage portion provided 70% of the DM of the diet.

According to Benchaar *et al.* (2007b) and Benchaar *et al.* (2006) DMI was not affected by the addition of EO to a TMR. Odongo *et al.* (2007) reported no effect on DMI with ionophore supplementation. In a meta-analyses Desnoyers *et al.*, (2009) found yeast products to increase DMI by 0.44 g/kg of BW: In general the effect of most feed additives on DMI is inconsistent, although ionophores will either depress DMI or have no effect. Sauvant *et al.* (2004) however found that yeast supplementation had no effect on DMI in their meta-analysis.

4.2.2 Milk yield, feed efficiency, milk composition, milk urea nitrogen and somatic cell count

4.2.2.1 Milk yield

Milk production and 4% fat corrected milk (FCM) tended ($P < 0.10$) to be higher for cows fed the EO treatment compared to the ionophore treatment but did not differ from the other treatments ($P < 0.05$).

From the data of the 12 studies analysed by Ipharaguerre and Clark (2003) they found that ionophores supplemented to cows either did not affect or increased milk production. The milk production from the studies which reported an increase in milk production ranged from 0.4 kg (2.6%) to 2.8 kg (11.2%) per day and averaged 1.5 kg (7%) per day when monensin was given in doses ranging from 80 to 350 mg per cow per day. These results were not dependant on the stage of lactation with the onset of the ionophore treatment. The responses were however dependant on the type of diet fed to the cows. Cows fed high forage diets had a bigger increase in milk yield with the addition of ionophores compared to cows fed low

forage diets. These observations might be because cows fed low forage diets containing high concentrate portions gave much higher milk yields before the addition of ionophores compared to cows fed high forage diets. Duffield *et al.* (1999) added to this by making another important observation. In their study they found that the magnitude of the milk production increase is to a large extent determined by the BCS of the cows at the onset of the ionophore treatment. Their results concluded that cows classified as thin were not significantly affected by ionophore administration. Cows classified as fair however had improved milk productions of about 0.85 kg per day and cows classified as fat had improved milk productions of about 2.5 kg per day.

Benchaar *et al.* (2003) observed no change in milk production, digestion and rumen fermentation when cows were supplemented with a dose of 750 mg/cow per day of an EO mixture containing thymol, eugenol, vanillin and limonene. This suggested that this dose may have been too low to alter ruminal fermentation and metabolism. A higher dose of 2000 mg/cow per day was supplemented in a study by Benchaar *et al.* (2006). No changes were however seen in milk production and 4% FCM with this higher dose. Benchaar *et al.* (2007b) also supplemented a specific EO mixture at a dose of 750 mg/cow per day and found that milk production was not affected by the EO.

The meta-analysis done by Desnoyers *et al.*, (2009) showed that yeast supplementation led to an average increase in milk yield of 1.2 g/kg BW. Milk yield was calculated per kilogram of BW to allow comparison between cattle and small ruminants. This increase in milk yield is in agreement with a study regrouping 22 experiments and showing an average increase in milk production of 7.3% for yeast-supplemented animals (Jouany, 1998). The meta-analysis of Sauvant *et al.* (2004) however indicated only a tendency toward increased milk production with yeast supplementation.

4.2.2.2 Feed efficiency

In the present study the feed efficiencies (FE) of the different treatments showed no significant differences between treatments ($P > 0.05$). This was expected since there were very little differences in milk production and DMI. This, however, is in contrast with studies which showed that the administration of ionophores to dairy cows caused a significant (Johnson *et al.*, 1988; Van Der Werf *et al.*, 1998; Green *et al.*, 1999; Phipps *et al.*, 2000) or slight increase (Weiss and Amiet, 1990; Ramanzin *et al.*, 1997; Erasmus *et al.*, 1999) in efficiency of milk production and never a decrease compared to control cows. The increase in the metabolic energy value of feeds caused by the effects of ionophores on rumen fermentation like higher feed digestibility and decreased methane production is the cause of the improved milk production efficiency of ionophore treated dairy cows (Erasmus *et al.*, 1999; Phipps *et al.*, 2000). The cows in our study, however, were in mid lactation where effects are less pronounced and FE generally lower.

Various studies showed an improved feed efficiency when dairy cows were supplemented with EO (Yang *et al.*, 2007; Benchaar and Greathead, 2001). The meta-analysis done by Poppy *et al.* (2012) indicated increased milk production efficiency when dairy cows were supplemented with yeast products.

The pressure to reduce environmental pollution from dairy cows keeps increasing and therefore the development of management strategies to increase milk production is essential.

4.2.2.3 Milk fat

A definite relationship between changes in the rumen VFA pattern and the decrease in milk fat production has been established for a range of diets. It was therefore suggested that reduced production of rumen acetate and butyrate will cause decreased synthesis of milk fat and contribute to milk fat depression (Emery, 1988; Thomas and Martin, 1988; Sutton, 1989). Sutton *et al.* (1988) determined that variations in molar proportions of ruminal VFA account for up to 80% of the variations in milk fat concentrations. Oldham and Emmans (1988) also concluded that there is a close relationship between the ratios of milk fat (acetate, butyrate and long chain fatty acids) and lactose (glucose and propionate) precursors and the fat content of milk. The latest research on milk fat depression however, downplays the role of VFA ratios as a major factor in milk fat depression and suggests that the role of trans fatty acids in inhibiting *de novo* milk fat synthesis is more important (Discussed later in this section).

Milk fat % was higher ($P < 0.05$) for the essential oil supplemented cow with a value of 4.52% compared to the control treatment with a value of 4.32%. The milk fat % for the ionophore and EO + Y treatment groups did not differ from the other treatments ($P > 0.05$). These results are presented in Table 4.2.

In many studies the administration of ionophores to dairy cows caused slight or significant depressions of milk fat content (Odongo *et al.*, 2007). The occurrence of this negative effect is not dependant on the type of diet fed, the BCS and stage of lactation at the start of the treatment or the dose administered. The ionophore dose however controls to a certain extent the magnitude of the effect (Ipharraguerre and Clark, 2003). In our current study monensin was supplemented at 300 mg per cow per day. In a study by Johnson *et al.* (1988) in which Jersey and Holstein cows were monitored, the cows fed more than 500 mg lasalocid per cow per day had the largest reduction in milk fat content. A study by Van Der Werf *et al.* (1998) contributes to these results. They found that when early lactation dairy cows were fed 0, 150, 300 and 450 mg monensin per cow per day it caused the milk fat concentration to be reduced progressively to 45.6, 45.5, 43.7 and 41.5 g/kg respectively. This effect however was only significant when monensin was administered at the highest dose. The reduced rumen production of acetate and butyrate which leads to a shortage of lipogenic precursors for fatty acid synthesis in the lactating mammary gland has frequently been seen as the cause of the milk fat depression by ionophores (Dye *et al.*, 1988; Van Der Werf *et al.*, 1998). Data from *in vitro* experiments alternatively indicate that ruminal biohydrogenation of long chain fatty acids can be inhibited by ionophores (Fellner *et al.*, 1997). This might in turn enhance the trans-10, cis-12 conjugated linoleic acid supply to the mammary gland (Bauman and Griinari, 2001). Increasing the availability of this trans fatty acid in the mammary gland (it is a potent inhibitor of the de novo synthesis of fatty acid (Bauman and Griinari, 2001)), can contribute to the mechanism which is responsible for the decreased milk fat concentrations of ionophore treated dairy cows. *In vitro* data of Jenkins and Fellner (2002) suggests that when ionophores are fed along with unsaturated plant oils the negative effect of ionophores on milk fat concentration might be enhanced. Several experiments

however proved that the decrease in milk fat concentrations caused by ionophores coincided with a milk yield increase, which implies a dilution effect could also be part of that mechanism.

Benchaar *et al.* (2006) and Benchaar *et al.* (2007b) found that EO supplementation had no effect on milk fat concentration. Santos *et al.* (2010) however found that milk fat production and milk fat proportion were higher in cows supplemented with an EO complex containing eugenol, geranyl acetate and coriander. These results were similar to the results of our study presented in Table 4.2.

The meta-analysis of Desnoyers *et al.* (2009) indicated a 0.05% increase in milk fat content when yeast is supplemented to lactating dairy cows. Poppy *et al.* (2012) also saw an increase in milk fat when yeast was supplemented.

4.2.2.4 Milk protein

In our study there were no differences in milk protein % between treatments ($P > 0.05$). These results were supported by various other studies. Essential oil supplementation had no effect on milk protein (Benchaar *et al.*, 2006; Benchaar *et al.*, 2007b) while ionophore treated cows had only slightly lower milk protein concentrations than that of untreated cows (Duffield *et al.*, 1999; Erasmus *et al.*, 1999; Heuer *et al.*, 2001; Ruiz *et al.*, 2001). Ionophores however caused significant milk protein decreases in certain studies (Hayes *et al.*, 1996; Green *et al.*, 1999; Phipps *et al.*, 2000). Odongo *et al.* (2007) reported a 4% decrease in milk protein with ionophore supplementation. Additionally the milk productions in these three studies were significantly increased by ionophore supplementation, which suggests that the milk protein concentration reduction might be a dilution effect. The above mentioned hypothesis is supported by the fact that in most experimental studies the decreased milk protein concentrations is a result of higher milk yields. The meta-analysis of Desnoyers *et al.* (2009) indicated that yeast supplementation had no influence on milk protein content while the meta-analysis of Poppy *et al.* (2012) found an increase in milk protein yield when yeast is supplemented.

4.2.2.5 Milk lactose

Sutton (1989) stated that dietary changes usually don't affect lactose concentrations and if dietary changes do cause changes they are inconsistent, small and of no significance. This supports the results of the present study which showed no differences in milk lactose percentages between the different treatments ($P > 0.05$). Sutton (1989) is supported by Jenkins and McGuire (2006) who reported that lactose concentration changes are only caused by severe feeding situations. In addition Benchaar *et al.* (2006) found that the addition of EO or ionophores did not affect milk lactose concentrations

In contrast Benchaar *et al.* (2007b) found that the milk lactose concentration was higher in cows receiving EO than those not receiving EO. Duffield *et al.* (2008b) reported no change in milk lactose percentage when ionophores are supplemented. In general however, feed additive supplementation does not affect milk lactose content.

4.2.2.6 Milk urea nitrogen (MUN)

There were no differences in MUN concentration between the treatments. This was expected since the cows were fed the same basal diet and DMI did not differ ($P < 0.05$). These results are supported by studies where cows were supplemented with EO (Benchaar *et al.*,2006; Benchaar *et al.*,2007b), ionophores (Odongo *et al.*,2007) and yeast products (Chaucheyras-Durand *et al.*, 2008).

4.2.2.7 Somatic cell count (SCC)

The SCC values were all below 100 000 which indicates excellent herd mastitis control. No differences were expected since the additives affect rumen fermentation patterns and generally have no effect on the immune status of cows.

4.2.3 Body weight and body condition score

Body weight (BW), body weight changes, body condition score (BCS) and body condition score changes is shown in Table 4.3. Body weight and BCS at the beginning and end of the study as well as the BW and BCS differences that occurred were not affected by treatment. Due to the design of the experiment and the similar basal diet the results were not unexpected.

It is stated by the NRC (2001) that changes in BW don't reflect tissue weight changes. This is because in early lactation there is an increased DMI which lead to gut fill which masks the decrease in tissue mass caused by tissue mobilisation. BW is therefore difficult to quantify and tissue mass cannot be truly reflected by it because 15% of the BW is made up of gut fill. This, in addition added to the lack of a response on BW in our study.

Benchaar *et al.* (2006) reported that BW gain was higher for cows fed EO than for those fed no EO. In a study by Duffield *et al.* (2008b) a positive BW change was recorded when ionophores were added to a dairy cow diet.

Duffield *et al.* (2008b) reported that ionophores included in dairy cow diets led to an improved BCS. Santos *et al.* (2010) observed only small BCS improvements with the addition of EO to the diet of dairy cows. These studies cannot be compared to our results since these studies were long term production studies with long experimental periods.

Table 4.3 The effect of different feed additive treatments on average body weight and body condition score of cows fed a total mixed ration (n = 8)

	Treatments[1]				
Parameters	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
BW start	412.1	418.5	418.5	410.5	4.41
BW end	432.3	426.9	433.1	430.5	3.63
BW difference	20.1	8.38	14.6	20.0	7.44
BCS start	2.25	2.31	2.25	2.31	0.04
BCS end	2.38	2.44	2.31	2.38	0.06
BCS change	0.13	0.12	0.06	0.07	0.62

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean, BW start= average cow body weight at the start of the study; BW end = Average cow body weight at the end of the study; BW difference = difference between the average start and end body weights; BCS start = average cow body condition score at the start of the trial; BCS end = average body condition score at the end of the trial.

4.2.4 TMR particle size analysis

The percentage distribution of particle sizes on the different trays and bottom pan is shown in Table 4.4. The general recommendation by Heinrichs (1996) is 6-10% on the top sieve, 30-50% on the middle sieve and 40-60% on the bottom pan. Comparing our results to the recommendation indicates too much longer particles and not sufficient medium particles. Results of the feed orts indicated that cows did not sort feed due to too much long fibre particles, butterfat was not reduced due to too much fine particles and no acidosis occurred. We therefore did not change our mixing time based on the particle size results. After all, the PSFPS is just one of many tools being used in troubleshooting.

Table 4.4 Average mass and percentage of TMR particles on each of the upper sieve, middle sieve and bottom pan of the penn state particle size separator (n = 16)

	Treatments[1]				
	Control	**Ionophores**	**EO**	**EO + Y**	**Target for TMR**
Upper sieve mass (g)	67.3	61.2	54.3	76.4	
Middle sieve mass (g)	20.5	14.4	19.2	13.4	
Bottom sieve mass (g)	175.0	238.6	234.3	242.3	
Total Mass (g)	262.8	314.2	307.8	332.2	
Upper sieve %	27.0	19.6	18.1	22.9	6-10
Middle sieve %	7.60	5.11	6.67	4.05	30-50
Bottom sieve %	65.4	75.8	75.7	73.0	40-60

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.

4.3 Rumen fermentation and *in sacco* study

4.3.1 Rumen pH

Rumen pH was measured with either a handheld pH meter or continuous pH data loggers. The handheld measurements were done at 08:00, 14:00, 20:00 and 02:00 in all four experimental periods and data is presented in Table 4.5. The only differences occurred at 08:00 and 14:00 ($P < 0.05$). At 08:00 the ionophore, EO and EO + Y treatments resulted in similar pH values which were higher than the control treatment pH ($P < 0.05$). The mean rumen pH over all four measured times tended ($P < 0.10$) to be higher for the ionophore and EO + Y treatments compared to the control treatment. The mean rumen pH did not differ ($P > 0.10$) between the EO and the control treatment. Figure 4.1 illustrates the pH fluctuations obtained with the handheld pH meter between treatments with different collection times.

Rumen pH is affected by feeding time and eating pattern and therefore the data loggers recorded pH every 10 minutes over a 72 hour period in order to illustrate the diurnal variation. The three measurements within each 30 minutes were averaged to present one measurement every half hour as illustrated in Figure 4.2. The lowest and highest pH measured for cows on the control treatment were 5.91 at 20:30 and 6.51 at 06:30 respectively. The essential oil treated cows revealed that the lowest and highest pH measurements were 6.06 at 19:30 and 6.57 at 06:30 respectively. The ionophore treated cows experienced their lowest and highest pH measurements at 19:30 (5.99) and 07:00 (6.63) respectively. The EO + Y treated cows revealed that their lowest and highest pH measurements were 5.89 at 19:30 and 6.50 at 07:00 respectively. These results suggested that all the treatments had their highest pH at around 07:00 and their lowest at around 20:00. This is similar to the yeast study done by Guedes *et al.* (2008) who reported that the rumen pH is normally the highest just before the cows are fed in the morning and lowest two to four hours after the cows are fed in the afternoon. In the present study this was approximately four to five hours after the afternoon feeding. The mean pH measurements obtained with the pH loggers showed no significant differences between treatments ($P > 0.05$) but it was still noted that the numerically highest pH was measured in the cows receiving the ionophore and essential oil treatments and the lowest pH in the cows receiving the control treatment.

In Table 4.6 is shown the mean pH logger values, only for the time periods when there were differences ($P < 0.05$) or tendencies for differences ($P < 0.10$) between treatments. In general, effects on pH after supplementation of these additives have been inconsistent. Essential oil supplementation increased rumen pH in comparison with the control diet in some studies (Benchaar *et al.*, 2003; Benchaar *et al.*, 2006) but not in others (Castillejos *et al.*, 2006). Ionophore supplementation of dairy cows caused either an increase in ruminal pH (Plazier *et al.*, 2000; Benchaar *et al.*, 2006) or no effect (Ramanzin *et al.*, 1997; Broderick, 2004).

Cow's receiving a well-balanced diet should maintain a rumen pH ranging between 5.8 and 6.4. This was true for all the treatment groups in the present study. All the important microbial species were accommodated by this pH range (Ishler *et al.*, 1996). The fibre

digesters and the starch digesters are the two most common groups of bacteria operating at different pH levels. The fibre digesting bacteria which are better known as the cellulolytic bacteria function best at a pH of 6.2 to 6.8. A reduction of cellulolytic and methanogenic bacteria is normally seen with a pH drop below 6. The starch digesting bacteria are more acid tolerant and function at a lower pH ranging from 5.2 to 6.0 (Ishler *et al.*, 1996). Energy is the first limiting nutrient for microbial growth and microbes deal with a large amount of energy becoming available in different ways (Rode, 2000). The excess energy is either stored by the microbes as intra or extracellular polysaccharides or the bacteria changes their fermentation pathway towards lactate production rather than acetate or propionate production (Rode, 2000).

In the presence of ruminally produced acids the extent of the pH decrease is dependant on the rate of acid production, the total amount of acids produced, the absorption rate of these acids across the rumen wall and the ability of the salivary secretions to neutralize the acids through their buffering capacity (National Research Council, 2001).

Table 4.5 Effect of different feed additive treatments on mean rumen pH measured with a handheld pH meter at 08:00, 14:00, 20:00 and 02:00 for cows fed total mixed rations (n=16)

	Treatments[1]				
Time	**Control**	**Ionophore**	**EO**	**EO+Y**	**SEM[2]**
08:00	6.20^{b}	6.52^{a}	6.43^{a}	6.47^{a}	0.06
14:00	5.79^{ab}	6.13^{b}	5.75^{a}	5.83^{ab}	0.10
20:00	5.52	5.60	5.65	5.67	0.09
02:00	5.71	5.85	5.80	5.76	0.09
average	5.80^{d}	6.02^{c}	5.91^{cd}	5.93^{c}	0.04

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean
[ab] Means in the same row without a common superscript differs (P<0.05)
[cd]Means in the same row without a common superscript tend to differ (P<0.1)

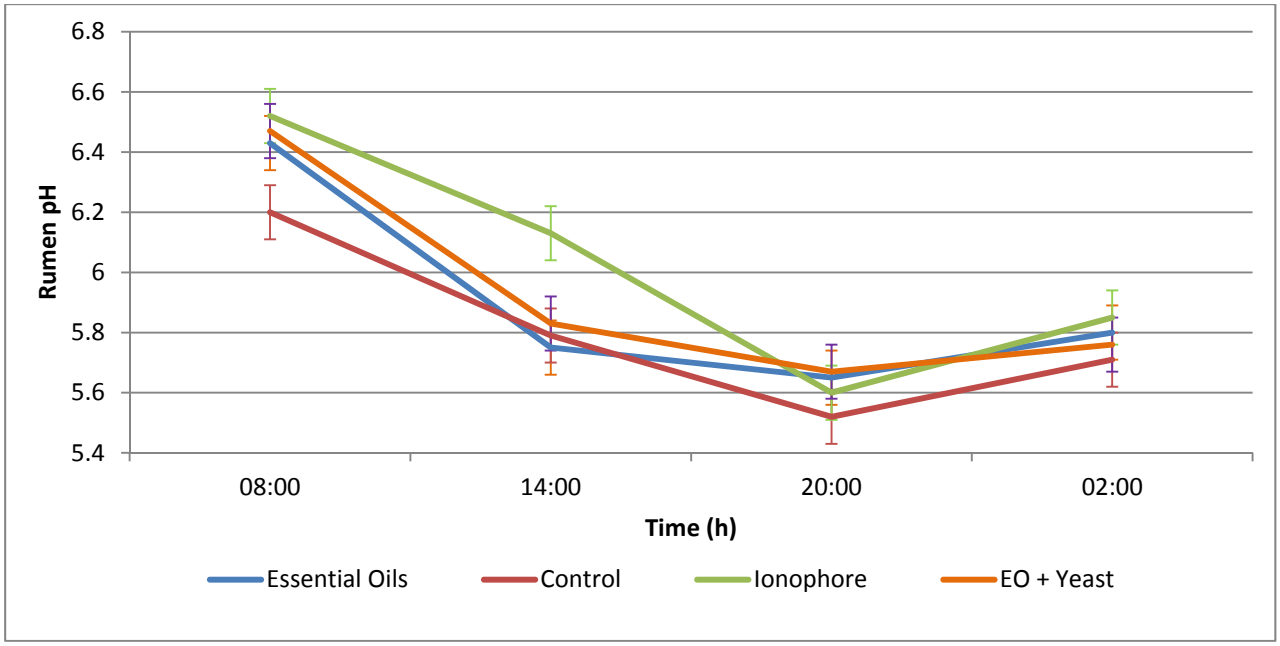

Figure 4.1 Fluctuations of the rumen pH measured in cows with a handheld pH meter at different sampling times (n = 16) receiving TMR diets supplemented with different feed additives, error bars represent SEM (Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day)

Table 4.6 Effect of different feed additive treatments on mean rumen pH measured with a pH logger in Jersey cows fed total mixed rations, only the measurements indicating a difference or a tendency to differ are included in the table (n = 16)

	Treatments[1]				
Time	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
13:00	6.02^{d}	6.28^{c}	6.30^{c}	6.10^{cd}	0.09
18:00	6.00^{cd}	6.14^{c}	6.13^{c}	5.94^{d}	0.07
21:30	5.92^{b}	6.10^{ab}	6.14^{a}	5.97^{ab}	0.06
(22:00)	5.96^{d}	6.10^{cd}	6.17^{c}	5.99^{d}	0.06
average	6.15	6.29	6.30	6.17	0.09

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean
[ab] Means in the same row without a common superscript differs (P<0.05)
[cd]Means in the same row without a common superscript tend to differ (P<0.1)

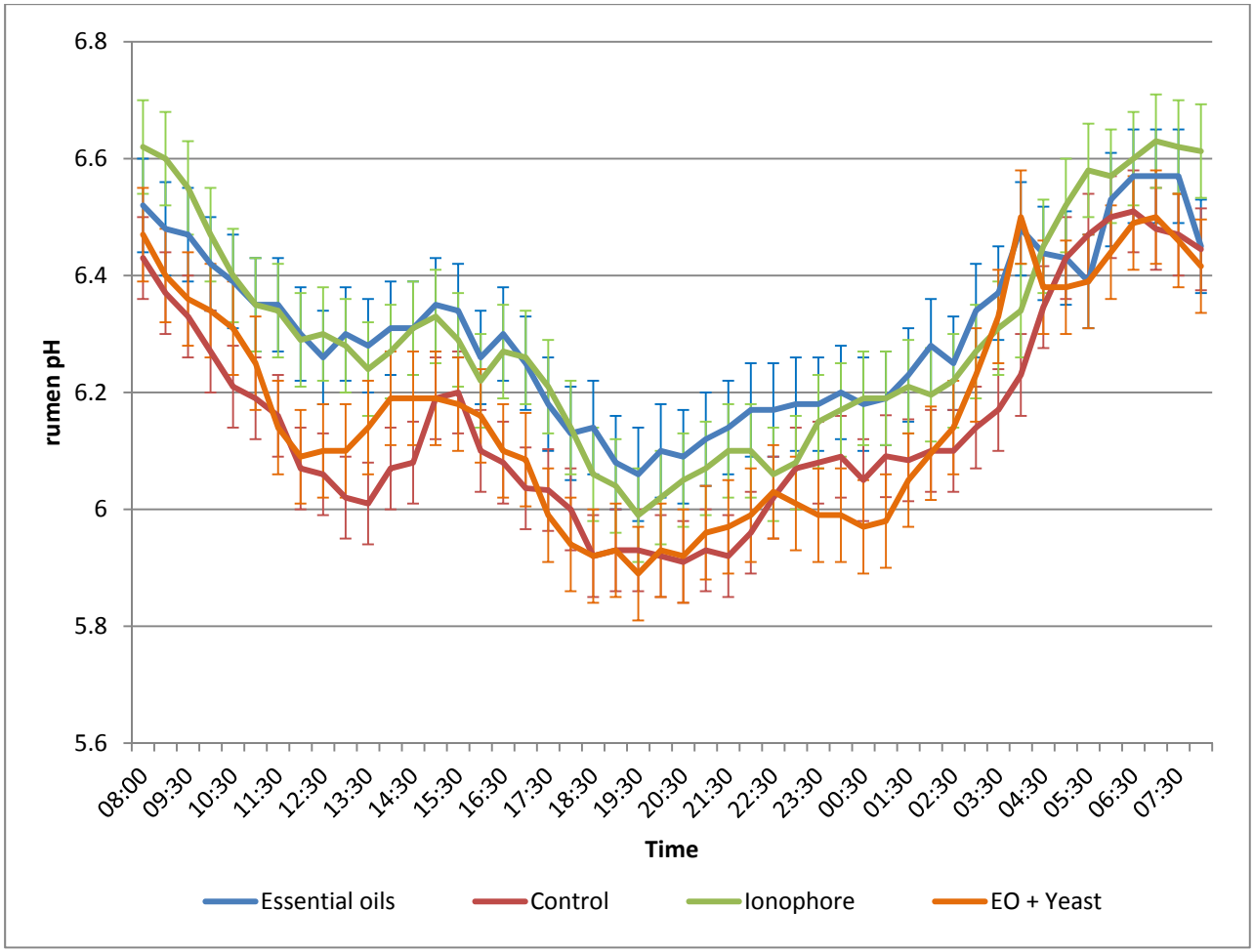

Figure 4.2 Diurnal fluctuations of the rumen pH of cows (n = 16) receiving TMR diets supplemented with different feed additives, error bars represent SEM (Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day)

Fibre digestion is optimised at pH 6.2 (Shriver *et al.*, 1986). As such a decrease in pH below 6.0 leads to a substantial depression in fibre digestion (Hoover, 1986). At pH 5.8 the digestion of NDF decreased to as low as 8.1% and overall activity of micro-organisms was reduced by 40% (Hoover, 1986). Growth of certain bacterial species also ceases below pH 6.0 (Hoover, 1986). The severity of low pH values (5.8-6.2) depends on the duration of time spent at such low pH. Cyclic drops in pH which last for a short duration of time are less severe and do not have any long lasting effects on microbial activity (Mould *et al.*, 1983; Hoover, 1986). Calsamiglia *et al.* (2002) determined that the fibrolytic and cellulolytic micro-organisms were able to maintain activity during transitory periods of low pH, where as an overall continuous low rumen pH negatively affected activity, lowering the rate of NDF and ADF digestion. A continuously lower pH results in micro-organisms expending more energy on maintenance and less replication will take place and the population size will begin to suffer (Russel and Dombrowski, 1980). When the pH is allowed to rise to an acceptable level the population recovers and activity levels will increase (Russel and Dombrowski,

1980). Calsamiglia *et al.* (2002) found that a drop in pH below 6.0 for less than four hours had negligible effects on rumen activity.

Subacute rumen acidosis (SARA) is characterized by repeated bouts of depressed rumen pH values between 5.2 to 5.6 (Duffield *et al.*, 2004). The disorder often results from a large intake of rapidly fermentable carbohydrates that leads to the accumulation of organic acids in the rumen (Duffield *et al.*, 2004). When assessing the significance of ruminal pH, it is important to consider not only the mean pH, but also the postprandial fluctuations, and in particular the time duration of suboptimal pH which is pH < 5.6 (Nagaraja & Titgemeyer, 2007). The number of hours which the rumen pH spent below 6.2, 6.0 and 5.8 during the 72 hours that the pH loggers were recording the pH is presented in Table 4.7. There were no differences between treatments (P > 0.05).

In a study by Keunen *et al.* (2002) the pH profile of TMR fed dairy cows were investigated. They found that the average pH values of control cows without SARA and SARA cows differed and were 6.25 and 6.11 respectively. The time per day the rumen pH spent below 6.0 was 10.7 and 5.3 hours for cows with- and without SARA respectively. The time per day the rumen pH spent below 5.6 was 2.6 and 1.8 hours for cows with- and without SARA respectively (Keunen *et al.*, 2002). The pH values of our present study compared well with the pH values of the control cows without SARA which indicates that our cows were not likely to have SARA.

Table 4.7 Mean time in hours per day that the rumen fluid spent below a specific pH (6.2, 6.0 and 5.8) of cows receiving TMR's supplemented with different feed additives (n = 16)

	Treatments[1]				
pH	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
< 6.2	14.0	10.2	11.1	13.8	5.08
< 6.0	7.97	6.53	8.00	8.70	3.82
< 5.8	3.50	3.83	4.87	2.70	5.76

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean
[ab] Means in the same row without a common superscript differs (P<0.05)
[cd]Means in the same row without a common superscript tend to differ (P<0.1)

4.3.2 Volatile fatty acids

Individual and total VFA concentrations (mmol/L) as well as individual molar percentages are represented in Table 4.8. Volatile fatty acids were measured at 08:00, 14:00, 20:00 and 02:00 in each experimental period. All these measurements were combined and averaged to represent the results shown in Table 4.8. The only differences (P < 0.05) in VFA's were found in butyric acid and tendencies to differ (P < 0.10) were found in iso-butyric and valeric acid. This will be discussed in more detail later in this chapter.

Table 4.8 Effect of different feed additive treatments on daily rumen volatile fatty acid production, ammonia nitrogen concentration and lactic acid concentration of cows fed a total mixed ration (n = 16)

	Treatments[1]				
Parameters	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
Total VFA (mmol/L)	117.8	110.7	120.5	122.5	6.16
Acetic acid (mmol/L)	74.1	71.9	76.6	77.2	4.34
Propionic acid (mmol/L)	26.3	24.0	24.4	26.6	1.86
Butyric acid (mmol/L)	15.1^{ab}	12.6^{b}	17.2^{a}	16.3^{a}	1.07
Iso butyric acid (mmol/L)	0.62	0.73	0.68	0.67	0.06
Valeric acid (mmol/L)	1.64	1.48	1.72	1.79	0.09
VFA Molar %					
Acetic acid %	63.1	65.2	63.6	63.2	1.05
Propionic acid %	22.0	21.3	20.1	21.4	1.19
Butyric acid %	13.0^{ab}	11.4^{b}	14.4^{a}	13.4^{a}	0.69
Iso butyric acid %	0.53^{c}	0.70^{d}	0.58^{cd}	0.56^{cd}	0.05
Valeric acid %	1.40^{cd}	1.33^{c}	1.42^{cd}	1.46^{d}	0.04
Acetic: Propionic	2.99	3.17	3.22	3.03	0.19
NH3-N (mg/dL)	9.79	9.45	11.3	10.0	0.87
Lactic acid (mg/dL)	1.79^{c}	1.83^{c}	1.55^{cd}	1.43^{d}	0.13
Lactic Acid (mmol/L)	0.20^{d}	0.20^{d}	0.17^{cd}	0.16^{c}	0.01

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean
[ab] Means in the same row without a common superscript differs (P<0.05)
[cd]Means in the same row without a common superscript tend to differ (P<0.1)

4.3.2.1 Total volatile fatty acids

The mean rumen VFA concentration (mmol/L) sampled at 08:00, 14:00, 20:00 and 02:00 over all the periods is shown in Table 4.9 and illustrated in Figure 4.3. No significant differences occurred between treatments (P > 0.05) and the only tendency to differ (P < 0.1) occurred with the 14:00 sampling. The VFA concentration of cows fed the EO treatment tended to be higher than the ionophore treatment but did not differ from the control. There were no differences between the other treatments.

No effect of ionophores was observed on ruminal total VFA concentration (Callaway *et al.*, 2003; Benchaar *et al.*, 2006), however, a study by Ponce *et al.* (2012) found that total volatile fatty acid concentrations were decreased for ionophore supplemented animals. The total VFA concentrations were not affected by EO supplementation (Newbold *et al.*, 2004; Benchaar *et al.*, 2006), however, an increase in total VFA concentrations were reported by Castillejos *et al.* (2005) and a decrease in total VFA concentrations were reported by Macheboeuf *et al.* (2008) when an EO mixture was supplemented. In the meta-analysis of Desnoyers *et al.* (2009) it was concluded that yeast supplementation led to an increased total VFA concentration.

The time of sampling after feeding was proven by Abd El-Ghani (2004) to have a significant effect on the VFA concentration. The studies by Andrighetto *et al.*, (1993) and Doreau and Jouany (1998) agreed with these results. In the present study the highest VFA concentrations were found in the samples taken at 14:00 and 20:00 which were 6 and 4 hours after feeding. Lower VFA concentrations were found in the samples taken at 02:00 and 08:00 which were 10 and 16 hours after feeding. The 08:00 measurements had significantly lower VFA concentrations compared to all the other sampling times. These results are supported by Andrighetto *et al.* (1993) who found that intense fermentation takes place up to three hours after feeding which causes great variation in VFA concentrations and thereafter the VFA concentration decrease until the next feeding.

Table 4.9 Effect of different feed additive treatments on total rumen volatile fatty acid concentration (mmol/L) measured at 08:00, 14:00, 20:00 and 02:00 for cows fed total mixed rations (n = 16)

	Treatments[1]				
Time	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
08:00	106.8	95.4	107.6	102.5	10.06
14:00	120.6[cd]	106.4[d]	128.1[c]	121.7[cd]	6.52
20:00	122.1	127.5	125.2	136.5	10.36
02:00	121.8	113.7	121.0	129.3	6.16

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean
[cd]Means in the same row without a common superscript differ (P<0.1)

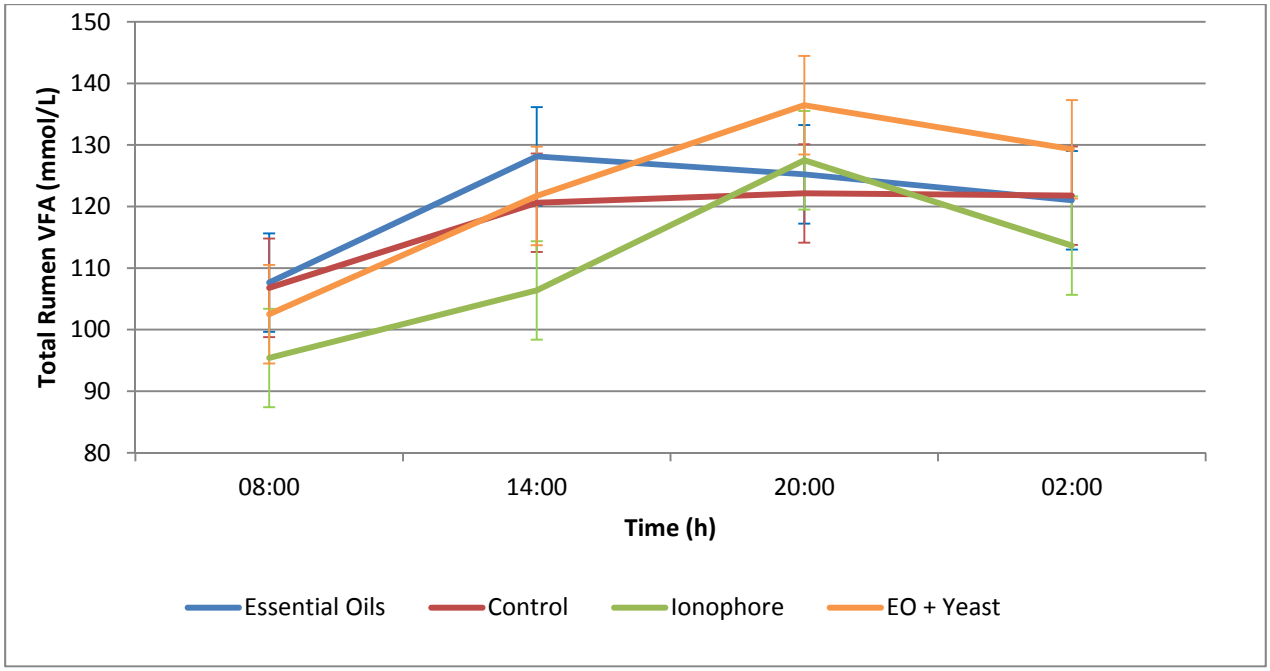

Figure 4.3 Diurnal variation in total rumen VFA concentrations (mmol/L) at different sampling times in cows (n = 16) receiving TMR diets supplemented with different feed additives, error bars represent SEM (Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day)

4.3.2.2 Acetic acid

Compared to other VFA's the rumen of animals consuming high fibre diets produce mostly acetate (Ishler *et al.*, 1996). Dairy heifers consuming a low concentrate diet had a significantly higher molar percentage of acetate in comparison with a high concentrate diet (Lascano and Heinrichs, 2009). After being produced the acetate is absorbed and utilised for fatty acid synthesis and deposited in adipose tissue as fat or used to produce milk fat (Ishler *et al.*, 1996).

In this present study the rumen acetic acid concentrations (mmol/L) measured at 08:00, 14:00, 20:00 and 02:00 were averaged over all the periods and presented in Table 4.10. No differences occurred between treatments ($P > 0.05$) and the only tendency to differ ($P < 0.1$) occurred with the 14:00 measurement. The ionophore treatment tended to have a higher rumen acetic acid molar percentage than the control treatment at 65.2% and 63.07 respectively. There were no differences between the other treatments. A study by Bargo *et al.* (2002) reported similar results of concentrations of acetic acid. In their study three different feeding systems were examined and it was found that the three systems resulted in similar molar percentages of acetic acid and was therefore averaged into one value of 63.1%. These values agree with the data from our study and falls within the range for acetic acid of 55-70% of total VFA (Hutjens, 2008). The fatty acids deposited in the milk in the mammary gland are a direct result of the absorbed rumen acetic acid produced from fibre fermentation in the rumen (Ishler *et al.*, 1996).

In a study by Ponce *et al.* (2012) it was found that molar proportions of acetate and total volatile fatty acid concentrations were decreased for all ionophore supplemented animals compared with control. Duffield and Bagg (2000) found similar results with ionophore supplementation. They observed that monensin changes the VFA ratio in the rumen, increasing propionic acid and reducing the molar percentages of acetic and butyric acid. The meta-analysis done by Duffield *et al.* (2008a) agrees with these studies and indicates that monensin supplementation led to decreased acetate production. Benchaar *et al.* (2008) observed that certain EO and their components shift molar proportions of volatile fatty acids in much the same way as ionophores by reducing acetate and increasing propionate proportions. Mohammed *et al.* (2004) found the same results both *in vitro* and *in vivo* with cyclodextrin encapsulated horseradish. They reported a decrease in acetate and increase in propionate proportion. McGuffey *et al.* (2001) also reported decreased acetate and increased propionate proportions when certain EO and components were added to ruminant diets. Fonty and Chaucheyras-durand (2006) observed that certain strains of *Streptococcus cerevisiae* caused a decrease in the volatile fatty acid proportion of acetate. The meta-analysis of Desnoyers *et al.* (2009) however, indicated that yeast supplementation had no influence on the acetate concentration.

Figure 4.4 illustrates the changes in acetic acid concentration (mmol/L) of the different treatments at the different collection times. The acetic acid concentrations appeared to be numerically the highest at 20:00 and the lowest at 08:00 for all the treatments.

Table 4.10 Effect of different feed additive treatments on average acetic acid concentration (mmol/L) measured at 08:00, 14:00, 20:00 and 02:00 for cows fed total mixed rations (n = 16)

	Treatments[1]				
Time	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
08:00	69.1	63.8	70.2	66.3	7.12
14:00	76.6^{cd}	69.8^{d}	82.1^{c}	77.3^{cd}	3.72
20:00	75.1	81.8	78.6	84.3	7.36
02:00	75.8	72.0	75.3	80.8	3.44

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean
[cd]Means in the same row without a common superscript tend to differ (P<0.1)

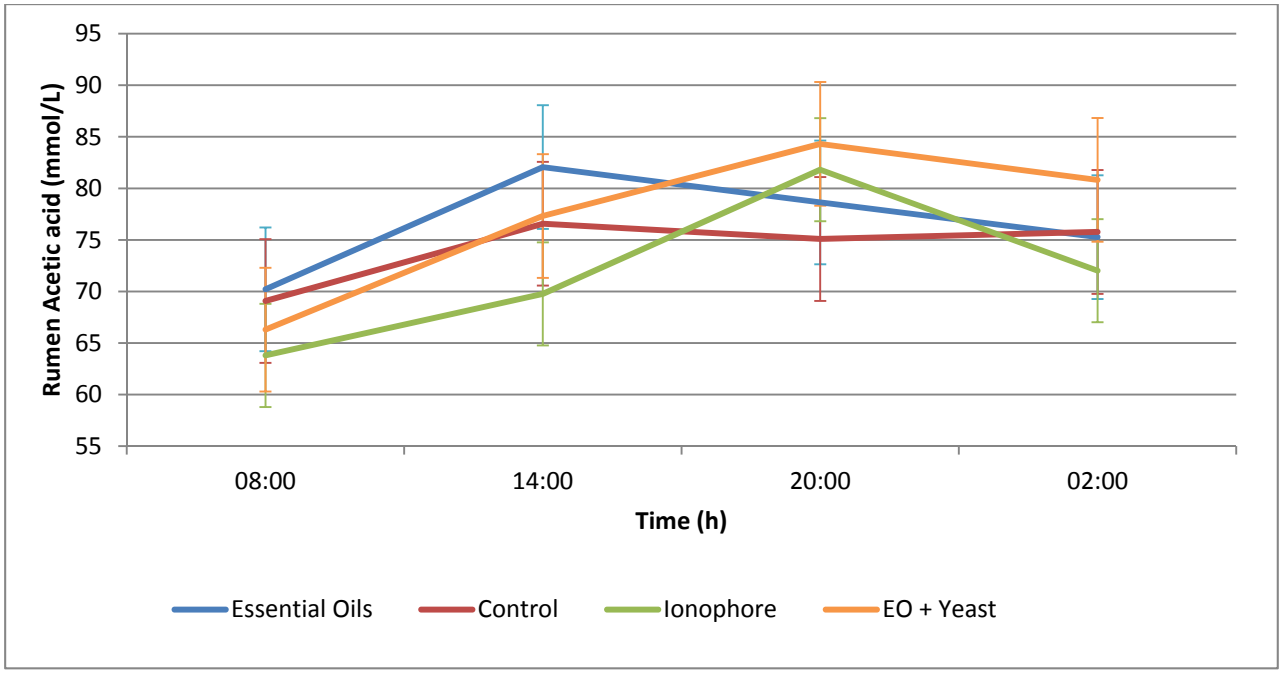

Figure 4.4 Diurnal variation in rumen acetic acid concentration (mmol/L) at different sampling times of cows (n = 16) receiving TMR diets supplemented with different feed additives, error bars represent SEM (Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day)

4.3.2.3 Propionic acid

When concentrate mixes or diets high in grain are fed then rumen propionic acid production predominates (Ishler *et al.*, 1996; Eastridge, 2006; Lascano and Heinrichs, 2009). The liver utilises propionic acid as a precursor for glucose synthesis which is used as an energy source and is also a precursor for synthesis of milk lactose (Ishler *et al.*, 1996).

Ionophores alter the ion flux across the membranes of gram positive bacteria. This changes the rumen bacteria population and favours propionic acid synthesizing bacteria which leads to higher propionic acid levels when ionophores are added to the diet (Dubuc *et al.*, 2009). Ponce *et al.* (2012) also found that the molar proportions of propionate were increased for the average of all ionophore supplemented animals. These results are also supported by the meta-analysis done by Duffield *et al.* (2008a) which indicates that monensin changes the VFA ratio by increasing the propionic acid production and by decreasing the acetic and butyric acid production. Benchaar *et al.* (2008) observed that specific EO and their components shift molar proportions of volatile fatty acids in much the same way as ionophores by reducing acetate and increasing propionate proportions. Mohammed *et al.* (2004) found the same results both *in vitro* and *in vivo* with cyclodextrin encapsulated horseradish. They reported a decrease in acetate and increase in propionate proportion. McGuffey *et al.* (2001) also reported decreased acetate and increased propionate proportions when certain EO and components were added to ruminant diets. Chaucheyras-durand *et al.* (2008) observed that certain yeast strains like *Propionibacterium spp.* may cause an increase in propionate production. The meta-analysis of Desnoyers *et al.* (2009) as well as the meta-

analysis of Sauvant *et al.* (2008), however, found that no influence of yeast supplementation was determined on the propionate concentration.

The rumen propionic acid concentration (mmol/L) measured at 08:00, 14:00, 20:00 and 02:00 were averaged over all the periods and shown in Table 4.11. No significant differences occurred between treatments at any of the sampling times ($P > 0.05$). Figure 4.5 illustrates the diurnal variation in propionic acid concentration (mmol/L) of the different treatments at the different collection times. The propionic acid concentration did not differ between treatments ($P < 0.05$) but were numerically the highest at 20:00 and the lowest at 08:00, similar to acetic acid concentrations.

Table 4.11 Effect of different feed additive treatments on average propionic acid concentration (mmol/L) measured at 08:00, 14:00, 20:00 and 02:00 for cows fed total mixed rations (n = 16)

	Treatments[1]				
Time	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
08:00	20.0	17.4	18.7	18.7	1.88
14:00	26.1	22.3	25.6	25.6	2.86
20:00	30.1	29.2	26.9	32.2	2.46
02:00	29.0	27.3	26.5	29.7	2.18

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean

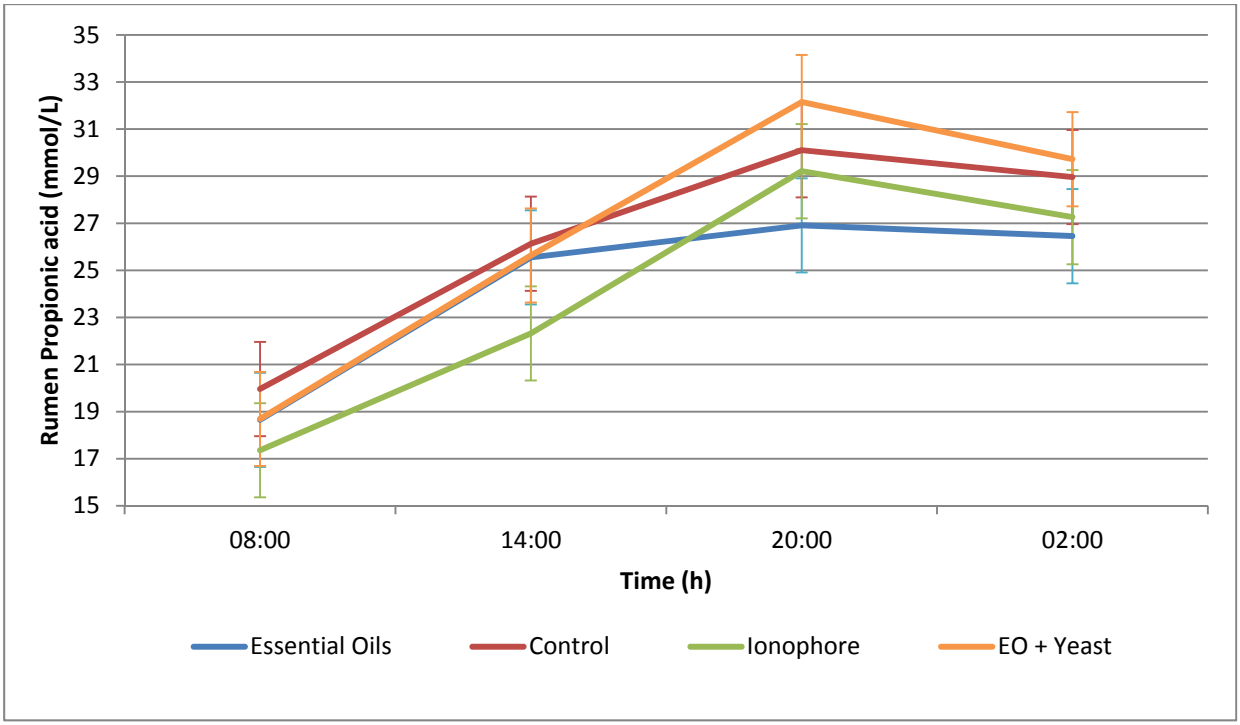

Figure 4.5 Diurnal variation in rumen propionic acid concentration (mmol/L) fluctuations at different sampling times of cows (n = 16) receiving TMR diets supplemented with different feed additives, error bars represent SEM (Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day)

4.3.2.4 Butyric acid

The rumen epithelium converts butyric acid to beta-hydroxybutyrate during absorption. During this conversion the butyric acid also provides energy to the rumen wall and is the primary source of energy for papillae development (Baldwin *et al.*, 2004). The beta-hydroxybutyrate is a ketone which is used for fatty acid production, to be stored in the adipose tissue (Ishler *et al.*, 1996).

The rumen butyric acid concentration (mmol/L) measured at 08:00, 14:00, 20:00 and 02:00 were averaged over all the periods and is presented in Table 4.12. At 14:00 the essential oil treatment was higher than the ionophore treatment ($P < 0.05$) but there were no other differences between treatments in terms of butyric acid concentration. At 08:00 and 02:00 the essential oil treatment tended to have a higher butyric acid concentration than the ionophore treatment ($P < 0.10$). There were no differences between the other treatments.

Odongo *et al.* (2007) and Ipharraguerre and Clark (2003) found that ionophores cause a decreased ruminal production of butyrate. They also found it causes decreased acetate production and this can contribute to milk fat depression. The major cause of milk fat depression, however, is due to changes in rumen biohydrogenation of unsaturated fatty acids and passage of specific intermediates of biohydrogenation out of the rumen, eg. Trans-10 cis-12 CLA. These intermediates reduce milk fat synthesis in the mammary gland by altering the expression of genes involved in fat synthesis. It is therefore not a single factor such as low

acetic acid concentration, by any combination of factors that can alter the rumen environment in addition to the rumen unsaturated fatty acid load that leads to milk fat depression (Lock *et al.*, 2012). The meta-analysis of Duffield *et al.* (2008a) also indicated that ionophore supplementation caused reductions in the molar percentages of butyric acid. Busquet *et al.* (2005b) observed that molar proportions of butyrate increased when EO from cinnamaldehyde and garlic oil was added to the ruminant diet. Busquet *et al.* (2006) also found that garlic oil and benzyl salicylate increased butyrate proportions. Chaves *et al.* (2008a) found that EO (cinnamon leaf oil) containing eugenol caused an increase in butyrate production. Fonty and Chaucheyras-durand (2006) observed that certain strains of *Streptococcus cerevisiae* caused an increase in volatile fatty acid proportions of butyrate. Lynch and Martin (2002), however, found that neither a *Streptococcus cerevisiae* culture nor live cells had an effect on butyrate concentration. The results from these studies were similar than the results of our current study.

In Figure 4.6 is illustrated the diurnal variation in the butyric acid concentration (mmol/L) changes of the different treatments at the different collection times.

Table 4.12 Effect of different feed additive treatments on average butyric acid concentration (mmol/L) measured at 08:00, 14:00, 20:00 and 02:00 for cows fed total mixed rations (n = 16)

	Treatments[1]				
Time	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
08:00	15.5^{cd}	12.1^{d}	16.4^{c}	15.3^{cd}	1.42
14:00	15.7^{ab}	12.1^{b}	17.9^{a}	16.3^{a}	1.11
20:00	14.7	14.0	17.3	17.5	1.39
02:00	14.7^{cd}	12.3^{d}	17.0^{c}	16.2^{c}	1.39

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean
[ab] Means in the same row without a common superscript differs (P<0.05)
[cd]Means in the same row without a common superscript tend to differ (P<0.1)

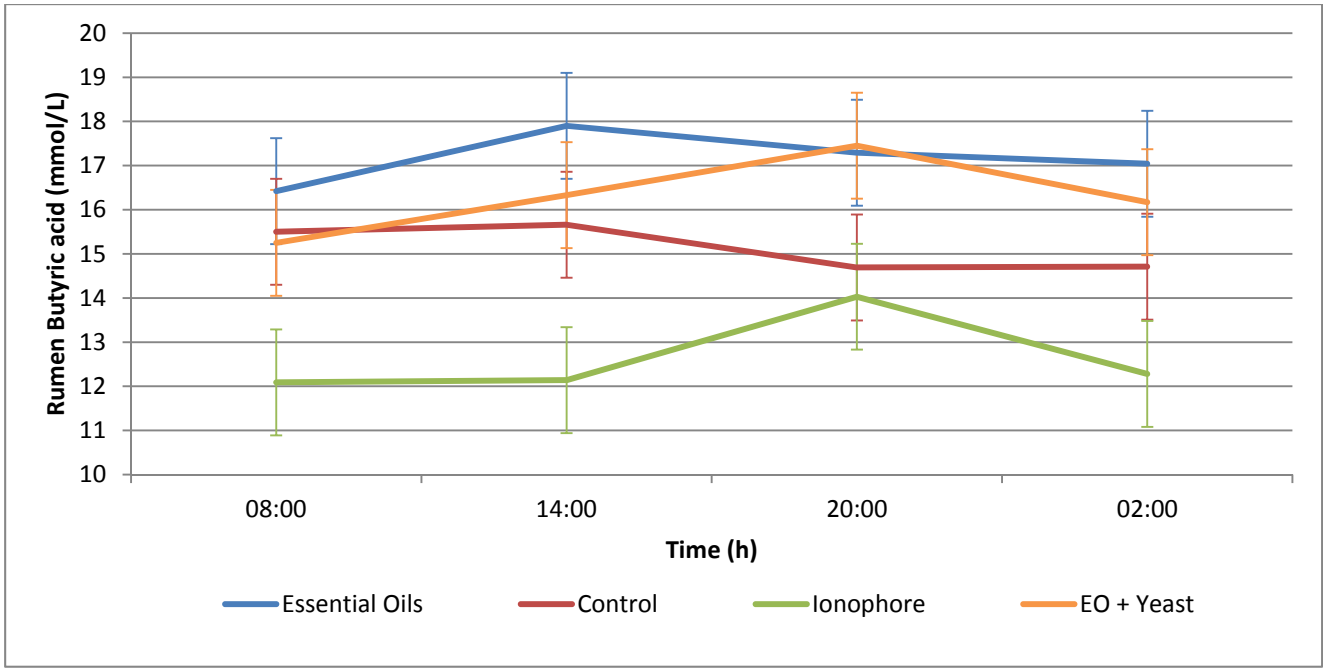

Figure 4.6 Diurnal variation in rumen butyric acid concentration (mmol/L) at different sampling times of cows (n = 16) receiving TMR diets supplemented with different feed additives, error bars represent SEM (Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day)

4.3.2.5 Isobutyric acid

Isobutyric acid is additional VFA's formed in the rumen from valine, generally in small quantities by deamination of amino acids (McDonald *et al.*, 2002).

The rumen isobutyric acid concentration (mmol/L) measured at 08:00, 14:00, 20:00 and 02:00 were averaged over all the periods and shown in Table 4.13. No differences occurred between treatments at any of the measurement times ($P > 0.05$). The isobutyric acid concentrations were all numerically higher at 08:00 compared to the measurements at the other collection times.

Figure 4.7 illustrates diurnal variation in the isobutyric acid concentration (mmol/L) of the different treatments at the different collection times.

Castillejos *et al.* (2006) indicated that the iso-butyric acid concentration was decreased by an EO mixture containing 500 mg/L thymol ($P < 0.05$). They also observed that smaller doses of 5 to 50 mg/L thymol EO did not change the iso-butyric acid concentration. Similarly an EO mixture containing 500 mg/L eugenol decreased the isobutyric acid concentration, while a 5 to 50 mg/L concentration had no effect. A study by Domescik and Martin (1999) indicated that ionophore supplementation did not alter the isobutyric acid concentrations even though the total VFA concentrations were decreased. Castillejos *et al.* (2006) also found that supplementing ionophores at 10 mg/L had no effect on the isobutyric acid concentration. Lascano and Heinrichs (2009) found that yeast supplementation to dairy cows caused increased ruminal iso-butyric acid concentrations. However, Robinson and

Garret (1999) found that the iso-butyric acid concentration is unaffected by yeast supplementation.

Table 4.13 Effect of different feed additive treatments on average isobutyric acid concentration (mmol/L) measured at 08:00, 14:00, 20:00 and 02:00 for cows fed total mixed rations (n = 16)

	Treatments[1]				
Time	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
08:00	0.74	0.95	0.87	0.80	0.08
14:00	0.58	0.66	0.68	0.60	0.06
20:00	0.56	0.68	0.60	0.63	0.09
02:00	0.60	0.64	0.57	0.63	0.05

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean

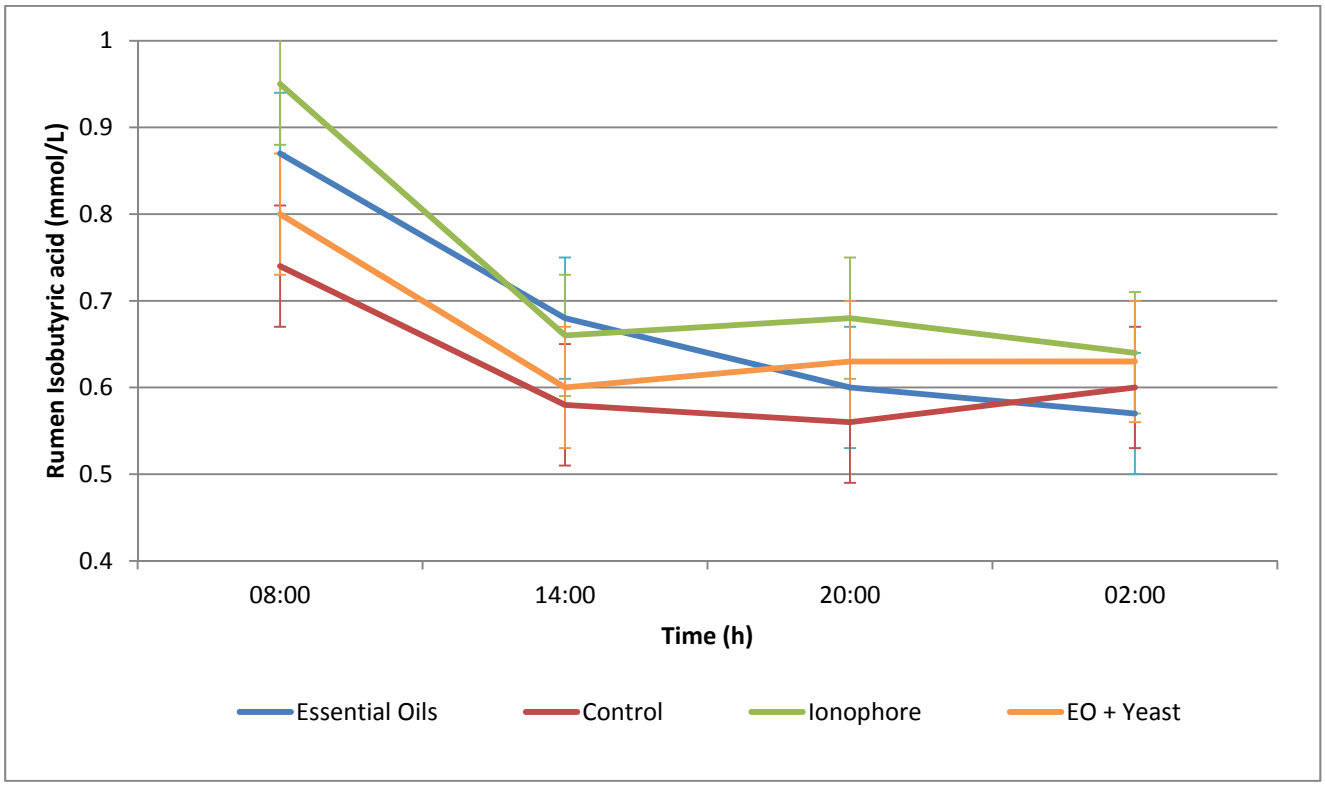

Figure 4.7 Rumen isobutyric acid concentration (mmol/L) fluctuations at different sampling times of cows (n = 16) receiving TMR diets supplemented with different feed additives, error bars represent SEM (Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day)

4.3.2.6 Valeric acid

Valeric acid is a VFA produced from fermentation of both structural and non-structural carbohydrates (Ishler *et al.*, 1996).

The rumen valeric acid concentration (mmol/L) measured at 08:00, 14:00, 20:00 and 02:00 were averaged over all the periods and shown in Table 4.14.

At 14:00 and 02:00 there were differences between treatments ($P < 0.05$). At the 14:00 measurement the essential oil treatment showed a higher valeric acid concentration compared to the ionophore treatment ($P < 0.05$). With the 02:00 measurement the EO + Y treatment had a significantly higher valeric acid concentration compared to the ionophore treatment ($P < 0.05$).

In Figure 4.8 is illustrated the diurnal variation in valeric acid concentration (mmol/L) for the different treatments at the different collection times. Similar to the other VFA's, except iso-butyric acid, the valeric acid concentration was numerically the lowest at the 08:00 sampling time.

A study by Castillejos *et al.* (2006) indicated that the valeric acid concentration was reduced by an EO mixture containing 500 mg/L thymol. They also found that smaller doses of 5 to 50 mg/L thymol EO had no effect on the valeric acid concentration. The same study by Castillejos *et al.* (2006) indicated that 500 mg/L eugenol EO caused an increase in the valeric acid concentration ($P < 0.05$), while a concentration of 5 to 50 mg/L eugenol EO had no effect on the valeric acid concentration. Domescik and Martin (1999) observed that ionophore supplementation increased the valeric acid concentration when compared to control ($P < 0.05$). Castillejos *et al.* (2006) found similar results which indicated that ionophores supplemented at 10 mg/L led to increased valeric acid concentrations ($P < 0.10$). Yeast supplementation leads to increased ruminal valeric acid (Dawson *et al.*, 1990) and *in vitro* valeric acid concentrations (Sullivan and Martin, 1999; Miller-Webster *et al.*, 2002). Lascano and Heinrichs (2009) also found that yeast products lead to increased valerate concentrations. Some studies however found that the valeric acid concentration is unaffected by yeast supplementation in dairy cows (Erasmus *et al.*, 1992; Putnam *et al.*, 1997; Robinson and Garret, 1999; Erasmus *et al.*, 2005).

Table 4.14 Effect of different feed additive treatments on average valeric acid concentration (mmol/L) measured at 08:00, 14:00, 20:00 and 02:00 for cows fed total mixed rations (n = 16)

	Treatments[1]				
Time	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
08:00	1.51	1.20	1.48	1.46	0.13
14:00	1.67[ab]	1.48[b]	1.95[a]	1.84[a]	0.09
20:00	1.68	1.78	1.78	1.90	0.15
02:00	1.71[ab]	1.47[b]	1.69[ab]	1.96[a]	0.14

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean
[ab] Means in the same row without a common superscript differs (P<0.05)

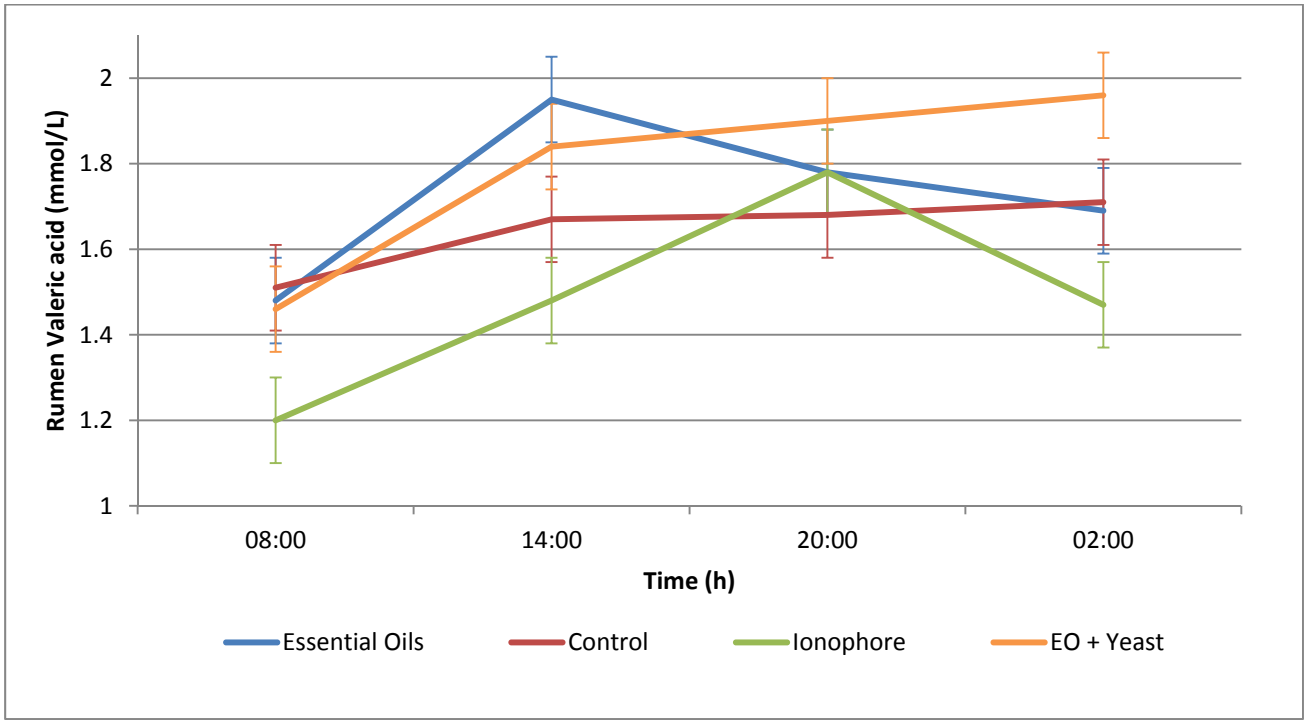

Figure 4.8 Diurnal variation in rumen valeric acid concentration (mmol/L) at different sampling times in cows (n = 16) receiving TMR diets supplemented with different feed additives, error bars represent SEM (Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day)

4.3.2.7 Acetic:Propionic ratio

The rumen acetic acid: propionic acid ratio (A:P) measured at 08:00, 14:00, 20:00 and 02:00 were averaged over all the periods and presented in Table 4.15. No differences occurred between treatments at any of the measurement times ($P > 0.05$).

In Figure 4.9 is illustrated the acetic: propionic acid ratio of the different treatments at the different collection times. All the treatments had the highest A:P ratio at 08:00, but decreased throughout the day until 20:00 where it stayed mostly constant till 02:00.

An A:P ratio below 2.2:1 generally indicates milk fat depression. The rumen VFA data supports the production data in the sense that the acetic: propionic ratio was never below 2.2:1 and no milk fat depression occurred (Bauman and Griinari, 2003).

Ipharraguerre and Clark (2003) reported that ionophores cause a shift in the acetate to propionate ratio toward less acetate and more propionate production and thereby decrease the A:P ratio. Similar results were reported by DiLorenzo (2010) and Nagaraja *et al.* (1982). Ionophore supplementation leads to an increase in total VFA production by increasing propionate production while having no effect on acetate production. This leads to a lower A:P ratio (Ponce *et al.*, 2012). From the current study it can be seen that the ionophore treatment did result in a lower A:P ratio.

Because EO have similarities with ionohores in terms of its antimicrobial action and mode of action, it may be expected that one of the main effects of EO on ruminal fermentation would be a shift in the proportions of volatile fatty acids towards a reduced A:P ratio (DiLorenzo, 2010). A decrease in the A:P ratio was found with essential oil addition in some studies (Busquet *et al.*, 2006; Cardozo *et al.*, 2006). This is however not always the case and some studies have reported no effects on volatile fatty acid molar proportions (Benchaar *et al.*, 2006; Benchaar *et al.*, 2007b; Meyer *et al.*, 2009). In a study conducted by Benchaar *et al.* (2008) they found that cinnamon essential oil and its main component cinnamaldehyde caused higher acetate to propionate ratios at a pH of 7. They however found that cinnamon essential oil and cinnamaldedhyde resulted in lower acetate to propionate ratios at a pH of 5.5. Santos *et al.* (2010) found similar results indicating that eugenol caused a sharp *in vitro* increase in the acetate to propionate ratio. Castillejos *et al.* (2007) also reported an increase in the A:P ratio when EO were fed. From the current study it can be seen that the essential oil treatment had a slightly higher A:P ratio which agrees with the observations of some of the above authors. The A:P ratio response to EO is diet dependant, which explains the different results of the different studies (Santos *et al.*, 2010)

The meta-analysis of Desnoyers *et al.* (2009) indicated that yeast supplementation had no influence on the A:P ratio. Yeast supplementation also failed to affect the A:P ratio in various other studies (Erasmus *et al.*, 1992; Erasmus *et al.*, 2005; Moya *et al.*, 2009). Even though the acetate and propionate molar concentrations increased significantly, the acetic to propionic acid ratio were not different between treatments (Lascano and Heinrichs, 2009). Pinos-Rodriguez *et al.* (2008), however found that yeast supplemented calves had a significantly higher A:P ratio compared to control. Conversely, yeast supplementation caused

decreased A:P ratios in certain studies (Besong *et al.*, 1996; Hinman *et al.*, 1998; Guedes *et al.*, 2008).

Table 4.15 Effect of different feed additive treatments on the average acetic:propionic acid ratio measured at 08:00, 14:00, 20:00 and 02:00 for cows fed total mixed rations (n = 16)

	Treatments[1]				
Time	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
08:00	3.55	3.88	3.84	3.63	0.24
14:00	3.09	3.32	3.23	3.07	0.27
20:00	2.62	2.81	2.95	2.71	0.16
02:00	2.71	2.67	2.87	2.74	0.14

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean

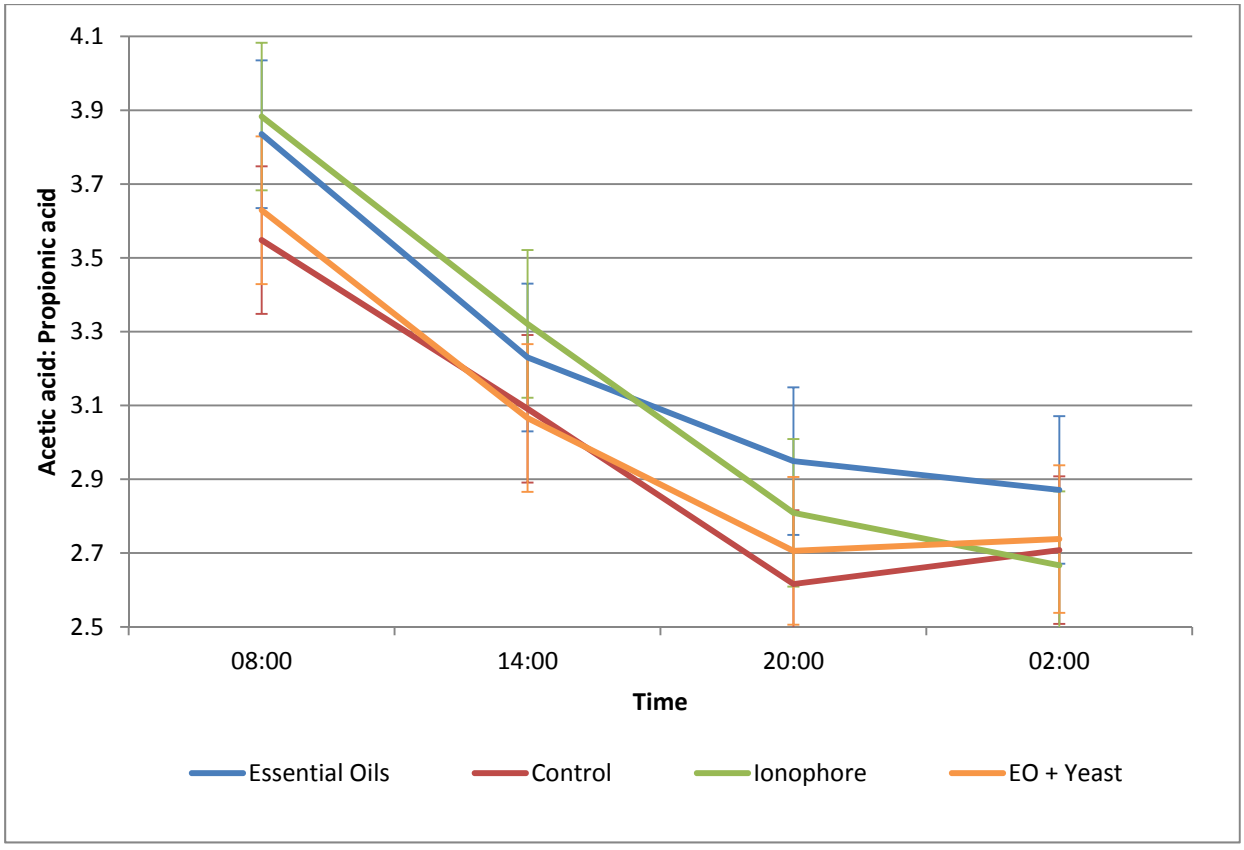

Figure 4.9 Diurnal variation in rumen acetic to propionic acid ratio at different sampling times of cows (n = 16) receiving TMR diets supplemented with different feed additives, error bars represent SEM (Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day)

4.3.3 Ammonia Nitrogen

The optimal ammonia concentration of rumen fluid may be defined as that which results either in the maximum rate of fermentation in the rumen or that which allows the maximum production of microbial protein per unit of substrate fermented (Mehrez *et al.*, 1977). Concentrations of rumen ammonia are affected by the fermenting ability of the diet as well as the endogenous and recycled N to the rumen (Olsen *et al.*, 1994). A reduced N loss during digestion of high quality pasture is a result of the inclusion of non-structural carbohydrates in the diet due to the dilution effect of N intake (Kolver *et al.*, 1998). Several *in vitro* studies found that to not compromise microbial production a minimum rumen ammonia nitrogen concentration of 5-6 mg/dL rumen fluid is required (Satter and Roffler, 1974; Satter and Slyter, 1974). However, the *in vivo* study by Mehrez *et al.*, (1977) has shown the rumen ammonia concentration needed for maximum digestion is 19.4 mg/dL. Erdman *et al.* (1986) suggested that rumen ammonia nitrogen needed for maximum degradation depends on the fermentability of the feed, higher fermentable feeds needing higher rumen NH_3-N concentrations. The equation derived from their studies: (minimum rumen NH_3-N (mg/dL) = 0.452 fermentability % - 15.71). From this equation they determined that the minimum rumen NH_3-N concentrations needed for TMR diets are 9-13 mg/dL. The present study resulted in optimum rumen NH_3-N concentrations which ensured that microbial production was not compromised. Adequate MUN levels in the diets of this study further determine that there are adequate rumen NH_3-N concentrations which will ensure optimal microbial protein production.

Sampling time determines to a large extent the rumen ammonia nitrogen concentration (Bargo *et al.*, 2002). In our study the rumen ammonia nitrogen concentration (mg/dL) measured at 08:00, 14:00, 20:00 and 02:00 were averaged over all the periods and presented in Table 4.16. No differences occurred between treatments and the only tendency to differ occurred at 02:00, where the essential oil treatment had a higher ammonia nitrogen concentration than the ionophore treatment ($P < 0.1$). Peak ammonia nitrogen concentrations are generally found two to four hours after feeding (Guedes *et al.*, 2008). Abd EL-Ghani (2004) performed a study in which the lower concentrations were found about six hours after feeding. In contrast, our study showed no differences between sampling times. A study by Bargo *et al.* (2002) showed the peak ammonia nitrogen concentrations were measured after concentrate ingestion which was set at 13:00 and 21:00. The peak values found by Bargo *et al.* (2002) were much higher than the values obtained by the present study. Our study however had set time intervals and periods for taking NH_3-N samples and the ammonia nitrogen concentrations not within these specific samples are unknown and could possibly have been higher or lower than the measured values.

Wessels *et al.* (1996) reported that ionophores did not affect ruminal concentrations of NH_3-N. In contrast to this Ali Haimoud *et al.* (1995) reported a decrease in ruminal NH_3-N concentration when ionophores were supplemented. Busquet *et al.* (2006) observed that certain EO like cade oil, anise oil, capsicum oil, clove, cinnamon oil, garlic oil, ginger oil, tea tree oil and oregano and their main components like eugenol, cinnamaldehyde, carvacrol, salicylate, carvone, anethol and benzyl salicylate clearly inhibited NH_3-N concentration at

high doses, effects were only minor with moderate doses and no effects were observed at low doses. Calsamiglia *et al.* (2007) also found that EO supplementation resulted in lower NH_3-N concentrations. Higher NH_3-N concentrations were found in studies when live yeast (*Saccharomyces cerevisiae*) (Pinos-Rodriguez *et al.*, 2008) as well as a yeast culture (Arcos-Garcia *et al.*, 2000) were supplemented. However the yeast culture supplementation resulted in higher ruminal NH_3-N concentrations than what was measured with the live yeast supplement. This difference in NH_3-N concentrations may be a product of higher proteolytic bacterial populations (Arcos-Garcia *et al.*, 2000). Lascano and Heinrichs (2009), however, found that the addition of yeast in all diets resulted in a lower NH_3-N concentration when compared with control. Other studies found a lack of effect of a yeast supplement on the NH_3-N concentration (Erasmus *et al.*, 2005; Guedes *et al.*, 2008; Longuski *et al.*, 2009).

Figure 4.10 illustrates the ammonia nitrogen concentration (mmol/L) changes of the different treatments at the different collection times.

Table 4.16 Effect of different feed additive treatments on rumen ammonia nitrogen concentration (mg/dL) measured at 08:00, 14:00, 20:00 and 02:00 for cows fed total mixed rations (n = 16)

	Treatments[1]				
Time	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
08:00	9.77	10.6	11.1	10.8	1.01
14:00	9.07	8.92	12.0	9.08	1.18
20:00	9.84	10.5	10.9	9.38	1.52
02:00	10.5^{cd}	7.87^{d}	11.2^{c}	10.9^{c}	1.00

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean
[cd]Means in the same row without a common superscript tend to differ (P<0.1)

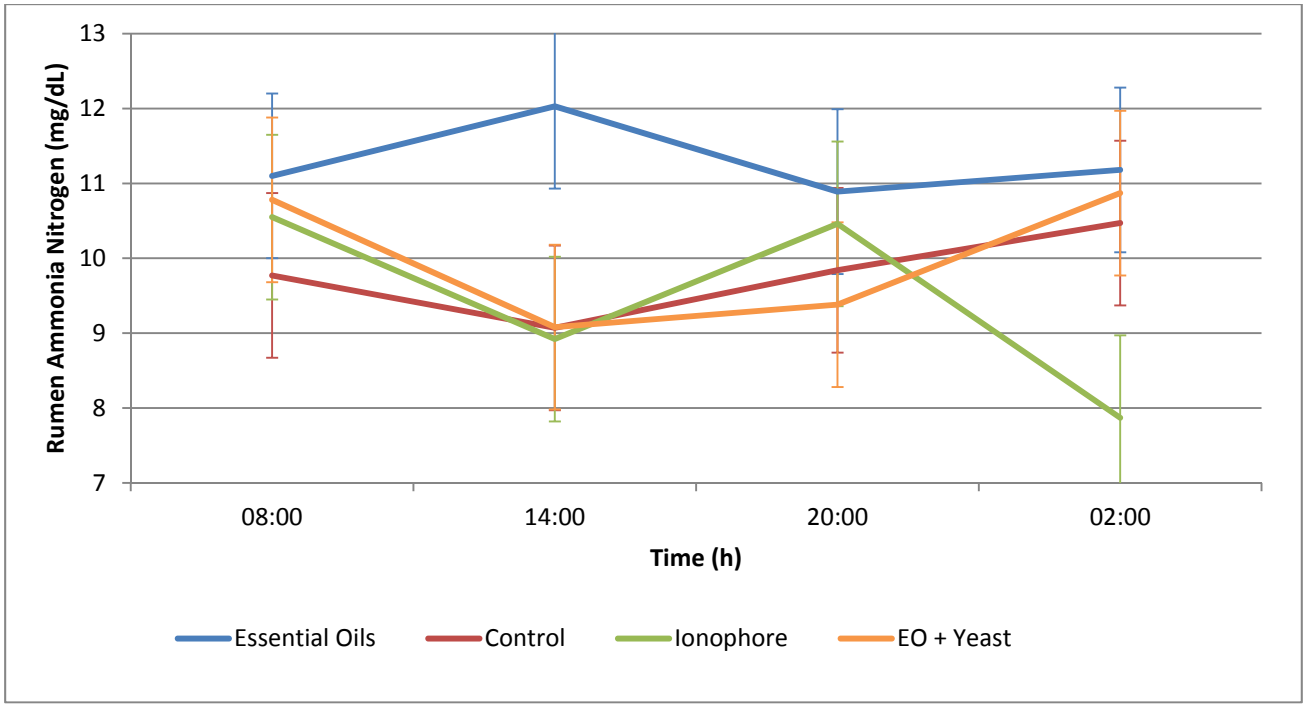

Figure 4.10 Diurnal variation in rumen ammonia nitrogen concentration (mg/dL) at different sampling times of cows (n = 16) receiving TMR diets supplemented with different feed additives, error bars represent SEM (Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day)

4.3.4 Lactic acid

Lactic acid is a much stronger acid compared to acetic acid and in the present study high levels of lactic acid were likely to occur because of the high amount of concentrate in the diet. Large increases in lactic acid leads to a decrease in the pH of the rumen and this can lead to acidosis (Tajima *et al.*, 2000). Streptococcus bovis is the major cause of lactic acidosis by fermenting starch and producing lactate (Tajima *et al.*, 2000). With high levels of rumen lactic acid the lactic acid can enter the bloodstream of the animal and cause increased blood pressure. In severe cases this can lead to damaged blood vessels in the feet of the animal which can eventually lead to lameness (Sayers *et al.*, 2003). Meijs (1986) concluded that by reducing the amount of easily fermentable substrates like starch in the diet leads to decreased lactic acid formation in the rumen and thereby decreases lactic acidosis.

Morgante *et al.* (2007) investigated 10 dairy farms with a high average milk production. The cows investigated were in early lactation, housed in free stalls and fed a TMR. They found that the lactate concentration ranged from 0.36 to 3.67 mmol/L between the different farms and that the average lactate concentration was 1.4 mmol/L. It was however concluded that the lactate concentration differs significantly under different conditions and is clearly diet dependant. The low fibre high energy portion of the diet especially affected lactate concentration.

The rumen lactic acid concentration (mmol/L) measured at 08:00, 14:00, 20:00 and 02:00 were averaged over all the periods and presented in Table 4.17. At 08:00 there were differences between treatments ($P < 0.05$), the ionophore treatment had a higher lactic acid concentration compared to the essential oil treatment with values of 0.23 and 0.14 mmol/L respectively. This differed from most other studies in which ionophore treatment decreased the lactic acid concentration (Nagaraja *et al.*, 1982; Duffield and Bagg, 2000). The overall mean lactic acid concentrations of ionophore supplemented cows however were the same as the control and EO treatments, but the combination of EO and Y were lower than the ionophore treatment. No other literature is available with similar additive comparisons.

In contrast to these results Nagaraja *et al.* (1982) found that cows with acidosis given monensin had a significantly lower lactate concentration and a higher rumen pH compared to cows not receiving monensin. They found that monensin fed cows had an average L(+) and D(-) lactate concentration of 400 mg/dL and 53 mg/dL respectively compared to control cows which had an average concentration of 238 mg/dL and 99 mg/dL respectively. They also found that cows receiving monensin had an average pH of 5.9 and those not receiving monensin had an average pH of 5.3. These findings were however only true at the 0.65 and 1.3 mg/kg body weight dosages. Duffield and Bagg (2000) reported that ionophores decreased lactic acid fermenting bacteria and increased lactic acid utilizing bacteria and thereby cause reduced lactic acid concentrations. EO and plant extracts have been found to cause different results concerning the lactic acid bacteria. Some extracts lead to the inhibition of these bacteria and some resulted in enhanced growth (Deans and Ritchie, 1987). In contrast to the results found in the present study Calsamiglia *et al.* (2007) reported that thymol which is one of the most well researched active components of EO caused a decrease in lactic acid concentrations.

In Figure 4.11 is illustrated the lactic acid concentration (mmol/L) of the different treatments at the different collection times.

Table 4.17 Effect of different feed additive treatments on rumen lactic acid concentration (mmol/L) measured at 08:00, 14:00, 20:00 and 02:00 for cows fed total mixed rations (n = 16)

	Treatments[1]				
Time	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
08:00	0.21[ab]	0.23[b]	0.14[a]	0.16[ab]	0.02
14:00	0.17	0.15	0.16	0.14	0.01
20:00	0.20	0.21	0.20	0.17	0.02
02:00	0.22	0.21	0.18	0.17	0.02

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean
[ab] Means in the same row without a common superscript differs ($P<0.05$)

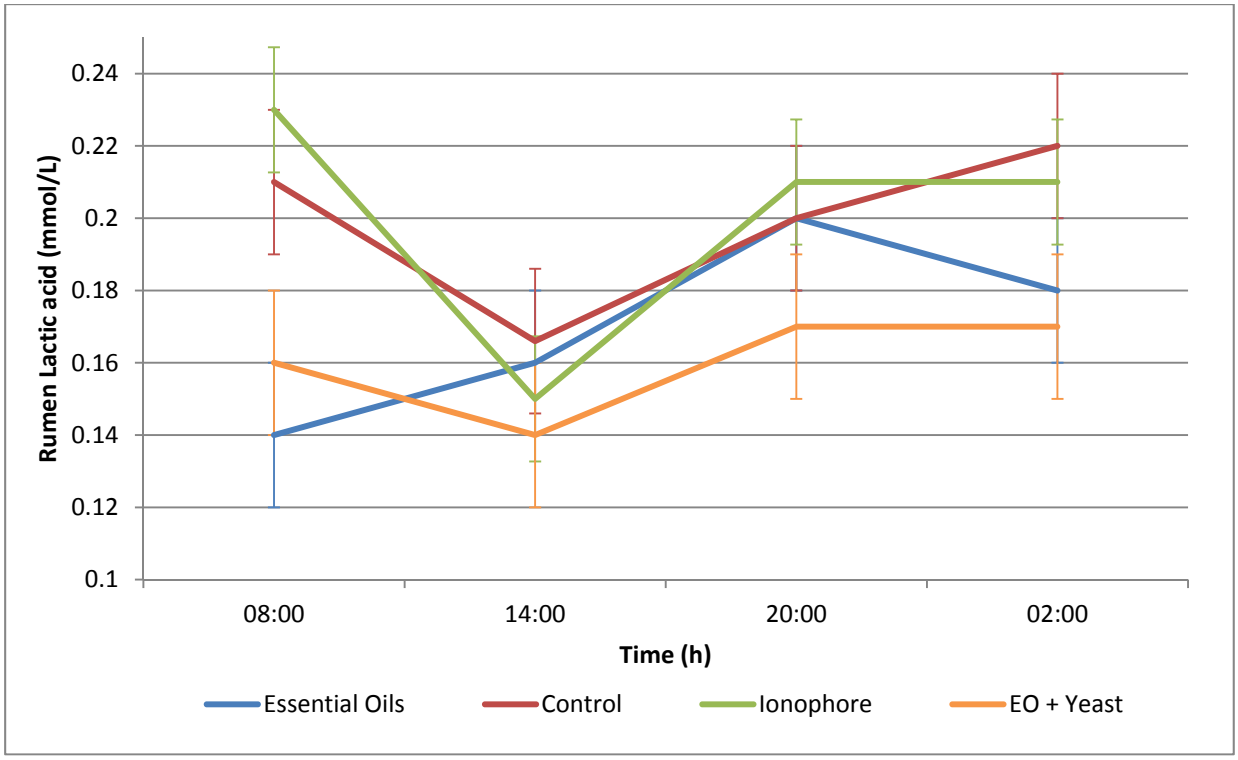

Figure 4.11 Diurnal variation in rumen lactic acid concentration (mmol/L) at different sampling times of cows (n = 16) receiving TMR diets supplemented with different feed additives, error bars represent SEM (Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day)

4.3.5 *In sacco*

The mean percentage *in sacco* disappearance of NDF, starch and N of lucerne and control TMR at 12 and 24 hours rumen incubation are averaged over all the periods combined and presented in Table 4.18. The NDF disappearance was estimated by incubating lucerne in the rumen and starch and N disappearance was calculated by incubating control TMR in the rumen. After 12 hours of incubation none of the parameters evaluated for disappearance showed a difference between treatments ($P > 0.1$).

After 24 hours of incubation all the parameters showed a difference or a tendency to differ between treatments. Neutral detergent fibre disappearance after 24 hours of incubation tended to be higher in the essential oil treatment with a value of 54.67% compared to the control and EO + Y treatments both with a value of 50.6% ($P < 0.1$). Starch disappearance was significantly higher with the essential oil treatment with 95.72% disappearance compared to the control and EO + Y treatments with values of 90.52% and 90.19% respectively ($P < 0.05$). Nitrogen disappearance was higher with the ionophore and essential oil treatments with values of 85.08% and 84.80% respectively compared to the EO + Y treatment with a value of 82.83% ($P < 0.05$).

Fraser *et al.* (2007) found that the 48h disappearance of NDF from barley silage tended ($P = 0.11$) to decrease while that of concentrate was significantly reduced ($P < 0.01$) by EO

from cinnamon leaf. Similarly the study by Martinez *et al.* (2006) showed a decrease in NDF degradability with the addition of EO and similar results with the addition of monensin. The study by Jalc *et al.* (1992) also showed a decrease in the *in vitro* NDF degradability after feeding monensin to animals. An *in vivo* study by McGuffey *et al.* (2001) however showed that NDF degradation was not affected by monensin. The reason for this is the expression of such an effect in an *in vivo* ruminal medium is more complex, which leads to the inhibition of certain bacteria like lactic acid producing Streptococcus bovis. Thereby stopping a pH reduction and favouring cellulolytic bacterial activity. It can therefore be hypothesised that the results on the degradability of EO can be different between *in vivo* and *in vitro* studies. Some studies however found that EO (Benchaar *et al.*, 2003; Castillejos *et al.*, 2005; Benchaar *et al.*, 2006) and ionophore (Benchaar *et al.*, 2006) supplementation had no effect on NDF disappearance. Plata *et al.* (1994) found that yeast products supplemented to Holstein steers caused the *in situ* NDF percentage disappearance of oat straw based diets to be higher compared to control. They suggested that this increase in percentage NDF disappearance was due to a significantly higher protozoal concentration. The study by Guedes *et al.* (2008) supported this theory by indicating that an increase in the level of live *Saccharomyces cerevisiae* supplementation from 0.3 to 1 g/day significantly increased the degradation of NDF for the low fibre degradation group of maize silages compared to the control and the 0.3 g supplemented cows. Roa V *et al.* (1997) found that the potentially digestible NDF increased from 46.6% to 55% when yeast was added to complete diets fed to steers. However, other studies showed that yeast supplementation had not improved NDF digestibilities (Doreau and Jouany, 1998; Miller-Webster *et al.*, 2002; Lehloenya *et al.*, 2008).

In a study by Benchaar *et al.* (2006) the apparent digestibility of starch was significantly ($P < 0.05$) higher in cows supplemented with EO compared to cows fed diets without EO. Benchaar *et al.* (2006) also observed no effect on the apparent digestibility of starch with ionophores supplementation which agreed with the results of Ali-Haimoud *et al.* (1995) who found no change in total tract digestibility of starch in lactating cows supplemented with ionophores. They did however observe that the sites of digestion were altered, the rumen digestion percentage were lower while the small intestine digestion percentage were higher. Some studies found that EO caused a reduction in starch digestibility (Hart *et al.*, 2008; Patra and Saxena, 2010). Beauchemin *et al.* (2003) observed that yeast products had no effect on the site or extent of starch digestion. However, Khetarpaul and Chauhan (1990) found that yeast supplementation improved the starch digestibility of pearl millet flour.

Ali-Haimoud *et al.* (1995) observed that ionophore supplementation caused no change in the total tract digestibility of nitrogen. Cho *et al.* (2006) observed no difference in nitrogen digestibility ($P > 0.05$) when animals were supplemented with EO. Fiems *et al.* (1993) indicated that nitrogen digestibility and balance were not affected by yeast supplementation.

Figure 4.12 illustrates the mean percentage *in sacco* disappearance of NDF, starch and N of lucerne and control TMR at 12 and 24 hours rumen incubation

Table 4.18 Effects of different feed additive treatments on the average percentage disappearance *in sacco* of neutral detergent fibre (NDF), starch and nitrogen (N) of lucerne and control TMR at 12 and 24 hours of ruminal incubation for cows fed a total mixed ration (n = 16)

	Treatments[1]				
Parameter[6]	**Control**	**Ionophore**	**EO**	**EO + Y**	**SEM[2]**
NDF Diss[3] 12	47.4	47.0	47.4	44.3	1.55
NDF Diss 24	50.6[d]	51.8[cd]	54.7[c]	50.6[d]	1.35
Starch Diss[4] 12	70.7	69.4	73.5	70.8	3.47
Starch Diss 24	90.5[b]	93.1[ab]	95.7[a]	90.2[b]	1.55
N Diss[5] 12	73.8	73.8	74.9	72.4	1.40
N Diss 24	84.5[ab]	85.1[a]	84.8[a]	82.8[b]	0.50

[1]Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day.
[2]Standard error of the mean
[3]NDF disappearance was calculated by incubating lucerne
[4,5]Starch and N disappearance were calculated by incubating control TMR
[6]NDF Diss 12 = Percentage NDF that disappeared from the *in sacco* bag 12 hours after insertion; NDF Diss 24 = Percentage NDF that disappeared from the *in sacco* bag 24 hours after insertion; Starch Diss 12 = PercentageStarch that disappeared from the *in sacco* bag 12 hours after insertion; Starch Diss 24 = Percentage Starch that disappeared from the *in sacco* bag 24 hours after insertion; N Diss 12 = Percentage N that disappeared from the *in sacco* bag 24 hours after insertion; N Diss 12 = Percentage N that disappeared from the *in sacco* bag 24 hours after insertion
[ab] Means in the same row without a common superscript differs (P<0.05)
[cd]Means in the same row without a common superscript tend to differ (P<0.1)

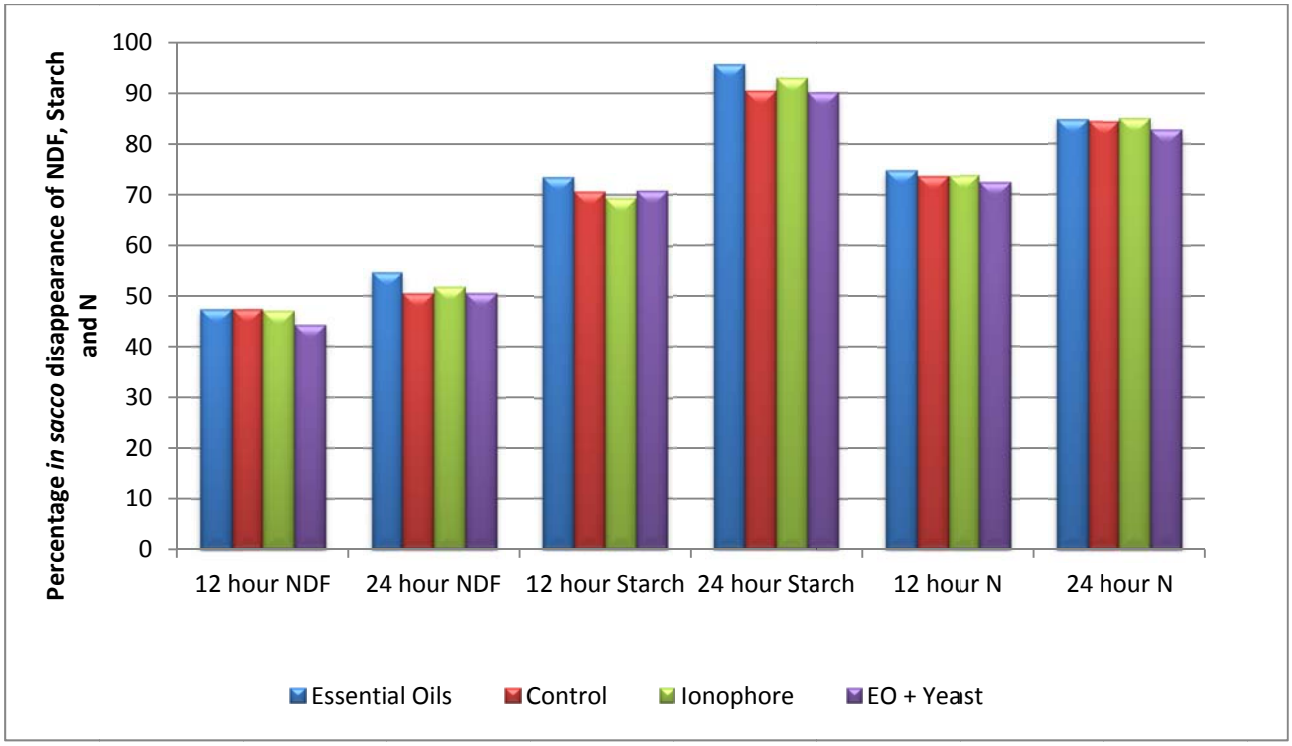

Figure 4.12 The effect of different feed additives on *in sacco* NDF, starch and nitrogen disappearance of lucerne and control TMR incubated for 12 and 24 hours in Jersey cows fed a total mixed ration (n = 16) (Control: diet contains no feed additive; Ionophore: diet contains monensin at 15 ppm; EO: diet contains essential oil at 167 g/ton fed at 1 g/cow/day; EO + Y: diet contains essential oil at 167 g/ton fed at 1 g/cow/day as well as yeast at 167 g/ton fed at 1 g/cow/day)

CHAPTER 5

CONCLUSION

Lactating Jersey dairy cows supplemented with EO or EO + Yeast tended ($P < 0.10$) to produce more milk than cows supplemented with ionophores (23.2 and 23.4 vs 22.4 kg/day) but did not differ from control cows (22.9 kg/day). Ionophore supplementation tended ($P < 0.10$) to suppress DMI when compared to cows supplemented with EO, but did not differ from EO + Y or control cows. The only milk component that was affected was milk fat % which was increased ($P < 0.05$) with EO supplementation when compared to the control treatment but did not differ from milk fat of cows supplemented with EO + Y or ionophores respectively. In addition, feed additive supplementation did not affect BW or BCS change between treatments ($P < 0.05$).

Only four cows in a 4 x 4 Latin square were used, and therefore the focus of this study was not so much on the production parameters, but more on the fermentation dynamics of the cows. Ruminal pH was measured with either handheld or individually continuous pH dataloggers and there were no differences in the mean pH between treatments ($P > 0.05$). Total VFA production and molar % of acetic and propionic acid did not differ between treatments; butyric acid production however was increased in the EO and EO + Y treatment cows when compared to cows supplemented with ionophores. This suggests that EO might play an important role in papillae development during early lactation when the rumen is still in an adaptation phase during the transition from high roughage to high concentrate diets.

Another interesting observation was that 24 hour *in sacco* N and starch disappearance of the control TMR were lower ($P < 0.05$) in the EO + Y treatment cows compared to EO only supplemented cows. This suggests a possible negative interaction when both EO and Y is supplemented. This interaction can however be advantageous by increasing the bypass of protein and starch from rumen degradation and therefor merits further investigation. It can be concluded that essential oil supplements has the potential to be a natural alternative to ionophore antibiotics in dairy diets. Results should however be interpreted with caution because the cows were not in early lactation and the 4x4 Latin square model is not the ideal design to evaluate milk production responses. Further research is therefor necessary under conditions where the rumen is challenged more by feeding higher starch and lower NDF diets.

CHAPTER 6

CRITICAL EVALUATION

6.1 Production study

6.1.1 Experimental design

A 4 x 4 Latin square design is not the appropriate design for milk production studies because of the short duration of the experimental periods and low animal numbers. The focus of this study, however, was on rumen fermentation dynamics, and it is well accepted that a 4 x 4 Latin square design is an appropriate design for rumen fermentation studies.

6.1.2 Yeast treatment

In the original protocol there was a yeast treatment, however the sponsor of the study requested that the yeast treatment be removed. It is the opinion of the researchers that a yeast treatment would have added much value to the study, especially with interpretation of results.

6.2 Rumen study

6.2.1 Rumen fluid sampling

Rumen fluid was collected only four times in each experimental period. With each rumen fluid sampling 400ml of rumen fluid was collected from each cow. This was then filtered through 4 layers of cheesecloth and only about 60 ml fluid was then used for analyses. These samples were taken from Jersey cows with an approximate rumen volume of 80L. The questions that then arise are: Are these rumen samples representative of the entire rumen content and are four samplings enough to describe the fermentation patterns accurately?

Incorporating more sampling times especially shortly after feeding could have led to a better description of the effect of the concentrate on rumen activity. Due to budget constraints and labour cost we had to follow the protocol described.

6.2.2 *In sacco* incubation

There were only 2 incubation periods. They were 12 and 24 hours. Additional incubation periods of 0, 2, 4, 8, 12, 18, 24, 36 and 72 hours could have helped to determine and compare the degradation rate of the different treatments more accurately. Six hour incubation intervals would alternatively have made it possible to use the Van Amburgh rate calculator to estimate the NDF disappearance rate. The use of only the two time intervals was a direct result of budget constraints.

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APPENDIX A

Selection of cows

All the cannulated cows in the herd of the Outeniqua Research Farm were considered for selection for participation in the trial. Cows that were not functionally sound, first lactation cows, cows with behavioural problems, cows with very high somatic cell count and cows that were too late in lactation were not considered. The dry cows in the herd were also considered and those close to calving were also included in the group that would be considered for participation. After the four cows for the trial were selected the four treatments were randomly allocated to the cows.

Table A1 Cows used for the study, their body weight, body condition, lactation number, days-in-milk, milk production per day and somatic cell count as it was at the start of the trial on 28 March 2012.

Cow nr.	Cow	BW	BC	Lact nr.	DIM	Milk yield (kg)	SCC
1	Bella 137	421	2.25	6	173	12.0	360 000
2	Mart 178	322	2.00	8	177	13.9	111 000
3	Mart 169	378	2.00	5	191	13.1	135 000
4	Firefly 52	399	2.25	4	228	11.0	205 000
Avg		380	2.13	5.75	192	12.5	202 700

Avg = Average
BW = Body Weight
BC = Body condition
Lact nr. = Lactation number
DIM = Days in milk
SCC = Somatic cell count

Table A2 Milk composition and production analysis of each cow used in the trial on each treatment

Cow	Treatment[1]	Period	% Fat	% Protein	% Lactose	SCC (x1000)/ml	MUN	Mpavg
Bella137	1	1	4.19	3.77	4.90	86.8	13.4	22.7
Mart178	2	1	4.01	3.77	5.01	40.5	12.5	20.1
Mart169	3	1	4.36	4.17	4.74	105.8	11.25	18.3
Firefly52	4	1	4.48	4.05	4.85	87.3	10.7	19.2
Bella137	2	2	4.06	3.77	5.02	105.0	17.8	25.3
Mart178	3	2	4.17	3.77	4.97	56.5	20.5	20.8
Mart169	4	2	4.52	4.07	4.74	129.5	19.3	22.3
Firefly52	1	2	4.57	4.03	4.97	39.8	14.2	22.4
Bella137	3	3	4.23	3.66	5.02	62.0	15.6	26.4
Mart178	4	3	4.27	3.77	5.02	51.8	20.8	24.9
Mart169	1	3	4.55	4.09	4.67	132.3	16.0	22.6
Firefly52	2	3	4.66	4.11	4.90	113.5	14.4	23.0
Bella137	4	4	4.57	3.75	4.96	60.0	15.4	27.2
Mart178	1	4	4.80	3.74	4.97	53.3	19.1	25.3
Mart169	2	4	4.56	4.09	4.69	117.5	13.8	23.1
Firefly52	3	4	5.04	4.10	4.90	56.3	16.7	23.5

[1]Treatment: 1 = Control TMR + Essential oils; 2 = Control TMR; 3 = Control TMR + Ionophores; 4 = Control TMR + Essential Oils + Yeasts
SCC = Somatic cell count
MUN = Milk Urea Nitrogen
Mpavg = Average daily milk production