# *IN VITRO* EFFECTS OF *MEGASPHAERA ELSDENII* NCIMB 41125 AND *SACCHAROMYCES CEREVISIAE* 1026 ON RUMEN FERMENTATION IN EARLY LACTATING COWS

by

Mulaudzi Thendo

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Supervisor: Dr MC Muya

Co-supervisor: Prof. KR Mbatha

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## DECLARATION

I Thendo Mulaudzi declare that this dissertation submitted for MSc Agriculture (Unisa) titled "*In vitro* evaluation of the effect of *Megasphaera elsdenii* NCIMB 41125 and *Saccharomyces cerevisiae* 1026 on rumen fermentation in early lactating cows" is my own original work and has not been previously submitted to any university for any qualification

I further declare that I have properly referenced all the sources used or quoted in-text and by a comprehensive reference list. This work was not plagiarised in accordance with the Unisa policy on plagiarism

Name: Ms Thendo Mulaudzi

Student number: 56598939

Signature:……

Date:……

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## ABBREVIATIONS AND DEFINITIONS

A: acetate

A: Pr: acetate to propionate ratio

A: Pr+B: acetate to propionate + butyrate ratio

B: butyrate

CFU: Colony Forming Units

$CH_4$: methane

DFM: direct-fed microbials

EBAL: energy balance

FDA: Food and Drug Administration

GRAS: generally recognized as safe

HC: high concentrate diet

LC: low concentrate diet

LUB: lactic acid-utilizing bacteria

LY and *S. cerevisiae*: *Saccharomyces cerevisiae* 1026

Me and M. *elsdenii*: *Megasphaera elsdenii* NCIMB 41125

Me+LY: *Megasphaera elsdenii* NCIMB 41125 plus *Saccharomyces cerevisiae* 1026

NEBAL: negative energy balance

P: significance

Pr: propionate

SARA: subacute ruminal acidosis

VFAs: volatile fatty acids

## PREFACE

This dissertation has been written in the format of the dissertation and most in accordance with the required format for the submission at the University of South Africa, College of Agriculture and Environmental Sciences, Department of Agriculture and Animal Health for the degree Master of Science in Agriculture. This study is the original work of the author and has not been submitted for any qualification to another university. Any reference to the work of others has been acknowledged in the text and listed in a comprehensive reference list. This dissertation is comprised of 6 chapter which covers the content, reference list with all the works referenced in-text and the appendix which cover miscellaneous stuff.

The dissertation follows a sequential format preface detailing the research project from conceptualisation, experiment and report and analysis of findings and conclusions. Chapter 1 provides the research project background, justification and objectives. Followed by the literature review on the subject matter compiled in chapter 2. Chapter 3 details the research methodology followed and materials used to conduct the research experiment. The finding is then reported in chapter 4 and discussed in comparison to the previous works of others in chapter 5. Finally, chapter 6 covers the general conclusions and recommendations for future research, followed by a list of references.

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## ABSTRACT

This study was conducted to evaluate the effect of microbial feed additives *Megasphaera elsdenii* NCIMB 41125 and *Saccharomyces cerevisiae* 1026, individually and combined on rumen pH,ammonia-N and volatile fatty acids (VFAs) fermentation.

An *in vitro* batch fermentation was conducted using rumen fluid from two early lactating Holstein cow donor fed the TMR for lactating cows which was evaluated under two diets, differing in concentrate to forage ratio. The diets were high concentrate, a 60:40 concentrate to forage ratio diet (HC) and low concentrate, a 40:60 (LC) concentrate to forage ratio diet. The treatments were; Control (diet with no additives), Me (diet with *M. elsdenii* NCIMB 41125 10mm ($10^8$ CFU/ml)), LY (diet with live yeast, *S. cerevisiae* 1026), and Me+LY (diet with mixture of *M. elsdenii* and *S. cerevisiae*).

The average rumen pH was 5.8 and ammonia nitrogen was not affected by Me and LY supplemented separately or in combination (Me+LY) in both low and high concentrate diets. Total VFAs were increased with the addition of LY alone and in combination Me+LY+Me) in high concentrate diet only but the addition of Me had no effect in both diets.

Acetate, lactate and A: Pr were decreased ($P<0.05$) by all the treatments (Me and LY alone and in combination) on both diets, except in high concentrate diet where the addition of Me tended to decrease ($P<0.07$) acetate and had no effect on lactate. Propionate was increased by all the treatments in low concentrate diet and tended to

increase (P<0.08) by addition of Me and Me+LY in high concentrate diet. In a low concentrate diet, butyrate was increased by LY but tended to be decreased by Me, however, all the treatments lacked effects on high concentrate diet.

Live yeast appears to act differently compared to Me by showing two times more effects on high than low concentrate diets.This *in vitro* study showed that both Me and LY had a tendency to modify rumen fermentation and that might indicate their potential to mitigate the metabolic challenges and improve energy status of Holstein dairy cows during the transition and early lactation period. However, there is a need for further research that will include *in vivo* study.

**Keywords:** Dairy, Holstein cows, Microbial feed additives, Ruminant nutrition, Transition period, VFAs

# CHAPTER 1 INTRODUCTION

## 1.1. Background

The period starting at parturition and ending 70 days post-partum, early lactation is a very challenging production phase in dairy nutrition (Erasmus et al. 2000). It is the phase when peak milk production is expected (Erasmus et al. 2000) and occurs concurrently with the majority of metabolic health disorders (Mulligan & Doherty 2008) and negative energy balance (NEBAL) (Erasmus et al. 2000, Opsomer 2015,).

Metabolic disorders result from the cow's inability to cope with the metabolic demands of high production (Mulligan & Doherty 2008). Their occurrence during the early lactation production phase has adverse financial implications (Mulligan & Doherty 2008) that extend to mid lactation lactation phase, 70 to 140 days post partum.

However, studies have shown that the use of feed additives have the potential to mitigate some metabolic challenges and improve feed efficiency (Mutsvangwa et al. 1992, Meissner et al. 2010, McAllister et al. 2011). This was achieved by manipulating rumen fermentation. Some examples of these additives are *Megasphaera elsdenii* (M. *elsdenii*) and live yeast *Saccharomyces cerevisiae* (*S. cerevisiae*). These additives are direct-fed microbials (DFM) additives and have been used for years in ruminant production (McAllister et al. 2011).

The rumen bacteria *M. elsdenii* is a gram-negative coccus bacterium native to the rumen of cattle and sheep (Marounek et al. 1989, Rossi et al. 2004). It is a strictly anaerobic lactic acid-utilizing bacteria (LUB) that is able to convert lactate to weaker

acids (volatile fatty acids) and hence increase pH (Horn et al. 2009, Meissner et al. 2010). Carbohydrates and organic acids can be utilised by *M. elsdenii* and it is one of the principal organisms that catabolise lactic acid and deaminate amino acid (Marounek et al. 1989). It is able to convert lactate to propionate and butyrate (Marounek et al. 1989, Drouillard et al. 2012) and glucose to butyrate (Henning et al. 2010, Muya et al. 2015).

On the other hand, *S. cerevisiae*, is a single cell eukaryotic fungal microorganism (Sontakke 2012) with properties that are very different from *M. elsdenii*. The *S. cerevisiae* has been reported to decrease methane production by reducing hydrogen available for methanogenesis based on stoichiometric principles (Bakker et al. 2001). Observed effects that would reduce hydrogen availability to methanogens include a shift in fermentation towards butyrate or propionate (Erasmus et al. 2005), reduction in protozoal numbers (Newbold et al. 1998) and promotion of acetogenesis as a sink for hydrogen (Chaucheyras et al. 1995).

A higher production of total volatile fatty acids (VFAs) especially propionate was observed in bulls fed barley diet with *S. cerevisiae* than those fed the same diet without *S. cerevisiae* (Mutsvangwa et al. 1992). Furthermore, the ruminal ammonia production was not affected but the ruminal pH was highly depressed by the addition of *S. cerevisiae* (Mutsvangwa et al. 1992).

Alteration of rumen fermentation is expressed by the change of VFAs, the end products of carbohydrate fermentation in the rumen and the cow's main energy source

(Siedlecka et al. 2008). The changes also affect rumen microbe population and the breakdown of rumen protein as well as the resulting ammonia.

## 1.2. Justification

Feed additive, *M. elsdenii* is able to control the accumulation of lactic acid and the decline of rumen pH in adult ruminants fed high level of dietary concentrate (Meissner et al. 2010). Through its lactate utilising ability, *M. elsdenii* facilitates the rate and direction of lactic acid fermentation to predominantly propionate and other VFAs. On the other hand, *S. cerevisiae* has the ability to stabilise ruminal pH (Chaucheyras-Durand & Fonty 2002) and the potential to reduce hydrogen availability for methanogenesis which may lead to reduced production of methane.

It is speculated that the effects of *M. elsdenii* and *S. cerevisiae* would positively affect the rate and pattern of rumen fermentation. The lactate utilising ability of *M. elsdenii* with the potential of *S. cerevisiae* to stabilise pH and reduce hydrogen availability would increase production of propionate in the rumen. Propionate will then be converted to blood glucose in the liver, proving energy in early lactation (Ishler et al. 1996). This is beneficial to early lactating cows by improving energy status due to propionate's glucogenic properties.

There is relatively extensive research on using *M. elsdenii* and *S. cerevisiae* in ruminant nutrition separately, however, research on their synchronous use is limited. The current research will contribute knowledge to the synchronous use and interaction between *M. elsdenii* and *S. cerevisiae* which can potentially address some of the

challenges of early lactation in dairy cattle. Taking into account that the effects of additives on ruminant performance can vary with the type of diet being fed (Ruiz et al. 2001). It is therefore, important to evaluate *M. elsdenii* , *S. cerevisiae* and their synergetic offect on different feeding scenarios, for instance, concentrate to forage ratio.

## 1.3. Objectives

To evaluate the effect of *M. elsdenii* and/or *S. cerevisiae* on rumen fermentation rate and patterns in different diet concentrate levels

1. To evaluate the effects of *M. elsdenii* NCIMB 41125 and *S. cerevisiae* 1026 on the rumen pH, production of ammonia nitrogen and volatile fatty acids in a high concentrate diet.
2. To evaluate the effects of *M. elsdenii* NCIMB 41125 and *S. cerevisiae* 1026 on the rumen pH, production of ammonia nitrogen and volatile fatty acids in a low concentrate diet.

## 1.4. Hypotheses

I There are no effect of *M. elsdenii* and *S. cerevisiae* on rumen pH, ammonia nitrogen, and volatile fatty acids in a high concentrate diet.

II There are no effect of *M. elsdenii* and *S. cerevisiae* on rumen pH, ammonia nitrogen and volatile fatty acids in a low concentrate diet.

# CHAPTER 2 LITERATURE REVIEW

## 2.1. Challenges in early lactating dairy cows

One of the most important factors in dairy production is operating at optimum milk production levels if it is to be profitable (Harrison et al. 1990). This has led to biased intensive selection for high milk yield, without equivalent selection for dry matter intake capacity and feed efficiency (Opsomer 2015). High milk yielders are able to produce large quantities for a prolonged time by 1) breaking down more body energy stores to support milk yield, 2) efficiently partitioning ME from feed to milk production and/or 3) acquiring more metabolizable energy (ME) from feed (Bell 1995, Opsomer 2015).

According to Goff (1999), the dry cow is fed a high forage, less energy dense diet which is higher in neutral detergent fibre than the lactation diet. The rumen physiological adaptation to dry period diet identified in (Goff 1999) are firstly, increment of the cellulolytic and methane-producing bacteria due to the high forage content and secondly, the reduction of the lactate producing bacteria due to reduced readily fermentable starches. Consequently reducing the lactic acid-utilizing bacteria (LUB) mainly *M. elsdenii* and *Selenomonas ruminantium* which convert lactate to VFAs. Lastly, reduction of papillae length and ruminal mucosa absorptive capacity of VFAs because of a low energy diet in early dry period.

The change from the dry cow diet to a lactation diet low in forage and high in rapidly fermentable carbohydrates is necessary at parturition although it disrupts the rumen microbial population (DeVries et al. 2009). Naturally, adaption to the high energy-

yielding lactation diet is imperative for the success in coping the metabolic challenge it presents, failure of which can lead to metabolic disorders (discussed in section 2.3) which are catastrophic to the health and productivity of the dairy cow.

During the early lactation phase, the lactating cow requires more energy for milk production and maintenance than for the gravid uterus and maintenance during the preceding late pregnancy (Bell 1995, Remppis et al. 2011). Lactating cows like all mammal prioritise mammary energy supply over maintenance of body functions for the sake of the newborn's nutrition (Opsomer 2015). Although this is desirable for milk yield it usually leads to health complications e.g ketosis mostly for high yielders' cow (Opsomer 2015).

The high energy demand of early lactation exceeds the energy that can be consumed and this gives rise to a state of negative energy balance (NEBAL) (Baumgard et al. 2006, Remppis et al. 2011). This energy state is further reinforced by reduced feed intake (Grummer 1995) caused by parturition inducing endocrine changes, parturition and other factors that influence feed intake.

After parturition dry matter intake increases slowly while the nutrient requirements are on a rapid increase due to milk synthesis (Reynolds et al. 2003). The NEBAL tends to be a common condition in this phase of lactation. Thus, other ways of availing more energy and avoiding loss have to be explored. Persistent NEBAL can be a cause of the drop in milk yield, fertility problems and occurrence of metabolic diseases (Remppis et al. 2011).

According to Baumgard et al. (2006) the approaches that have been attempted to deal with energy balance (EBAL) are 1) supplemention with fats, 2) addition of concentrates, 3) reduced milking frequency (i.e. 1x/d), 4) propylene glycol, 5) monensin and 6) conjugated linoleic acid-induced milk fat depression (CLA-MFD). Limitations observed from the first four approaches were palatability, acidosis and mammary functions that created difficulties when their effect on EBAL was evaluated.

The energy loss in ruminants can occur through methane eructation. The energy lost as methane ($CH_4$) in ruminants can range from 2-12 % of the gross energy intake (Johnson & Johnson 1995). Factors that influence rate of $CH_4$ production include level of feed intake, type of dietary carbohydrate, feed processing, dietary addition of lipids or ionophores, organic acids, and changes in ruminal microbial flora plus microflora (Johnson & Johnson 1995, Boadi et al. 2004, Khampa & Wanapat 2007). In addition, $CH_4$ emission has a huge impact on global warming which is an issue causing a lot of public concern. According to Nguyen et al. (2013), $CH_4$ emissions from cows are the main contributor (52%) to the climate change impact of milk production.

## 2.2. Rumen fermentation and animal performance

The rumen is the largest of the four compartments in the adult ruminant stomach (other compartments are; reticulum, omasum and abomasum) (Figure 2.1). The inside lining of the rumen consists of tiny projectiles and papillae which increase the surface area and allow better absorption of nutrients (Moran 2005). The rumen and reticulum are collectively referred to as the reticulorumen because their functions are very similar and anatomically they are only separated by a small muscular fold of tissue (Parish et

al. 2009). The inside lining of the reticulum mirrors the honeycomb (Parish et al. 2009). The reticulorumen is home to billions of microorganisms (bacteria, fungi, protozoa) (Moran 2005), some of which digest starch and sugars while others digest cellulose.

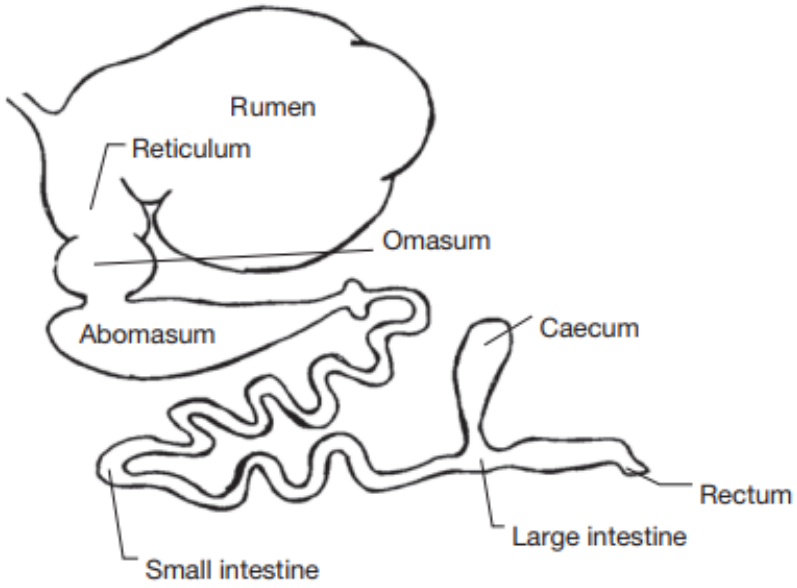


Figure 2.1: Digestive system of the dairy cow (Moran 2005)

The rumen functions include: 1) Behaving as a fermentation vat which is the primary host of microbial fermentation (Moran 2005). 2) Fermentating and breaking down of fibrous feed portions (plant cells) to their carbohydrate functions. They are then used to produce VFAs such as propionate, acetate, and butyrate which are used as the main energy source for animal (Parish et al. 2009, Moran 2005). This is achieved through the action of rumen microorganisms (Moran 2005). 3) Digestion of a large percentage of starch and soluble sugars (50-65%) by the ruminant (Parish et al. 2009). 4) Lastly, the syntheses of protein from non-protein nitrogen and B vitamins and vitamin K take place in the rumen (Parish et al. 2009).

The nutrients and VFAs produced in the rumen are absorbed straight into the bloodstream by rumen papillae lining the rumen wall (McDonald et al. 2011). Fibrous feedstuffs can remain in the rumen for up to 48 hours for further fermentation (Parish et al. 2009). The primary function of the reticulum is collecting smaller particles from the rumen and move them to the omasum. Heavy objects, primarily non-food objects consumed by the ruminant are confined in the reticulum.

The omasum has many folds resembling pages of a book and are called laminae (Moran 2005). These folds increase the surface area for the efficient absorption of nutrients from digested feed and fluids (Parish et al. 2009) and to grind the feed further (Moran 2005). The abomasum is the true stomach because it is similar, the stomach of monogastric or nonruminants (Parish et al. 2009)., Similary, the abomasum produces hydrochloric acid and digestive enzymes (Parish et al. 2009, Moran 2005).

It is of importance to take care of rumen microorganisms for the sake of the ruminant's nutrition. They are responsible for the fermentation of almost all of the soluable sugars and startch in the feed of adult ruminants fed high forage diet as well as being a source of protein (microbial protein) (Ishler et al. 1996). Without rumen microbes, the ruminant's digestive system would shut down leading to starvation and death. The rumen is a complex ecosystem that is anaerobic in nature.

The normal rumen pH is 6.5>pH<7.0 and is a good indicator of the rumen condition (Penner 2015). It is influenced by type and size of feed, type of fermentation end product, rate of VFAs production and absorption and saliva flow rate. When rumen pH

drops to between 5.2 and 5.6, clinical signs of subacute ruminal acidosis (SARA) may manifest (Chiquette 2009). This may impair proper ruminal and physiological functions (Meissner et al. 2010).

Probiotics are defined as live microbial feed supplements which beneficially affect the host animal by improving its microbial balance (Fuller, cited in Jouany & Morgavi 2007). They can be used to enhance the establishment of rumen flora and fauna in calves (Jouany & Morgavi 2007). In mature ruminants, probiotics can be used to mitigate adverse consequences (e.g metabolic disorders) of metabolic challenging phases such as diet change from forage-based to a cereal-rich diet where the microbial population balance could be disrupted (Jouany & Morgavi 2007).

## 2.3. Metabolic disorders

Despite extensive research on the physiology and nutrition of cows in the transition period, production and metabolic challenges remain a perplexing and problematic management aspect of dairy farms (Erasmus et al. 2008). The energy requirements of an early lactation dairy cows exceed the energy obtainable from the diet (Goff 2001, Goff 1999) due to low dry matter intake. The sudden increase in nutrient demand for milk synthesis brought by parturition (Erasmus et al. 2008), necessitates the feeding of a high energy-yielding concentrate diet.

Metabolic disorders mainly occur in early lactation when peak production is expected (Erasmus et al. 2000). This have a significant negative effect on productivity and profitability of a dairy enterprise (Opsomer 2015). Even with good farming practices

metabolic disorders still occur warranting scientific intervention. Metabolic disorders are a major challenge in early lactation (Bell 1995) when feed efficiency and production efficiency are of paramount importance.

The metabolic and production related diseases perceived as important in dairy populations include rumen acidosis, ketosis and fatty liver disease, laminitis, hypocalcemia/milk fever, displaced abomasums, and reproductive inefficiency (Garry 2001). Milk fever, ketosis, retained placenta and displaced abomasum occur within two weeks of the onset of lactation (Goff 1999).

Ruminal acidosis is a digestive disorder when the rumen pH is more acidic (pH<5.8) than normal rumen pH of 6.5>pH<7.0 (Penner 2015). There are two types of ruminal acidosis; namely acute acidosis/clinical acidosis and subacute acidosis/subclinical acidosis. Acute acidosis is usually experienced in the first 2 to 3 days of concentrate feeding in unadapted cows while subacute acidosis is experienced for longer periods with adverse metabolic and productivity outcomes. Subclinical acidosis is more prevalent than clinical and has more economic impact by reducing milk fat content, feed conversion efficiency, fibre digestion and compromising the animal health (Lean et al. 2007, Meissner et al. 2010, Penner 2015).

Acidosis can be instigated by one or a combination of the following: sudden dietary change, high-energy diet coupled with insufficient good roughages, rapid switch to high grain rations, rapid intake of high quality forages and low fibre in the diet (Lean et al. 2007, Meissner et al. 2010, Penner 2015). Symptoms of acidosis include reduced milk yield, laminitis , liver abscessation (Owens et al. 1998), scouring , a higher

incidence of left and right displacements of the abomasum, damage to the hooves tissue and ridges as well as weight loss (Penner 2015).

The use of direct-fed microbials (DFM) feed additive *M. elsdenii* and *S. cerevisiae*, which can reduce the accumulation of lactic acid in the rumen through fermentation can prevent ruminal acidosis (Nagaraja & Titgemeyer 2007, Meissner et al. 2010, Al Ibrahim et al. 2012). Penner (2015) identified the inclusion of prebiotics, probiotics, and/or yeasts as viable methods of preventing acidosis by introducing or stimulating the growth of good rumen microorganisms that mitigate the accumulation of strong acids. Ionophores can inhibit the growth of acid producing bacteria in the rumen, hence preventing acidosis (Penner 2015).

Ketosis is the accumulation of ketones in the blood, urine and/or milk of a cow and that is usually coupled with reduced blood glucose (Goff 2001). The occurrence of ketosis is predominant in the first month of lactation followed by the second month and most rare in the third month of lactation (Ingvartsen 2006).

To supplement energy obtained from feed, dairy cows are predestined mobilise body fat as an energy source in order to meet lactation energy requirements (Erasmus et al. 2000, Kokkonen 2005). The liver through the tricarboxylic acid cycle is able to completely oxidise a limited amount of fatty acids (Goff 1999). Thereafter remaining fatty acids are converted to ketones (Goff 2001). The appearance of these ketones enables diagnosis of ketosis. The fats that cannot be burned for energy start to accumulate in the liver as triglyceride (Goff 2001).

The primary treatment of ketosis is an injection of glucose intravenously coupled with an adjusted diet (Goff 2001, McDonald et al 2011). Another effective method is oral administration of propionate salts or propylene glycol (Goff 2001), which the liver can readily convert to glucose.

It is better to prevent metabolic diseases than opting for treatment once they manifest. The prevention of ketosis can be achieved by avoiding excessive lipid mobilization through increasing nutrient density 2-3 weeks prepartum, close up diet, overfeeding or feeding high starch diet for a limited period (Ingvartsen 2006, Mulligan & Doherty 2008). Another prevention measure is supplemental fat which works by suppressing mobilisation (Dreckley 1999).

## 2.4. Dairy cows nutrients requirement

Ruminants are able to consume fibrous feeds that are not suitable for humans and monogastric animals (Ishler et al. 1996) and convert them to milk and meat through the activity of rumen microbes. When non-structural carbohydrates are part of the diet lactic acid becomes readily available in the rumen (Ishler et al. 1996). This lactic acid should not accumulate in the rumen of dairy cattle fed a balanced diet. However, it accumulates due to slow absorption from unadapted cows having poorly developed rumen epithelia (Goff 1999).

Abrupt introduction of grains or high energy feed stimulates the proliferation of lactic acid producing bacterium so that it exceeds the growth rate of LUB (Bevans et al. 2005), this circumstances lead to lactic acid accumulation. The LUB are rumen

microbes specialising in the fermentation of lactate to VFAs such as acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, and traces of various other acids (Ishler et al. 1996). The VFAs proportions are largely influenced by the ratio of forage and concentrate in the diet. According to Ishler et al. (1996), the forage to concentrate ratio decrease is positively correlated to the acetate propionate ratio.

Acetate, the most abundant VFA predominates in high forage diets (Ishler et al. 1996). It is the main end product of fibre fermentation (Moran 2005). Acetate is essential for the production of milk fat (Moran 2005). Propionate concentration is favoured by a high grain diet (Ishler et al. 1996). Fermentations favouring propionate production produce less methane and carbon dioxide, hence propionate is considered to the more efficient energy source (Moran 2005). Butyrate is metabolised into ketones in the liver which are used as an energy source (Moran 2005). It provides energy for thickening the rumen wall and formation of papillae in calves (Muya et al. 2015) and fatty acid synthesis (Moran 2005).

The microbial degradation of dietary protein and nonprotein nitrogen, degradation of microbial crude protein and hydrolysis of recycled urea are ways through which ammonia is derived in the rumen (Ishler et al. 1996). This rumen ammonia can then be used by rumen bacteria as a source or nitrogen, absorption through rumen wall and flushing to the omasum (Ishler et al. 1996).

The recommended dairy cow nutritional requirements are designed to meet all the lactation and body functions maintenance nutritional demands during lactation.

Table 2.1: Nutritional requirements in early lactation

| Nutrient | Quantity |
|---|---|
| Water | 45% moisture of TMR |
| Non-structural carbohydrates | 35-40% of dietary DM |
| Fibre | >18% ADF, 28% NDF |
| Protein | >18-19% of diet DM |
| Metabolizable energy | 11.3 MJ ME per kg DM |
| Fat | <7% of total diet DM, of which <4% from supplemental fat |
| Roughage | >1.5kg of roughage DM |
| Minerals | ~1% of concentrate mix |
| Urea | < 1% of concentrate mix |
| Salt | 0.5% of diet or 1%of grain mix |
| Vitamins | Supplement with A, D and E |

Adapted from (Erasmus et al. 2000)

## 2.5. The use of feed additives in dairy cows

### 2.5.1. Direct-fed microbial

Direct-fed microbials (DFM) are source of live naturally occurring microorganisms as defined by the US Food and Drug Administration (FDA). There are two types of DFM namely: bacterial (*Megasphaera elsdenii* and Selenomonas ruminantium) and fungal cultures (*Saccharomyces cerevisiae* and Aspergillus *oryzae*). Bacterial DFM are further classified as LUB and lactate producing bacteria. The main use of DFM in

ruminant nutrition is to manipulate the rumen microbial ecosystem to maximise production efficiency and minimise metabolic challenges (Yoon & Stern 1995).

Direct-fed microbials have been used as a safe alternative to antibiotics. The adoption of natural, growth-promoting feed additives has been due to increased public concern about safety, quality of animal products and environmental issues (Sontakke 2012). Thus, feed additives now have to at least meet the three standards (Sontakke 2012); 1) increase productivity, 2) reduce the risk of ruminant digestive carriage of human pathogens, and 3) decrease excretion of polluting outputs like nitrogen-based compounds and methane.

### 2.5.2. *M. elsdenii* NCIMB 41125

#### 2.5.2.1. Background and mode of action

The *Megasphaera elsdenii* (strain $CH_4$), is a biologically pure bacterial culture deposited at NCIMB, Aberdeen, Scotland. UK under NCIMB 41125 (Horn et al. 2009) commonly known as *M. elsdenii* NCIMB 41 125. It occurs naturally in the rumen and this particular strain was obtained and selected from the rumens of concentrate feed adapted dairy cows (Meissner et al. 2010). It is an efficient LUB that is able to proliferate at low pH (5.0 and as low as 4.5). It is resistant to ionophores inhibition and catabolite repression by the presence of sugars (Counotte et al. 1981). Methods used for the isolation of this strain of bacteria are modified pH-Auxostat, and spread plate method (Horn et al. 2009). The isolates were identified using phylogenetics based on 16S rRNA gene sequences.

The ability of *M. elsdenii* to utilise 40-90% lactate in the presence of sugars makes it ideal in facilitating the adaptation from a roughage diet to a high energy concentrate diet in ruminants (Horn et al. 2009). The invention of *M. elsdenii* was aimed at finding a treatment for acidosis that can be commercialised. The shortcoming preventing other strains of *M. elsdenii* from being commercialised were overcome through the invention of *M. elsdenii* NCIMB 41125 (Horn et al. 2009).

#### 2.5.2.2 Actions of *M. elsdenii* in dairy cows

Feed additive *M. elsdenii* is an efficient LUB that converts lactate to weaker acids (Meissner et al. 2010). It has shown ability to convert lactate to propionate and butyrate (Marounek et al. 1989, Drouillard et al. 2012), convert glucose to butyrate in mature ruminants (Henning et al. 2010) and calves (Muya et al. 2015). This process helps to control the build-up of lactic acid in the rumen during early lactation when dairy cows are fed high concentrate diet to support milk production (Meissner et al. 2010, Drouillard et al. 2012). In beef cattle, dosing with *M. elsdenii* at the commencement of the adaptation period has shown the reduction on the occurrences of subacute ruminal acidosis (SARA), other digestive disturbances, morbidity and mortalities (Leeuw et al. 2009, Meissner et al. 2010, Drouillard et al. 2012).

### 2.5.3. The *S. cerevisiae* 1026

#### 2.5.3.1 Background and mode of action

The *S. cerevisiae* strain is deposited in both the National Collection of Yeast Cultures (UK) with a designation NCYC 1026 and the Centraalbureau voor Schimmelcultures

(NL) with the accession number CBS 493.94.11 (Aquilina et al. 2014). For the purpose of this study, the designation NCYC 1026 will be used. The live yeast *S. cerevisiae* 1026, is a single cell eukaryotic fungal microorganism with properties that are very different from bacteria (Sontakke 2012). The method used to produce yeast cells is batch fermentation in a medium based on molasses mineral salts, the final medium includes hop oil as an excipient (Aquilina et al. 2014). Centrifugation is used to recover the cells to produce yeast cream, which is further dried and granulated to produce the final additive.

The *S. cerevisiae* is authorised by the European Union to be used in dairy cows, calves, cattle for fattening and horses as a feed additive. According to Sontakke (2012), the Food and Drug Administration (FDA) has considered *S. cerevisiae* as generally recognized as safe (GRAS) hence appropriate to use in animal feed. The ability of *S. cerevisiae* to convert sugars (i.e. glucose, maltose) into ethanol and carbon dioxide has made it an industrially important yeast (Sontakke 2012). Sontakke (2012) stated that the *S. cerevisiae* "nutritive value is high and rich in enzymes, fatty acids, vitamin B complex, unknown growth factors and amino acids (more than 40% of total dry matter)" making desirable for use in ruminant nutrition.

#### 2.5.3.2 Action of *S. cerevisiae* in ruminants

The *S. cerevisiae* has been reported to decrease methane production by reducing hydrogen availability for methanogenesis based on stoichiometric principles (Bakker et al. 2001). Observed effects that would reduce hydrogen availability to methanogens include a shift in fermentation towards butyrate or propionate (Erasmus et al. 2005),

reduction in protozoal numbers (Newbold et al. 1998) and promotion of acetogenesis as a sink for hydrogen (Chaucheyras et al. 1995).

A significantly higher production of total VFAs and propionate were observed in bulls fed a barley diet with *S. cerevisiae* than those fed the same diet without (Mutsvangwa et al. 1992). The *in vitro* studies using Menke gas test (Menke et al. 1979) showed a reduced methane production after 12 hours of adding S. cerevisiae. Furthermore, the ruminal ammonia production was not affected but the ruminal pH was significantly depressed by the addition of *S. cerevisiae* (Mutsvangwa et al. 1992).

The ability of *S. cerevisiae* to alter rumen microorganisms (bacteria, protozoa and fungi) has been observed in several studies (Wallace & Newbold 1995, Hučko et al. 2009). According to Callaway & Martin (1997), *S. cerevisiae* stimulated the growth of LUB and cellulolytic bacteria by providing soluble growth factors (organic acids, B vitamin and amino acids) which stimulate their growth. Hučko et al. (2009) also observed an increase in the number of cellulolytic bacteria. Furthermore, Newbold et al. (1998) observed an increase of 38% in total variable bacterial count, 48% increase in the cellulolytic population and an increase in LUB *Selenomonas ruminantium* in a medium containing ruminal fluid and sugars *in vitro*.

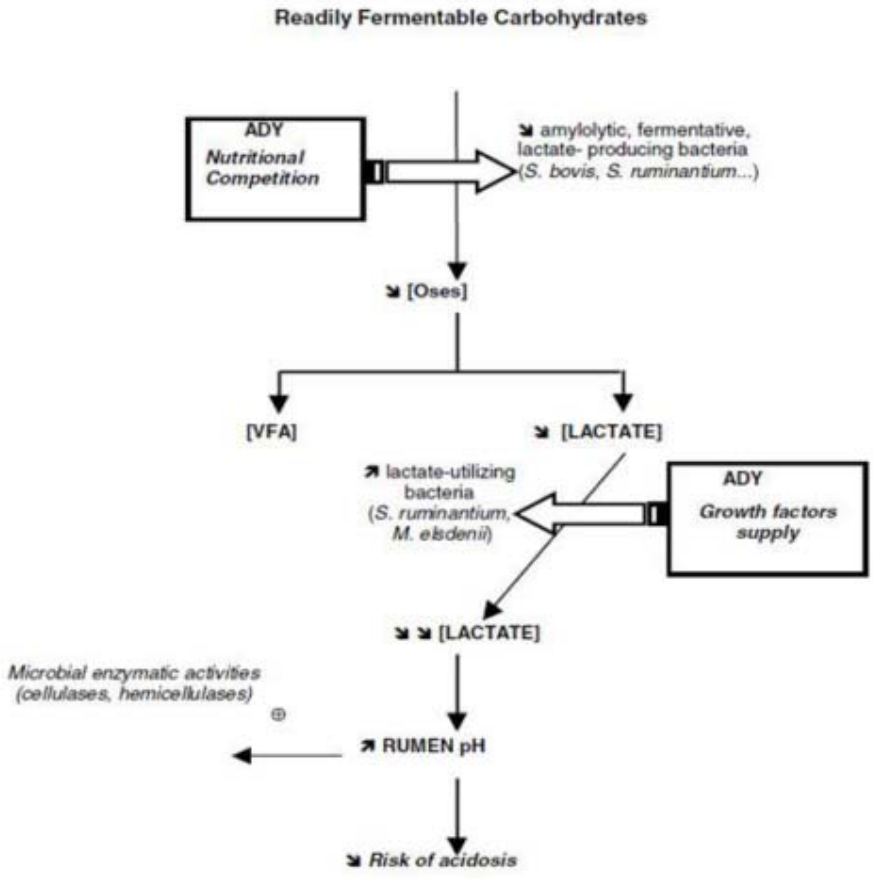


Figure 2.2: Mode of action of an active dry yeast on lactate metabolism and rumen pH (Sontakke 2012).

According to Harrison et al. (1988) an increase in the variable bacterial count is preferential to cellulolytic bacteria. However, Newbold et al. (1998), showed a reduction in rumen ciliate protozoa while Wallace & Newbold (1995) observed a reduction in protozoal numbers. Erasmus et al. (2005) observed an increase in propionate production leading to a decrease in the ruminal acetate to propionate ratio. Contrary to Newbold et al. (1998) where propionate production decreased in favour of acetate.

The *S. cerevisiae* has been used to mitigate methane production by increasing propionate production, which competes with methanogenesis for hydrogen (Mutsvangwa et al. 1992). The mitigation was also possible through enhancing acetogenesis by stimulating acetogenic utilisation of hydrogen (Chaucheyras et al. 1995). Furthermore, by reducing the number of protozoa Newbold et al. (1998) and Wallace & Newbold (1995), which are assumed to have a symbiotic relationship with methanogens (Boadi et al. 2004). Johnson & Johnson, (1995) and Mutsvangwa et al. (1992) stated that mainly, the fraction of propionic acid produced relative to acetic acid has a major impact on methane production.

In ruminant nutrition and management of *S. cerevisiae* been used to prevent rumen microorganisms disorders and disturbances, especially when high energy concentrates feed is consumed (Sontakke 2012). The desired outcomes of yeast inclusion in ruminant diets are: an increase of dry matter consumption, utilization of fibre and other nutritive substances resulting in increase daily gains. Yeast can also improve digestibility and absorption of minerals such as phosphorus, magnesium, calcium, copper, potassium, zinc and manganese.

## 2.6. Limitations and application of an *in vitro* fermentation study in ruminants

The *in vitro* batch fermentation experiment simulate rumen fermentation patterns/pathways. The results can be used to assess and gauge the effects or impacts of feed type and/or feed additives *in vivo*. The usefulness of the results are limited due to lack of animal factors such as disappearance ratios, dilution rate and

passage from the rumen present *in vivo* (Meissner et al. 2010; 2014). The major advantages of *in vitro* experiments are that they provide an affordable alternative to *in vivo* experiments and that they mitigate adverse effects on the animal welfare.

# CHAPTER 3 MATERIALS AND METHODS

## 3.1. Study site

The study was conducted at the Agriculture Research Council-Animal Production Institute (ARC-API). Ethical approval was obtained from ARC-API Irene and the University of South Africa (Unisa) animal ethics committee (2016/CAES/009).

## 3.2. Additives

An existing commercial product containing *M. elsdenii* NCIMB 41125 was used. It was supplied by Afrivet, Newmark Estate/Office Park, 195 Dawie Street, Silver Lakes Road, Hazeldean 0081, South Africa. The product was provided in sachets with two compartments separated by a breakable seal, one contained 50 mL of inoculum and the other 200 mL of sterile growth medium. The preparation was done according to the manufacturer by breaking the seal between the compartments and mixing the contents, making sure the outer seal of the sachet remained intact to maintain anaerobic conditions. The final mixture contained $10^8$ CFU/mL of *M. elsdenii* NCIMB 41125. The bag was then incubated for 24h at 39 °C an incubator. The bag inflated during incubation to indicate the increase in the bacteria population. A syringe was used to withdraw 0.5 mL of the contents ($10^6$ CFU of *M. elsdenii* NCIMB 41125) from the bag. This was immediately added to the serum bottle containing diet and 100mL of rumen fluid/buffer solution under $CO_2$.

The product containing live yeast culture *S. cerevisiae* 1026 (LY) was commercially available as Levucell, the live yeast product contained $10^8$ CFU/g of *S. cerevisiae*,

supplied by VITAM, 142 South Street, Centurion, 0157, South Africa. Preparation was achieved by adding to the basal diet 0.25g of Levucell per kg of feed, to make diet+live yeast.

### 3.3. Diet and treatments

The effects of *M. elsdenii* NCIMB 41125 and *S. cerevisiae* 1026 and combination thereof were evaluated separately on two basal diets (High and low concentrate) formulated to fulfil the minimum nutrient requirement of an early lactating 600 kg Holstein cow producing 40 kg of milk with 3.5% fat and 3.3% protein (NRC, 2001). The formulated basal diets comprised of lucerne hay, ground maize, cottonseed meal, whole cottonseed (linted), sunflower meal, soybeans roasted, cane Molasses, brewers grains, Megalac, sodium chloride and vitamin/mineral premix. Their chemical compositions are shown in Table 3.1.

Treatments were:

1) Con: Basal diet with no additives (Control)
2) Me: Basal diet + $10^6$ CFU of *M. elsdenii* NCIMB 41125)
3) LY: Basal diet + 0.25 g/kg of *S. cerevisiae* 1026
4) Me +L Y: Basal diet + Me + LY

Below is the nutrient composition of the high concentrate and low concentrate diets used in the evaluation *M. elsdenii* and *S. cerevisiae* individually and in combination during batch fermentation.

Table 3.1: Chemical composition of the diets used in this study.

| **Items** | **High concentrate diet** | **low concentrate diet** |
|---|---|---|
| | **60:40 C:F** | **40:60 C:F** |
| Dry matter, (g/kg) | 695 | 598 |
| Organic matter | 940 | 933 |
| Crude protein | 173 | 173 |
| Readily undegradable protein | 372 | 331 |
| Neutral detergent fibre (NDF) | 337 | 303 |
| Forage NDF | 169 | 254 |
| Starch | 280 | 293 |
| Non-fibre carbohydrate (NFC) | 388 | 416 |
| Net energy for lactation (NEL), Mcal/kg | 16 | 162 |
| Calcium | 10 | 9.8 |
| Phosphorus | 0.36 | 0.35 |
| Magnesium | 0.25 | 0.25 |
| Potassium | 1.26 | 1.40 |

## 3.4. Rumen fluid donor

The ruminal fluid was obtained from rumen fistulized lactating Holstein cows fed total mixed ration once daily in the morning (08:00) at the University of Pretoria Experimental farm. The rumen fluid was collected two hours after feeding, squeezed through four layers of cheesecloth into pre-warmed flasks. The flask was closed tight and immediately transported to the lab. The rumen fluid was transferred into a pre-warmed blender (Waring blender; Waring Products, New Hartford, CT, USA) under continuous flushing with $CO_2$, and then blended at high speed for ten seconds, then placed in a 39 °C water bath ready to be used (Holden 1999).

## 3.5. Reduced buffer

The reduced buffer solution was constituted of the macro and micro mineral, the buffer and reducing solution (Table 3.2).

Table 3.2: Buffer composition

| Macro mineral | Reagents | 1L volume |
|---|---|---|
| | Distilled water (mL) | 1000 |
| | $Na_2HPO_4$ anhydrous (g) | 5.7 |
| | $KH_2PO_4$ anhydrous (g) | 6.2 |
| | $MgSO_4.7H_2O$ (g) | 0.59 |
| | NaCl (g) | 2.22 |
| Micro mineral | Reagents | 100mL volume |
| | Distilled water (mL) | 100 |
| | $CaCl_2.2H_2O$ (g) | 13.2 |
| | $MnCl_2.4H_2O$ (g) | 10 |
| | $CoCl_2.6H_2O$ (g) | 1 |
| | $FeCl_3.6H_2O$ (g) | 8 |
| Buffer solution | Reagents | 1L volume |
| | Distilled water (mL) | 1000 |
| | $NH_4HCO_3$ (g) | 4 |
| | $NaHCO_3$ (g) | 35 |
| | Resaruzin 0.1% (w/v) | |
| | Dissolve 0.1 g resaruzin | 100 ml $dH_20$ |
| Reducing solution | Reagents | 100mL volume |
| | Distilled water (mL) | 100 |
| | Cysteine hydrochloric acid (g) | 0.625 |
| | KOH pellets (g) | 10 |
| | Sodium sulphide non hydrate(g) | 0.625 |

Adapted from (Goering, Van Soest 1970)

## 3.6. *In vitro* ruminal fermentation

*In vitro* batch fermentation was used to evaluate the effect of Me, LY and Me+LY on rumen microbial fermentation of diet (Lila et al. 2004). The feed samples (0.5 g) was poured into 250 mL serum bottles. The buffered rumen fluid was prepared by mixing the reduced solution to rumen fluid at 4:1 ratio. Hundred mL of the buffered rumen fluid was then added to the serum bottles while flushing with $CO_2$. The bottles were closed tightly with rubber stoppers, crimp sealed to contain gas pressure and placed at 30 °C in a shaking water bath for 0; 12; 24 and 48 hours of incubation period. For each incubation period, 3 bottles of each treatment were prepared, the pH was measured and samples collected for the determination of ammonia nitrogen and volatile fatty acids as affected by the treatments. Immediately the samples were labelled and stored at -20 °C, they were kept at this temperature until they were sent to the lab for analysis.

## 3.7. The determination of rumen pH, ammonia nitrogen and volatile fatty acids

Immediately after each incubation time, the pH was measured using a standard pH meter and recorded. A sample from each bottle was collected immediately and stored at -20°C.pending analysis. At the laboratory, the ruminal fluid was thawed, centrifuged (15,000 x g, 4 °C for 15 min) and analysed for ammonia nitrogen and VFAs. Ammonia nitrogen was measured by phenol-hypochlorite reaction as described by (Weatherburn 1967) and total and individual VFAs analysed by gas chromatography (Hofirek & Haas 2001).

## 3.8 Data analysis

Mean values for low concentrate and high concentrate diets were subjected to analysis of variance (ANOVA) separately as a complete randomised design using the GLM procedures (SAS, 2009). The model included the fixed effects of treatments (additives) as main effects. Rumen pH, ammonia nitrogen and volatile fatty acids were variables. Significance if P≤ 0.05.

$Y_{it} = \mu + \alpha_i + \beta_t + T_{it} + e_{cit}$,

where $Y_{it}$ = an observation value for pH, ammonia nitrogen, total VFAs and molar molar proportion of individual VFAs obtained from treatment i at time *t*;

$\mu$ = overall mean for the population;

$\alpha_i$ = fixed effect of treatment i, where i = CON, Me, LY, or Me+LY;

$\beta_t$ = fixed effect of time *t*, where *t* = 0, 12, 24 or 48 hours;

$T_{it}$ = fixed interaction of effect of treatment i and time *t*;

$e_{cit}$ = error associated with each $Y_{it}$.

Significance was declared at P<0.05 and tendency was accepted if 0.10 >P> 0.05.

# CHAPTER 4 RESULTS

## 4.1. The effects of *M. elsdenii* and *S. cerevisiae* on rumen pH, ammonia nitrogen, volatile fatty acids in a high concentrate diet

The effects of Me, LY and Me+LY on rumen pH, ammonia-N and individual VFAs concentration and molar proportions in high concentrate diet (60:40) are presented in Tables 4.1 and 4.2.

### 4.1.1 The effects of addition of *M. elsdenii*

There were no effects of Me on rumen pH, ammonia-N and total VFAs (Table 4.1). However, acetate concentration (µmol) was decreased (P=0.0003) while molar % showed a tendency to decrease (P=0.07). Propionate and butyrate concentrations were not affected by Me, but their molar % tended (P=0.08) to decrease with Me. Isobutyrate and valerate concentrations and molar % were not affected by Me. Lactate concentration tended to decrease (P=0.07) with Me, but its molar % was not affected. The acetate to propionate ratio (A: Pr) was also decreased (P=0.02) with Me, but the Acetate to propionate plus butyrate (A: Pr+B) ratio was not affected.

When evaluated per incubation period in comparison with control (Table 4.2), the addition of Me did not affect rumen pH and the molar % of propionate at all incubation periods. The rumen ammonia-N was higher (P=0.002) at 0h with addition of Me, however, it showed a tendency to decrease (P=0.06) at 24h while a decrease (P<0.0001) was observed at 48h. The addition of Me lowered (P≤0.02) the molar % of

acetate from 0h to 48h, except at 24h where a tendency to decrease (P=0.06) was observed. The molar % of lactate showed a tendency to decrease (P=0.08) and a decrease (P=0.0003) at 24h and 48h, respectively.

### 4.1.2 The effects of addition of *S. cerevisiae*

The LY had no effects on rumen pH, ammonia-N and propionate (µmol) and molar %. The addition of LY increased (P=0.005) total VFAs concentration, Butyrate concentration and molar % (P=0.009 and P=0.02), respectively and decreased (P<0.0001) the acetate concentration and molar %. There were no effects of LY on valerate and lactate concentration, but their molar % tended to be decreased (P=0.05). Isobutyrate concentration showed a tendency to decrease (P=0.08) while molar % decreased (P=0.007) with LY. The addition of LY decreased (P<0.002) A: Pr and A: Pr+B ratios.

The rumen pH was lower (P=0.0002) with LY at 0h, but higher (P=0.008) at 48h, when evaluated per incubation period in comparison with control (Table 4.2). The rumen ammonia-N was lower at 48h with the addition of LY. The molar % of acetate was lower (P≤0.0002) from 0 to 48 h with the addition of LY. The molar % of propionate was lower (P=0.03) and (P=0.02) with LY at 24h and 48h, respectively. The addition of LY lowered (P<0.0001) the lactate molar % at 24h and 48h.

### 4.1.3 The effects of the combination of *M. elsdenii* and *S. cerevisiae* (Me+LY)

The combination of feed additives Me+LY had no effects on rumen pH, ammonia-N. and butyrate (µmol) and molar %. The lactate concentration and molar % was decreased ($P<0.01$) with addition of Me+LY. The addition of Me+LY significantly increased ($P=0.0007$) and decrease ($P=0.0001$) the total VFAs and acetate (µmol and molar %), respectively. There were no effects of Me+LY on valerate and isobutyrate and propionate concentration, but the molar % of valerate and isobutyrate were decreased ($P=0.009$) while the molar % of propionate, tended to decrease ($P=0.09$). The A: Pr and A: Pr+B ratios were decreased ($P=0.005$) and ($P=0.03$), respectively, with Me+LY.

When evaluated per incubation period (Table 4.2) and compared to the control, the addition of Me+LY had no effects on rumen pH at all incubation periods. Ammonia-N was higher ($P=0.0005$) at 0h but lower ($P=0.01$) at 48h, with Me+LY addition. The molar % of acetate and lactate were lowered ($P<0.0001$) and ($P\leq0.002$), respectively from 0 to 48h with the addition of Me+LY. The addition Me+LY tended to lower ($P=0.07$) and lowered ($P=0.01$) the molar % of propionate at 0h and at 24h, respectively.

Table 4.1: Effects of *Megasphaera elsdenii* (Me) and *Saccharomyces cerevisiae* live yeast (LY) on rumen fermentation of high concentrate dairy cattle diet.

| Parameter | Additives | | | | SEM[1] | Contrast, *p* | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Me | LY | Me+LY | | Control vs. Me | Control vs. LY | Control vs. Me+LY |
| pH | 5.78 | 5.74 | 5.77 | 5.76 | 0.064 | 0.64 | 0.86 | 0.82 |
| Ammonia-N, mg/L | 7.31 | 7.01 | 7.20 | 7.13 | 0.244 | 0.39 | 0.75 | 0.60 |
| total VFAs, µmole/L | 129.56 | 128.67 | 137.01 | 138.71 | 1.767 | 0.69 | 0.005 | 0.0007 |
| Acetate, µmole/L | 78.94 | 74.18 | 72.28 | 73.78 | 0.865 | 0.0003 | <0.0001 | 0.0001 |
| Propionate, µmole/L | 42.63 | 43.60 | 44.11 | 44.25 | 0.753 | 0.37 | 0.17 | 0.13 |
| Butyrate, µmole/L | 10.08 | 8.37 | 13.06 | 10.77 | 0.77 | 0.12 | 0.009 | 0.53 |
| Isobutyrate, µmole/L | 2.51 | 2.30 | 2.18 | 2.24 | 0.133 | 0.28 | 0.08 | 0.16 |
| Valerate, µmole/L | 0.81 | 0.71 | 1.08 | 0.84 | 0.133 | 0.57 | 0.16 | 0.86 |
| Lactate, µmole/L | 6.88 | 6.46 | 6.46 | 5.89 | 0.156 | 0.07 | 0.17 | <0.0001 |
| Acetate, % | 61.00 | 57.74 | 53.23 | 53.24 | 1.249 | 0.07 | <0.0001 | <0.0001 |
| Propionate, % | 32.88 | 33.91 | 32.23 | 31.89 | 0.405 | 0.08 | 0.27 | 0.09 |
| Butyrate, % | 7.75 | 6.48 | 9.45 | 7.72 | 0.499 | 0.08 | 0.02 | 0.96 |
| Isobutyrate, % | 1.93 | 1.79 | 1.58 | 1.58 | 0.088 | 0.29 | 0.007 | 0.009 |
| Valerate, % | 1.93 | 1.79 | 1.58 | 1.58 | 0.088 | 0.29 | 0.007 | 0.009 |
| Lactate, % | 5.30 | 5.03 | 4.73 | 4.27 | 0.155 | 0.22 | 0.01 | <0.0001 |
| A:Pr[2] | 1.86 | 1.71 | 1.65 | 1.67 | 0.045 | 0.02 | 0.002 | 0.005 |
| A:Pr+B[3] | 1.51 | 1.44 | 1.28 | 1.35 | 0.049 | 0.30 | 0.002 | 0.03 |

[1]Standard error of mean
[2]Acetate to Propionate ratio
[3]Acetate to propionate +butyrate ratio

Table 4.2: Change in selected rumen parameters as affected by addition of *Megasphaera elsdenii* (Me) and *Saccharomyces cerevisiae* live yeast (LY) to dairy cow's diet high (60:40) in concentrate to forage ratio.

| Parameter | Additives | | | | SEM[1] | Contrast, *p* | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Me | LY | Me+LY | | Control vs. Me | Control vs. LY | Control vs. Me+LY |
| **Rumen pH** | | | | | 0.09 | | | |
| 0h | 6.0 | 5.9 | 5.5 | 6.1 | | 0.40 | <0.001 | 0.90 |
| 12h | 5.8 | 5.9 | 5.7 | 5.7 | | 0.64 | 0.49 | 0.28 |
| 24h | 5.7 | 5.6 | 5.9 | 5.7 | | 0.55 | 0.13 | 0.96 |
| 48h | 5.6 | 5.5 | 6.0 | 5.6 | | 0.72 | 0.008 | 0.8 |
| **Rumen ammonia-N, mg/L** | | | | | | | | |
| 0h | 6.0 | 7.4 | 6.0 | 7.3 | 0.24 | 0.002 | 0.92 | <0.001 |
| 12h | 6.7 | 7.1 | 7.0 | 7.2 | | 0.33 | 0.43 | 0.17 |
| 24h | 7.4 | 6.7 | 7.5 | 7.1 | | 0.06 | 0.84 | 0.43 |
| 48h | 9.1 | 6.8 | 8.4 | 6.9 | | <.0001 | 0.04 | 0.01 |
| **Rumen acetate, %** | | | | | | | | |
| 0h | 67.1 | 60.9 | 62.4 | 55.9 | 0.80 | <.0001 | <0.001 | <0.001 |
| 12h | 60.2 | 57.6 | 54.8 | 55.0 | | 0.02 | <0.001 | <0.001 |
| 24h | 59.0 | 56.8 | 51.1 | 51.5 | | 0.06 | <0.001 | <0.001 |
| 48h | 57.7 | 55.7 | 44.6 | 50.5 | | 0.01 | <0.001 | <0.001 |
| **Rumen proionate, %** | | | | | | | | |
| 0h | 32.4 | 33.5 | 33.4 | 30.7 | 0.62 | 0.21 | 0.27 | 0.07 |
| 12h | 31.2 | 32.6 | 31.9 | 32.3 | | 0.11 | 0.46 | 0.22 |
| 24h | 33.6 | 33.9 | 31.6 | 31.3 | | 0.74 | 0.03 | 0.01 |
| 48h | 34.4 | 35.6 | 32.1 | 33.3 | | 0.16 | 0.02 | 0.24 |
| **Rumen lactate, %** | | | | | | | | |
| 0h | 5.5 | 5.5 | 5.5 | 4.9 | 0.13 | 1.00 | 0.72 | <0.001 |
| 12h | 5.5 | 5.4 | 5.1 | 4.8 | | 0.86 | 0.05 | <0.001 |
| 24h | 5.1 | 4.7 | 4.2 | 3.8 | | 0.08 | <0.001 | <0.001 |
| 48h | 5.1 | 4.4 | 4.1 | 3.6 | | 0.0003 | <0.001 | <0.001 |

[1]Standard error of mean

## 4.2. The effects of *M. elsdenii* and *S. cerevisiae* on rumen pH, ammonia nitrogen and volatile fatty acids in a low concentrate diet

Tables 4.3 and 4.4 presented the effects of Me, LY and Me+LY on rumen pH, ammonia-N and individual VFAs concentrations (µmol) and molar proportions (%) in low concentrate diet (40:60).

### 4.2.1 The effects of Me

The mean rumen pH, ammonia-N and total VFAs were not affected with addition of Me (Table 4.3). The addition of Me increased (P=0.001) and (P=0.009) the propionate concentration (µmol) and molar proportion (%), respectively. There was no effect of Me on the concentration of acetate and lactate, but their molar % were decreased (P<0.05). There were no effects on the concentration and molar % of butyrate, isobutyrate, valerate with Me. The addition of Me decreased the A: Pr (P=0.005) and A: Pr+B (P=0.03) ratios.

When evaluated per incubation period (Table 4.4) and compared to the control, rumen pH was lower (P=<0.0001) at 0h with addition of Me but, higher (P=0.004) and (P=0.003) at 24h and 48h, respectively. The addition of Me did not affect ammonia-N at all incubation periods. The molar % of acetate was higher (P<0.0001) at 0 h with addition of Me but was lower at 12h (P=0.03), 24h (P<0.0001) and 48 h (P<0.0001) compared to the control. The molar % of propionate was higher than the control at 12 h (P=0.02), 24h (P=0.01) and 48h (P=0.03) with Me. At 48h, the addition of Me lowered (P=0.03) the molar proportion of lactate.

### 4.2.2 The effects of addition of LY

The addition of LY did not affect mean rumen pH, ammonia-N and total VFAs concentration. The concentration and molar % of propionate increased ($P<0.05$) with addition of LY. The concentration of acetate and lactate were not affected by LY, but their molar % were decreased ($P<0.05$). The addition of LY did not affect the concentrations and molar % of butyrate, isobutyrate and valerate. The A: Pr and A: Pr+B ratios decreased ($P<0.05$) with LY.

The rumen pH was lower ($P=0.0002$) at 0 h with addition of LY but, was higher ($P=0.009$) at 12h compared to the control when evaluated per incubation period (Table 4.4). The molar proportion of acetate was lower ($P<.0001$) from 12h to 48h with LY. At 12h the molar % of propionate was higher ($P=0.01$) with addition of LY while the molar % of lactate was lower ($P<0.05$) at 24h and 48h with LY.

### 4.2.3 The effects of the combination of Me+LY

The addition of Me+LY increased ($P=0.05$) the rumen ammonia-N (mg/L) and total VFAs. The concentration and molar % of propionate were increased ($P<0.001$) by Me+LY. The addition of Me+LY did not affect rumen pH and valerate concentration (µmol) and molar %. However, increased ($P=0.01$) isobutyrate and tended to increase ($P=0.07$) butyrate concentrations while there was no effect on their molar proportions. The concentration of acetate and lactate were not affected with Me+LY, but, their molar proportions were decreased ($P<0.05$). The ratios A: Pr and A: Pr+B were also decreased ($P<0.0001$) with Me+LY.

When evaluated per incubation period (Table 4.4) rumen pH was lower (P=<.0001) at 0 h with addition of Me+LY but, higher at 12 h (P=0.04) and 24 h (P=0.01) compared to the control. The rumen ammonia-N was higher (P<0.05) at 0 h and 48h with addition of Me+LY. The molar proportion of acetate was higher (P=0.01) only at 0h with addition of Me but was lower (P<.0001) from 12h to 48h. The molar proportion of propionate was higher at 0 h (P=0.01), 12h (P=0.0006) and 48h (P=0.04) and, the molar proportion of lactate was lower at 24h (P=0.04) and 48h (P=0.006) with addition of LY.

Table 4.3: Effects of *Megasphaera elsdenii* (Me) and *Saccharomyces cerevisiae* live yeast (LY) on rumen fermentation of low concentrate dairy cow's diet.

| Parameter | Additives | | | | SEM[1] | Contrast, *p* | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Me | LY | Me+LY | | Control vs. Me | Control vs. LY | Control vs. Me+LY |
| pH | 5.84 | 5.93 | 5.77 | 5.81 | 0.092 | 0.47 | 0.63 | 0.87 |
| Ammonia-N, mg/L | 5.93 | 6.03 | 6.13 | 6.57 | 0.225 | 0.76 | 0.53 | 0.05 |
| total VFAs, µmole/L | 119.93 | 124.63 | 131.38 | 132.06 | 0.925 | 0.76 | 0.53 | 0.05 |
| Acetate, µmole/L | 74.08 | 74.13 | 74.63 | 72.90 | 0.788 | 0.96 | 0.62 | 0.88 |
| Propionate, µmole/L | 32.05 | 35.83 | 37.15 | 39.00 | 0.781 | 0.001 | <0.0001 | <0.0001 |
| Butyrate, µmole/L | 9.30 | 9.61 | 9.24 | 10.53 | 0.466 | 0.64 | 0.93 | 0.07 |
| Isobutyrate, µmole/L | 1.53 | 1.73 | 1.52 | 1.86 | 0.092 | 0.12 | 0.95 | 0.01 |
| Valerate, µmole/L | 0.83 | 0.83 | 0.92 | 0.86 | 0.092 | 0.95 | 0.49 | 0.8 |
| Lactate, µmole/L | 5.23 | 4.54 | 4.70 | 4.75 | 0.288 | 0.1 | 0.2 | 0.2 |
| Acetate, % | 61.78 | 59.54 | 56.80 | 56.04 | 0.788 | 0.05 | <0.0001 | <0.0001 |
| Propionate, % | 26.75 | 28.72 | 28.27 | 29.50 | 0.512 | 0.009 | 0.04 | 0.0004 |
| Butyrate, % | 7.76 | 7.70 | 7.03 | 7.94 | 0.337 | 0.90 | 0.13 | 0.70 |
| Isobutyrate, % | 1.28 | 1.39 | 1.14 | 1.40 | 0.07 | 0.28 | 0.16 | 0.24 |
| Valerate, % | 1.28 | 1.39 | 1.14 | 1.40 | 0.07 | 0.28 | 0.16 | 0.24 |
| Lactate, % | 4.36 | 3.64 | 3.60 | 3.62 | 0.236 | 0.04 | 0.03 | 0.03 |
| A:Pr[2] | 2.32 | 2.10 | 2.02 | 1.90 | 0.052 | 0.005 | 0.0002 | <0.0001 |
| A:Pr+B[3] | 1.80 | 1.65 | 1.62 | 1.51 | 0.468 | 0.03 | 0.008 | <0.0001 |

[1]Standard error of mean
[2]Acetate to Propionate ratio
[3]Acetate to propionate +butyrate ratio

Table 4.4: Change in selected rumen parameters as affected by addition of *Megasphaera elsdenii* (Me) and *Saccharomyces cerevisiae* live yeast (LY) to dairy cow's diet with low (40:60) concentrate to forage ratio.

| Parameter | Additives | | | | SEM | Contrast, *p* | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Me | LY | Me+LY | | Control vs. Me | Control vs. LY | Control vs. Me+LY |
| **Rumen pH** | | | | | 1.00 | | | |
| 0h | 6.6 | 5.9 | 6.05 | 5.8 | | <.0001 | 0.0002 | <.0001 |
| 12h | 5.6 | 5.8 | 5.99 | 5.9 | | 0.13 | 0.009 | 0.04 |
| 24h | 5.5 | 5.9 | 5.65 | 5.9 | | 0.004 | 0.34 | 0.01 |
| 48h | 5.6 | 6.0 | 5.40 | 5.6 | | 0.003 | 0.16 | 0.90 |
| **Rumen ammonia-N, mg/L** | | | | | 0.32 | | | |
| 0h | 5.0 | 5.0 | 5.40 | 6.3 | | 1.00 | 0.35 | 0.007 |
| 12h | 6.9 | 6.9 | 6.97 | 6.8 | | 0.94 | 0.94 | 0.72 |
| 24h | 5.9 | 6.0 | 5.93 | 6.2 | | 0.83 | 0.88 | 0.47 |
| 48h | 5.9 | 6.3 | 6.20 | 7.0 | | 0.47 | 0.56 | 0.02 |
| **Rumen acetate, %** | | | | | 0.59 | | | |
| 0h | 57.6 | 63.7 | 56.40 | 59.9 | | <.0001 | 0.15 | 0.01 |
| 12h | 62.2 | 60.4 | 57.23 | 56.7 | | 0.03 | <.0001 | <.0001 |
| 24h | 63.3 | 59.3 | 57.03 | 55.6 | | <.0001 | <.0001 | <.0001 |
| 48h | 64.0 | 54.7 | 56.53 | 52.0 | | <.0001 | <.0001 | <.0001 |
| **Rumen proionate, %** | | | | | 0.74 | | | |
| 0h | 25.3 | 26.3 | 26.60 | 28.13 | | 0.33 | 0.21 | 0.01 |
| 12h | 25.2 | 27.8 | 28.10 | 29.27 | | 0.02 | 0.01 | 0.0006 |
| 24h | 28.4 | 30.2 | 29.40 | 30.30 | | 0.01 | 0.33 | 0.08 |
| 48h | 28.1 | 30.5 | 29.00 | 30.30 | | 0.03 | 0.42 | 0.04 |
| **Rumen lactate, %** | | | | | 0.34 | | | |
| 0h | 4.5 | 4.1 | 4.3 | 4.10 | | 0.41 | 0.78 | 0.45 |
| 12h | 4.7 | 4.0 | 4.5 | 4.53 | | 0.18 | 0.78 | 0.78 |
| 24h | 4.2 | 3.5 | 3.0 | 3.20 | | 0.14 | 0.01 | 0.04 |
| 48h | 4.1 | 3.0 | 2.5 | 2.63 | | 0.03 | 0.003 | 0.006 |

## 4.3. Effect of *M. elsdenii, S. cerevisiae* and their combination on the lactate in high and low concentrate diets

Live yeast and Me were able to control the build-up of lactate and influence the concentration of rumen lactate and determine the development of acidosis. Figures 4.1; 4.2 and 4.3 show the decreasing effects of Me, Ly and Me+LY comparing it between high and low concentrate diets.

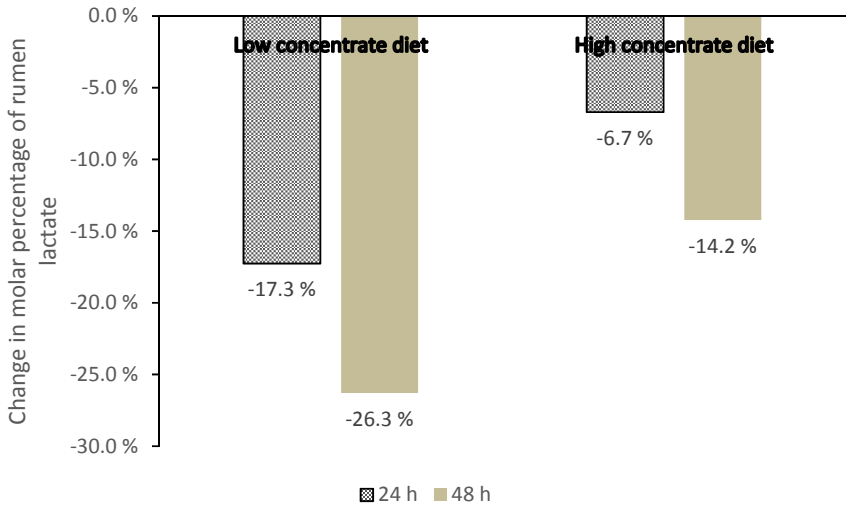


Figure 4.1: The effects of Me on lactate in low and high concentrate diets

Addition of Me decrease significantly the molar percentage of rumen lactate in low concentrate compared to high concentrate diet, see Figure 4.1.

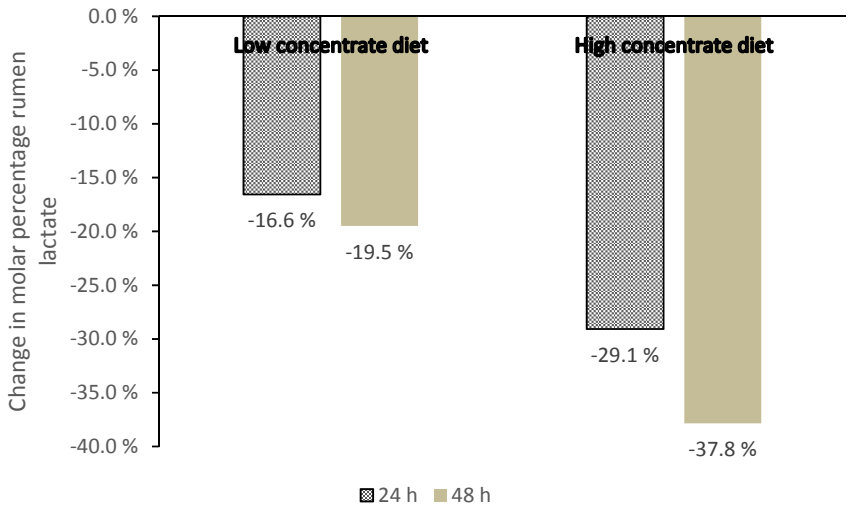


Figure 4.2: The effects of LY on lactate in low and high concentrate diets

As opposed to the effects of Me, addition of LY had a more decreasing effect on the molar % of rumen lactate in high compared to low concentrate diet, see Figure 4.2.

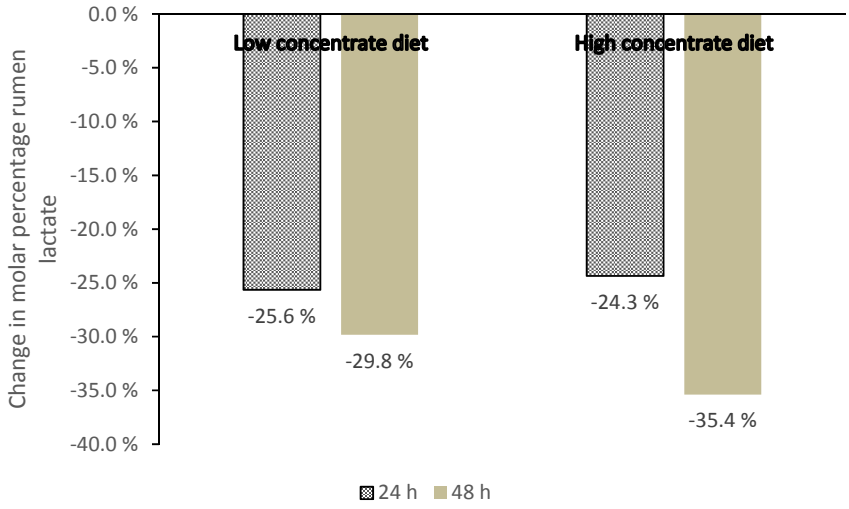


Figure 4.3: The effects of Me+LY on lactate in low and high concentrate diets

Addition of Me+LY decrease the molar percentage of rumen lactate almost similarly in high and low concentrate diets.

## 4.4. The linear relationship between additives and control evaluated in high and low concentrate diets and presented

In the high concentrate diet, there was a strong negative relationship between LY and Me+LY compared to the control for lactate molar percentage.

The equations were:

1) LY = -1.032 x + 11.956 ($R^2$= 0.84 ; P = 0.03)

2) Me+LY= -1.432 x + 13.735 ($R^2$= 0.78; P= 0.002)

While the addition of Me presented a strong but not significant relationship with the control. The equation was:

Me= -0.965 x + 10.843 ($R^2$= 0.69; P = 0.23)

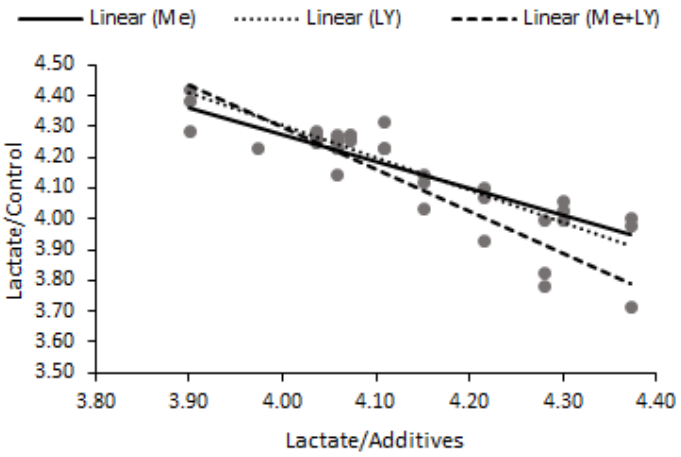


Figure 4.4: Relationship between additives and the control for lactate in high concentrate diet.

There was a strong negative correlation between LY and Me+LY compared to the control in the low Concentrate diet.

The equations were:

1) LY=-0.642 x + 8.786 ($R^2$= 0.63; P= 0.04)

2) Me+LY= -0.536 x + 8.432 ($R^2$= 0.78; P=0.01)

While the addition of Me presented a moderate but significant negative correlation with the control.

The equation was: Me= -0.543 x + 7.893 ($R^2$= 0.59; P=0.02)

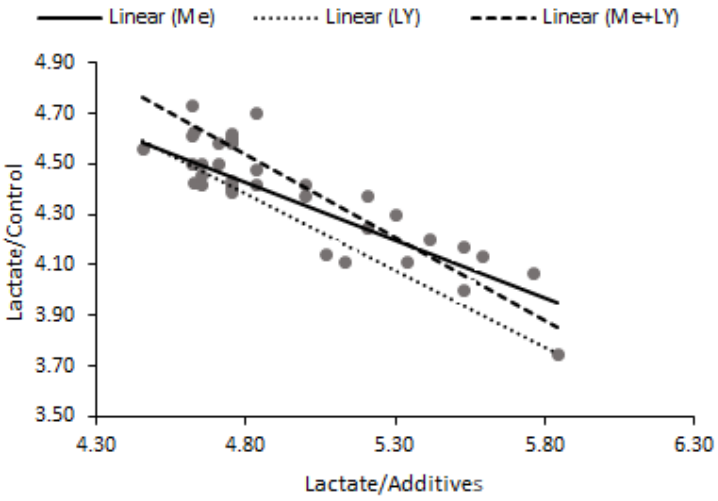


Figure 4.5: Relationship between additives and the control for lactate in low concentrate diet.

# CHAPTER 5 DISCUSSIONS

## 5.1. The effects of additives on pH and ammonia nitrogen

In the present study, the average rumen pH and ammonia nitrogen in both low and high concentrate diets was 5.8 and was not affected by Me and LY supplemented separately or in their combination (Me+LY). This lack of effects of Me on the pH is in agreement with previous studies (Aikman et al. 2009; 2011, Hagg et al. 2010, Zebeli et al. 2012), but contradicts with results of Henning et al. (2010). The latter author reported that *M. elsdenii* increased and decreased the rumen pH *in vivo* in Bonsmara steers and lambs, respectively. The *M. elsdenii* did not affect rumen pH in pre-weaned calves (Muya et al. 2015) and in steer (Henning et al. 2010). A 48 – 96 hours adaptation period has been suggested as critical for noticeable effects on stabilising pH with the strain *M. elsdenii* 41125 (Meisser et al. 2014). The noticeable competitive advantages of the strain *M. elsdenii* 41125 on pH and outgrowing other lactate utilising organisms have been reported more after a sudden increase in concentrate (McDaniel *et al.* 2009), which was not the case in the present study. The absence of effects of LY on pH agrees also with previous *in vitro* studies (, Zeleňák et al. 1994, Newbold et al. 1995, Newbold et al. 1998, Lila et al. 2004). Mutsvangwa et al. (1992) and Al Ibrahim et al. (2012) observed no effect of *S. cerevisiae* in the first period (morning) but in the second period (afternoon) decrease and increase , respectively. Contrary to our observations, a specific strain, *S. cerevisiae* 1026 decreased pH in Holstein cows (Angeles et al. 1998, Chung et al. 2011), which is attributed to the stimulation of metabolism and growth of bacteria that utilise lactate, such as *M. elsdenii* or *S. ruminantium* (Chevrauillard et al. 1996; Rossi et al. 2004). No critical pH condition was

prevailing in order to allow for these two additives to express their potential on stabilising pH. The increase in pH at 24 and 48 h by Me at 0 and 12 h by LY in low concentrate diet only is difficult to explain.

## 5.2. The effects of additives on total volatile fatty acids

The lack of effects of addition of Me alone in both high and low concentrate diets agree with Aikman et al. (2009) and Hagg et al. (2010). The changes in rumen VFAs after dosing early lactating cows with Me were not observed. In the present study, early lactating cows were used as rumen fluid donors. However, caution should always be taken when interpreting rumen VFAs concentration results, because the rumen VFAs level varies over time.

In high concentrate diet, total VFAs were increased with the addition of LY alone and in combination with Me (LY+Me). The increase in total VFAs in the present study with addition of LY to high concentrate diet is in agreement with some reports (Al Ibrahim et al. 2012, Arcos-García et al. 2000, Lila et al. 2004) but not with other researchers (Chung et al. 2011, Angeles et al. 1998). The observed increased VFAs by Me+LY was probably due to the increasing effect of LY since Me alone did not show an effect.

The lack of effects of these three additives on the mean total VFAs concentration in low concentrate diet is difficult to explain. A contrasting report by (Meissner et al. 2014) reported an increase in total VFAs.

## 5.3. The effects of additives on major volatile fatty acids

The concentration of VFAs produced in the rumen and the proportions (molar percentage) in which they are produced are important determinants of a ruminant's metabolism. However, the molar percentages are more appropriate and have greater utility for evaluation of treatments because it is not sensitive to ruminal liquid amount, which has great variability in rumen digesta liquid amounts (Hall et al. 2015). For this reason, the discussion on VFAs will focus mainly on molar percentage of individual VFAs.

As also reported previously by Aikman et al. (2009; 2011), in lactating cows, the acetate tended to decrease with Me as it is observed in the present study with both low and high concentrate diets. No effects were observed by other authors in young calves (Muya et al. 2015) and steer (Henning et al. 2010), suggesting that Me may act differently depending on the age and animal breed probably due to the stage of rumen development dynamic in the rumen. The tendency of increase in propionate in both diets, is concurring with observation by Aikman et al. (2011). In contrast, Henning et al. (2010) reported a decrease in the molar percentage of propionate. Generally, when pH decreases with Me, the fermentation shifts from propionic acid to butyric and valeric acid (Marounek et al. 1989). The change in fermentation observed in the present study is more favourable for dairy cows, as more propionic can enter the tricarboxylic cycle and generate more glucose.

As a result of the tendency to decrease and increase of acetate and propionate, respectively, in the present study, the A: Pr was reduced. Although the molar

percentage of VFAs at different incubation times was not statistically compared within treatments, numerically more propionate was produced with time at the expense of acetate. The decrease in the molar percentage of lactate in high concentrate diet by 6 and 14 % at 24 and 48 h, respectively, and by 26 % at 48 h in low concentrate diets, confirm the action of Me on converting lactate to others VFAs. The *M. elsdenii* is reported to be a major role player in the generation of branched chain of VFAs in the rumen (Wallace 1986) and converting lactate to propionate and butyrate as well as converting glucose to butyrate (Henning et al. 2010). This is supported by the decrease in lactate with Me. The decrease in the ratio of acetate to propionate (A: Pr) with Me was also reported by Aikman et al. (2009; 2011). However, Meissner et al. (2014) reported an increase but (Hagg et al. 2010) reported no effect. The current study shows that *M. elsdenii* NCIMB 41125 alters rumen fermentation patterns supportive of glucogenic propionate, which can potentially benefit energy balance, animal health and animal production (Aikman et al. 2009; 2011) in early lactation. However, Aikman et al. (2011) and Henning et al. (2010) reported a decrease in the production and molar proportion of propionate, respectively.

Addition of LY decreased acetate in both diets as observed by Chung et al. (2011) but not by Lila et al. (2004) and Al Ibrahim et al. (2012). Erasmus et al. (2005) reported an increase in acetate and Zeleňák et al. (1994) observed no effects of LY. This increase in acetate, increase in propionate in low concentration diet but not affected in high concentrate diet with LY also resulted in reduced A: Pr ratio. In addition to the increased butyrate in both diets, hence the A: Pr+B was also reduced. The general and well known effects reported in many studies with LY is the stimulation of increase in propionate at the expense of acetate (Erasmus et al. 2005), which was also

observed in the present study in both low and high concentrate diets. Lactate is not used as a substrate by *S. cerevisiae* (Williams et al. 1991). Therefore, it was suggested that the decrease in rumen lactate may be, amongst other, the result of the inhibition of lactate production or stimulation of lactate utilisation by other microbes (Williams et al. 1991). For Me, numerically more propionate was produced with time at the expense of acetate with LY. As observed in the present study, lactate was also reported to be decreased by yeast (Lila et al. 2004).

When added in combination, the two additives increased propionate (although only tendency was observed in high concentrate diet) at the expense of acetate, reducing the A: Pr. This effects were more pronounced in low than high concentrate diet. The decreasing effect of lactate appears to be greater when the two additives were added in combination.

In high concentrate diet, ruminal lactate is expected to increase and cause acidosis. In the present study, no cases of clinical acidosis were observed. All treatment groups had rumen pH >5.6 (a commonly used threshold to define subacute ruminal acidosis). Lactate level was elevated in high concentrate diet, which was expected because large amounts of starch and sugar stimulate bacteria that make lactic acid. This can also partially explain the greater extent of lactate decrease in low compared to high concentrate. In the presence of more LUB, more moles of existing lactate could have been converted to other VFAs.

Live yeast appears to act differently compared to Me by having a double the effects on high than low concentrate diets. The exact mechanism by which the yeast culture

exerts its potential in stimulating rumen bacteria is not well understood . Nonetheless, it is could be through the removal of oxygen from the rumen environment or the presence of unidentified growth factor delivered by the active live yeast cells (Jouany & Morgavi 2007). However, the greater effects on high concentrate diet in the present can be attributed to a selective stimulation of LUB as suggested by Callaway and Martin (1997).

When added separately to the diet, Me and LY acted differently on rumen lactate in low and high concentrate diets, but the decreasing effects of Me+LY on rumen lactate was almost similar in both low and high concentrate diets, which suggest a modest complementarity effect between the two additives. Associative effects of LY and other additives was previously reported (Erasmus et al. 2005). The general decrease in lactate by all additives observed in this study indicate their beneficial effect in early lactation period of dairy cows when animals are fed high concentrate and are at high risk of developing acidosis.

# CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

The use of feed additives and rumen modifiers in ruminant production will continue to play an essential role in improving nutrient efficiency and alleviating metabolic disorders, which more often occur in high producing animals. Improved energy metabolism and animal performance with feed additives as rumen manipulators are the main benefits reported in dairy production. Specific rumen condition appears to be the key driver of the expression on different additives and has led to different effects.

The present results support that dietary addition of *M. elsdenii* and *S. cerevisiae*, can shift the rumen fermentation patterns of a dairy cow's diet, mainly towards the production of more propionate and decrease of acetate molar proportion. The interaction of the two additives showed more pronounced effects on this shift with low concentrate diet. This is particularly important and can improve the energy balance health and productivity of cows fed low concentrate diets, which are known to provide less energy due to the glucogenic properties of propionate. A decrease in lactate was also found as a result of interaction between the two additives and was similar in both low and high concentrate diets. The control of lactate build-up is critical in high producing early lactating dairy cows. As reported in other studies, understanding these associative or complementary effects are important and can help animals and feed producers in decision making.

More research is warranted to document effects of these two additives *in vivo* and different feeding conditions.

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