#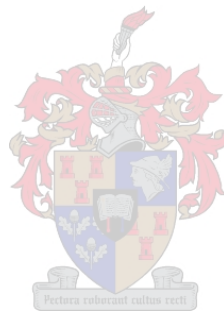 Sensitivity and improvement of *in vitro* methods to assess the effects of *Saccharomyces cerevisiae* on rumen fibre degradation

by

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Date: March 2017

DECLARATION

By submitting this thesis electronically I, Aimee Russouw, declare that the entirety of the work contained therein is my own original work and that I am the authorship owner thereof (save to the extent explicitly otherwise stated), and that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously, in its entirety or in part, submitted it for obtaining any qualification.

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GENERAL ABSTRACT

In this study, the effects of a yeast supplement, *Saccharomyces cerevisiae* CNCM I-1077, on *in vitro* neutral detergent fibre digestibility (NDFd) and rate of digestion of NDF(kd), organic matter digestibility (OMd), dry matter digestibility (DMd), pH and volatile fatty acids (VFA) concentration were investigated when different parameters were changed, within the *in vitro* system, in order to challenge the yeast's effectiveness. In the first experiment, the Kansas State, McDougall and Goering-Van Soest media where tested with 3 different yeast doses against a control (0, 1×10^5, 1×10^6 and 1×10^7 cfu/ml) using oat hay and wheat straw. The maximum *in vitro* NDFd, DMd, OMd as well as rate of digestion of NDFwere obtained with dose 1×10^6 cfu/ml and with the McDougall or Goering-Van Soest media, although differences between doses were not always significant. The Kansas State medium resulted in the lowest values compared to the other two media. There was a decrease in pH estimates as yeast dose increased which corresponded with an increase in VFA concentrations. However, although the differences in pH were not all significant, dose 1×10^7 cfu/ml related to lower estimates compared to the control and 1×10^5 (6.53 vs. 6.61 and 6.61, respectively). In the second experiment, the effect of yeast (one-dose or two-doses) was tested while oxygen and distilled water were injected into the flasks every 3 hours using lucerne and maize silage. Yeast did not have any significant effect on NDFd, kd, OMd and DMd, nor did it interacted with forage, oxygen or time. Oxygen stress, however, reduced NDFd, VFA concentrations as well as rate of digestion of NDFby about 5% while there was no effect seen on the pH estimates. Two-doses of yeast caused a more stable and controlled pH environment over time as there was no significant interaction between pH and two-doses at 12, 24 or 48 h (6.80 vs. 6.82 vs. 7.02, respectively). Lastly, in the third experiment, the effect of yeast (one-dose or two-doses) was tested while having a starch-stressed environment with lucerne and maize silage with certain samples receiving additional wheat-starch. There were no significant interactions between dose and forage, starch or time for NDFd, kd, DMd, OMd or VFA. Forage had a positive interaction with yeast, showing increased pH estimates for yeast compared to the control, although differences were small.

Yeast doses $1x10^6$ and $1x10^7$ cfu/ml showed increased digestibility as well as VFA concentrations demonstrating the yeast's ability to facilitate fibre digestion *in vitro* and by supplying the microorganisms with a better micro-environment, as also seen with the MD & GV media. The two media provided a better environment for fermentation than the KS medium, resulting in higher NDFd, rate of digestion, DMd, OMd, pH estimates as well as VFA concentrations. The pH estimates did not always result in significant differences when different stressors were placed on the *in vitro* environment which may be as a result of the excellent buffering capacity of the medium. The differences that were seen were very small (< 0.1) which may not be biologically important as pH remained within normal ranges for fermentation to occur. The decreases in pH were often accompanied by increases in VFA concentrations which were expected.

This study confirms the negative effect oxygen can have on the anaerobic rumen environment, while also showing how high levels of starch can cause significant decreases in rumen pH values. It also confirms the positive effects yeast has on pH by stabilizing the rumen environment; however, this may also be due to the medium used. More research is needed to study the mode of action of yeast supplements and to determine the optimal oxygen and starch concentrations that yeast can withstand. It is also important that care be taken when evaluating different fermentation parameters when yeast supplements are used, especially when comparing different variables such as media and forages.

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NOTES

The language and style used in this thesis are in accordance with the requirements of the *Journal of Dairy Science.* This thesis represents a compilation of manuscripts, where each chapter is an individual entity and some repetition between chapters is therefore unavoidable.

ABBREVIATIONS

AA	Amino acids
ADF	Acid detergent fibre
ADL	Acid detergent lignin
BCAA	Branch chain amino acids
CMCase	Carboxymethylcellulase
CP	Crude protein
DM	Dry matter
DMd	Dry matter digestibility
DMI	Dry matter intake
EE	Ether extract
GV	Goering-Van soest
iNDF	Indigestible neutral detergent fibre
kd	Rate of digestion
KS	Kansas State
MD	McDougall
ME	Metabolisable energy
NDF	Neutral detergent fibre
NDFd	Neutral detergent fibre digestibility
NDS	Neutral detergent solubles
NSC	Non-structural carbohydrates
OMd	Organic matter digestibility
pdNDF	Potentially digestible neutral detergent fibre
PPLO	Pleuropneumonia-like organisms
SC	Structural carbohydrates
TVFA	Total volatile fatty acids

VFA   Volatile fatty acids

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CHAPTER 1

1.1 GENERAL INTRODUCTION

Feed costs represent the highest expense, up to 50% of total costs, to any dairy cattle farmer (VandeHaar and St-Pierre, 2006) and it is thus of highest importance to lower feed costs while not compromising on feed quality or productivity. The *in vitro* and *in situ* techniques are important systems to determine the quality and characteristics of various forages and grains to enable nutritionists to formulate diets which offer optimal nutritional value to the ruminant without unnecessary wastage through excessive inclusion levels of certain feedstuff. Neutral detergent fibre (NDF) values are a good indicator of forage quality as well as the maturity of the forage (Oba and Allen, 1999). However, they should always be accompanied by NDF digestibility values to better describe the quality characteristics (Getachew *et al.*, 2004), because when comparing forage across species, NDF amount does not represent a good quality predictor alone anymore (Van Soest, 1994).

When it comes to an animal's ability to digest fibre, ruminants are the only animals capable of doing so, but more specifically, the microorganisms that reside in their rumen produce enzymes which ferment and degrade the fibre polymers. The end products of microbial fermentation include volatile fatty acids (VFA), vitamins and microbial protein (Rode, 2000; Russell and Rychlik, 2001). Microorganisms within the rumen need an optimal environment to produce enzymes and digest plant matter, but foremost to survive. The factors that can often affect microbial balance and fermentation include anaerobiosis, temperature, nutritional requirements and, most importantly, pH. Any changes or disruptions to these characteristics can result in an unbalanced rumen environment resulting in poor microbial growth, fermentation and digestion, and ultimately poor production by the ruminant.

In an attempt to prevent any unwanted changes in the rumen and to improve the environment for microbial fermentation and thus production, direct fed microbials (DFM) have been recognized as a safer alternative to antibiotics (Thrune *et al.*, 2009). *Saccharomyces cerevisiae* is the most common yeast species being used as a supplement for high producing dairy cows (Chaucheyras-Durand *et al.*, 2008; Opsi *et al.*, 2012; Thrune *et al.*, 2009). Although the exact mode of action is not completely known, yeast has the ability to stimulate the microorganisms within the rumen

through providing vitamins, nutrients and dicarboxylic acids (Newbold *et al.*, 1998) as well as through the removal of O_2 which enters the rumen via feed or water, etc. from the rumen fluid which may hinder the growth of strict anaerobic bacteria (Auclair, 2001).

In this study we looked at the effects of a yeast supplement, *Saccharomyces cerevisiae* strain CNCM I-1077, on the different *in vitro* fermentation parameters when incubated with different forages and different media as well as when the yeast is subjected to different stressors namely, oxygen and starch stress.

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CHAPTER 2

The rumen micro-environment and its use to measure fibre quality: a review

2.1 INTRODUCTION

Feed costs represent the highest expense, up to 50% of total costs, to any dairy cattle farmer (VandeHaar and St-Pierre, 2006) and it is thus of highest importance to lower feed costs while not compromising on feed quality or productivity. The *in vitro* and *in situ* techniques are routinely used, both experimentally and commercially, to determine quality and characteristics of various forages and grains. These techniques have therefore an invaluable importance since they enable the nutritionist to formulate diets which offer optimal nutritional value to the ruminant without unnecessary wastage through excessive inclusion levels of certain feedstuff. However, there are no standardized procedures for either technique which involve the rumen microorganisms. In *in vitro* systems the media and particle size are often two variables which differ between research papers while for *in situ*, bag pore size and bag material vary. These variables can often lead to inconsistent results through their differing interactions with the microorganisms in the rumen fluid. It would thus be essential to improve a standardized procedure for both techniques and therefore minimize the discrepancies between different studies. This review is aimed at looking at the rumen environment as well as the microorganism requirements and seeing how they are replicated *in vitro* or *in situ*. The review will identify discrepancies and similarities within each system with the main focus being of the *in vitro* technique.

2.2 The rumen environment

The rumen can be considered as one of the largest naturally occurring fermentation chambers with a large and diverse microbial population (Kamra, 2005; Krause *et al.*, 2003; Rode, 2000). The environment within the rumen is anaerobic, consisting of approximately 65% carbon dioxide (CO_2), 27% methane (CH_4), 7% nitrogen (N_2) and 0.2% hydrogen (H_2) with the remaining proportion of gases including carbon monoxide (CO), hydrogen sulphide (H_2S) and oxygen (O_2) (Mizrahi, 2013).The anaerobic environment of the rumen is accompanied by a negative redox potential which reflects the absence of O_2 and strong power of reduction (Marden *et al.*, 2005). The redox potential of the rumen falls in the range of between -185 to -228 mV

(Křížová *et al.*, 2011) which is similar to the range found by Marden *et al.* (2005). The redox potential was shown to fluctuate during the day, especially after feeding where it would increase until around 3 h after feeding and then slowly decrease again thereafter. Marden *et al.* (2005) attributed this fluctuation to the presence of oxygen in the rumen which is brought in via feed and water intake as well as mastication.

The pH of the rumen is maintained relatively constant in a range of 5.5 to 7.0 by means of saliva produced by the ruminant which acts as a buffer within the rumen (Mizrahi, 2013; Rode, 2000). However, when the pH drops below 6.0, the buffering capacity loses its ability to prevent further decreases in pH (Terry *et al.*, 1969) which could ultimately result in either subacute acidosis (pH 5.0 to 5.5) or even acute acidosis (< 5.0) (Nagaraja and Titgemeyer, 2007). Subacute acidosis is related to the accumulation of volatile fatty acids and acute acidosis is due to the accumulation of lactic acid, however, both ultimately stem from a high-concentrate diet (Nagaraja and Titgemeyer, 2007). The maintenance of an anaerobic environment with the presence of CO_2 within the rumen also plays a role in the maintenance of rumen pH. When CO_2 is dissolved within an aqueous medium, such as rumen fluid, a carbonic acid buffer (H_2CO_3) is produced. This buffer is responsible for establishing the correct pH and if CO_2 is allowed to escape from the rumen, it will cause a decrease in the production of H^+ ions and therefore an increase in pH (Marden *et al.*, 2005).

The temperature of the rumen remains relatively constant between 38°C and 40°C (Mizrahi, 2013) due to the animal's homeothermic metabolism (Theodorou and France, 2005). However, the temperature may increase to around 41°C after the animal has eaten (Brod *et al.*, 1982). The increase in rumen temperature can be due to the fermentation process by microbial activity which generates heat (Brod *et al.*, 1982; Theodorou and France, 2005; Tajima *et al.*, 2007). The heat that is produced in the rumen through fermentation activity is dissipated by convection to the body by means of the blood (Beatty *et al.*, 2008). Therefore, rumen temperature will be slightly higher than that of the animal's core temperature which is between 38 and 38.5°C.

2.3 Microorganism requirements

Microorganisms within the rumen need an optimal environment to produce enzymes and digest plant matter, but foremost to survive. Any changes or disruptions to these characteristics can result in an unbalanced rumen environment resulting in poor microbial growth, fermentation and digestion, and ultimately poor production by the ruminant.

The ruminal microorganisms are able to produce enzymes which aid in plant matter digestion (Krause *et al.*, 2003; Russell and Rychlik, 2001), while the ruminant is able to supply the microorganisms with an ideal anaerobic environment. Mammals themselves are unable to digest cellulose, however, the microbes within the rumen produce enzymes that are able to ferment and digest these polymers to produce end products which the ruminant is then able to utilise (Kamra, 2005; Rode, 2000; Russell and Wilson, 1996; Russell and Rychlik, 2001). The end products of microbial fermentation include volatile fatty acids (VFA), vitamins and microbial protein (Rode, 2000; Russell and Rychlik, 2001).This relationship that exists between the microbes and the ruminant is seen as symbiotic as both benefit from this cooperative system (Hungate, 1984; Kamra, 2005; Krause *et al.*, 2003; Russell and Rychlik, 2001).

2.3.1 Anaerobiosis

The majority of the ruminal microbes are anaerobes and thus require a constant anaerobic environment. An anaerobic environment is achieved through the production of gases by the ruminal microbes during the fermentation process such as carbon dioxide, methane and hydrogen. However, O_2 does enter the rumen through the feed and water that is consumed by the animal as well as mastication and thus the rumen microorganisms are also exposed to aerobic conditions (Marden *et al.*, 2005). It is believed that there are anaerobes present in the bacteria population that can tolerate aerobic conditions (Loesche, 1969; Ellis *et al.*, 1989) as well as in the protozoal population (Ellis *et al.*, 1989).

One of the experiments in an early study by Baldwin and Emery (1960) was to look at the effect of O_2 on the stability of the rumen environment *in vitro* and to see if there are any noticeable effects on fermentation. Between the treatments and control, there was no effect on volatile fatty acid or ammonia production, nor were there any

difference in the redox potential or any changes caused to the end products after fermentation. They concluded that the microorganisms within the rumen have the ability to maintain a constant environment and that the facultative anaerobes are capable of utilising highly oxidative compounds whereby maintaining a relatively constant anaerobic environment with a low redox potential (Baldwin and Emery, 1960).

Loesche (1969) performed a study to determine the sensitivity of various anaerobic bacteria to different levels of oxygen using agar plates containing either Brain Heart Infusion supplemented with 10% horse blood and 0.5 µg of menadione per ml or supplemented pleuropneumonia-like organisms (PPLO) medium. Loesche (1969) discovered that anaerobic bacteria have different degrees of sensitivity to molecular oxygen and could be divided into three groups, namely strict anaerobes, moderate anaerobes and microaerophiles. Strict anaerobes generally exhibited little or no growth when subjected to oxygen levels ranging from 0.4% to 0.7% when streaked on the PPLO medium. There were, however, two species (*Clostridium haemolyticum* and *Lachnospira multiparus*) from the strict anaerobes that were able to exhibit growth when oxygen levels were increased to 1% and even as high as 10% on the Brain Heart Infusion medium. The *Treponema* species, on the other hand, were the most sensitive to oxygen showing no growth when the pO_2 was greater than 0.1%. When looking at the moderate anaerobes, the growth of most species was still recorded when the oxygen level reached up to 3% on the PPLO medium. There were, however, differences between strains of certain species such as with *Bacteroides melaninogenicus* strain BEI which showed growth up to 6% pO_2, while strains 9GBK and 1GBK showed minimal growth when pO_2 reached levels greater than 2%. Microaerophiles were concluded as being bacteria which grew best at intermediate levels of oxygen. These species included *Vibrio sputorum* and *Vibrio fetus* with growth being observed at oxygen levels of 5 to 10% for *Vibrio sputorum*and and even higher for *Vibrio fetus.* Of all the bacteria testing, the ones being found in the rumen were *Selenomonas ruminatium, Butyrivibrio fibrisolvens, Succinivibrio dextrinosolvens and Lachnospira multiparus.* These bacteria are regarded as being strict anaerobes, however, interestingly *Lachnospira multiparus*was found to exhibit growth in the presence of increased oxygen.

Methanogens are known to be strict anaerobes and therefore oxygen that is present in the rumen must be utilised by other microorganisms. Williams (1986) reported that up to 7% of ruminal O_2 is utilised by holotrich protozoa which have been reported to have a high affinity for O_2 (Hillman *et al.,* 1985). Ellis *et al.* (1989) performed a study to investigate the effects of O_2 on the respiratory activities of mixed ruminal organisms, protozoa and bacteria. During the experiment, dissolved O_2 concentrations were increased from 0 to 51 µM in 5% intervals lasting 15 min. When oxygen concentrations were increased above the normal levels that occur in the rumen (<0.25 to 3 µM), all three populations were unable to utilise and consume O_2 with total inhibition for each population occurring when the concentration exceeded 7 µM. Table 2.1 shows that both protozoal and bacterial populations had similar rates for O_2 utilisation when the O_2 concentration was at the same concentration as that of the lower detected level of O_2 in the rumen (0.25 µM) as well as the higher concentration reached directly prior to feeding (1 to 1.5 µM).

Table 2.1. Contributions to ruminal O_2 consumption made by protozoa and bacteria[a] (Adapted from Ellis et al. (1989))

Ruminal population	Total respiratory activity (μM O_2/min per ml)[b]	O_2 recovered (%)
Mixed organisms	4.40	100
Protozoa	3.24	73.6
Bacteria	3.43	77.9

[a]Measurements taken at 1 µM O_2
[b]Values were recalculated from the diluted samples in order to represent an undiluted ruminal population

2.3.2 pH

The rumen is buffered at a pH of 5.7 to 7.3 by means of phosphate and bicarbonate that are present in the saliva produced by the ruminant through chewing activity, as well as by bicarbonate produced through the fermentation process in the rumen

(McDougall, 1948a; Rode, 2000). The diet of ruminants plays a large role in the pH of the rumen or the maintenance thereof, with pH being the major factor affecting fibre digestion (Hoover, 1986). The amount of saliva produced is correlated to the diet composition such as forage type and the amount of forage to concentrates in the diet as well as factors including rumination time, digestibility of the diet and rate of digestion (Mirzaei-Aghsaghali *et al.*, 2011).

Not only does the composition of the diet and feeding frequency affect the ruminal pH, diet also has a direct effect on the microbial ecosystem through the change in pH. Tajima *et al.* (2001) confirmed the shift in the microbial ecosystem from cellulolytic bacteria to more amylolytic bacteria as pH decreased due to a shift from a forage diet to grain diet. They found a 10- and 20- fold drop in *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*, respectively when the diet of a ruminant was changed from hay to grain while the number of amylolytic bacteria increased. *Streptococcus bovis* showed an increase of as much as 67- fold with the change in diet (Tajima *et al.*, 2001).

In a study by Franzolin and Dehority (2010), the effect that pH has on the presence of protozoa in the rumen of steers fed four different treatment diets over 17 weeks with two different levels of feed intake was researched. It was found that steers that became defaunated had a lower ruminal pH than faunated steers but that both faunated and defaunated steers had the highest pH before feeding and lowest 4 hours after feeding. The lower pH in defaunated steers could be attributed to the fact that protozoa engulf larger starch granules, via amylolytic bacteria, found in high concentrate diets, resulting in a decrease in the production of organic acids (Santra *et al.,* 2007). During the first 13 weeks steers were receiving a lower feed intake (4.6 kg DM/animal per day) and during the last 4 weeks intake was increased to 7.3kg DM/animal per day. In three of the four steers used, more defaunation was observed with the higher feed intake thus resulting in the conclusion that the daily intake has a larger effect than the type of diet being fed in determining the protozoal population (Franzolin and Dehority, 2010). With an increase in daily feed intake, bacterial fermentation was increased which results in a decrease in pH due to the increased production of organic acids (i.e. volatile fatty acids).

Although it has been reported that the rumen can reach a pH of 5.7, when the pH drops below 6.0 the digestion of fibre by bacteria is severely impaired (Rode, 2000).

This is due to the fact that the enzymes responsible for breaking down fibre polymers are unable to do so effectively when the pH is less than 6.0 and the bacteria responsible for these enzymes are unable to grow themselves (Rode, 2000). Hoover (1986) previously found that even when the pH is around 6.0 there was indeed a slight decrease in fibre digestion, however, the number of fibrolytic bacteria remained the same. It was only when pH dropped below 6.0 to between 5.0 and 5.5, that there was hindered growth rates as well as a decreased in the number of fibrolytic bacteria. Shiver *et al.* (1986) also found a decrease in neutral detergent fibre (NDF) digestibility by 33.1% to 8.1% as pH decreased from 6.2 to 5.8. This could be explained by the decrease in the number of bacteria tightly attached to the plant cell wall thus resulting in no obvious fibre digestion (Cheng *et al.*, 1984; Shriver *et al.*, 1986), however, the exact mechanism behind this is unknown. The decrease in protein and ammonia at these lower pH levels may also further contribute to the decrease in NDF digestibility as cellulolytic microbes growth may be hindered. However, Shiver and collaborators (1986) did not find a significant increase in organic matter or fibre digestion once the pH was increased to 6.2.

2.3.3 Temperature

Due to the ruminant's homeothermic metabolism being able to maintain the rumen temperature relatively constant (Theodorou and France, 2005), it might come as no surprise that Beatty *et al.* (2008) found that even with an increase in environmental temperature, the rumen- and core temperature remained relatively constant and with no effect on the difference between the two temperatures. The same was observed in muskoxen which were subjected to environmental temperatures as low as -40.9°C yet were able to maintain a rumen temperature average of +38.8°C (Crater and Barboza, 2007).

With the maintenance of rumen temperature being observed with various environmental temperatures, it was expected that Tajima *et al.* (2007) found no significant differences in the microbial population numbers when Holstein heifers were subjected to environmental temperatures of 20°C, 28°C and 33°C. Their findings also suggested that environmental temperature and humidity did not affect the general bacterial molecular diversity in the rumen, however, the bacterial composition

in the rumen was significantly affected (Tajima *et al.*, 2007). Heifers of 430 kg showed significant differences in bacterial composition between environmental temperatures of 20 vs. 33°C and 28 vs. 33°C. This could be attributed to the decrease in dry matter intake (DMI) at higher temperature levels which resulted in a decrease in VFA. The relationship between increased temperatures with decreased feed intake has been studied a number of times (Beatty *et al.*, 2008; Crater and Barboza, 2007; Tajima *et al.*, 2007). This relationship represents a mechanism to maintain homeostasis by decreasing microbial fermentation in the rumen, therefore decreasing the rumen temperature and subsequent core temperature of the cattle.

Unlike the previous studies mentioned, Roger *et al.* (1990) looked at the effect of temperature *in vitro* instead of *in vivo* on two species of dominant cellulolytic bacteria, *Ruminococcus flavefaciens* strain 007 and *Fibrobacter succinogenes* strain S85. Cultures of *R. flavefaciens* were prepared and left for 10 min at the desired temperature after which cellulose was added, the tubes shaken for 1 min then immediately centrifuged. *F. succinogenes* was prepared in a similar manner but left for 24 min and were not centrifuged. The adhesion of the bacteria was then determined and compared to a control which was untreated. While adhesion of both species was optimal at 38°C, *F. succinogenes* was most affected by the changes in temperature showing variation across all temperatures with the slowest adhesion being seen at 48°C with only 39% of bacteria being attached (Table 2.2). Interestingly, *R. flavefaciens* appeared to be unaffected by the changes in temperature even when exposed to 100°C, still showing 85% attachment of bacteria to cellulose (Roger *et al.*, 1990).

Table 2.2. Effect of temperature on adhesion of *F. succinogenes* S85 and *R. flavefaciens* 007 to cellulose Avicel (Adapted from Roger *et al.* (1990))

Temp (°C)	% of adherent cells[a]	
	R. flavefaciens	*F. succinogenes*
4	99 ± 7	71 ± 3
13	98 ± 5	81 ± 2
20	98 ± 7	84 ± 5
30	100	96 ± 3
42	100	98 ± 3
43	ND[b]	85 ± 6
44	ND	77 ± 3
46	ND	83 ± 6
48	ND	39 ± 4
52	93 ± 3	ND

[a]Percentage of adherent cells compared with a control tube at 38°C (average of four replicates ± standard deviation).
[b]ND, Not determined.

2.3.4 Nutritional requirements

Carbohydrates and proteins (i.e. nitrogen) are the most important nutrients required by rumen microorganisms, for microbial growth (Belanche *et al.*, 2012). The use of protein (amino acids and peptides) is dependent on the carbohydrates available, as carbohydrates in the ruminant's diet serves as an energy source to the microbial ecosystem (Bach *et al.*, 2005; Hackmann and Firkins, 2015). Amino acids (AA) can be transaminated or used directly for microbial protein synthesis if energy is available or, if energy is limited, AA will be deaminated (Bach *et al.*, 2005).

Rumen microorganisms obtain nitrogen (N) from five main sources, namely rumen degradable protein, microbial protein, peptides, AA and ammonia, with each

microorganism species having a preferred source (Belanche *et al.*, 2012). The CNCPS system divides the rumen microbial system into two groups depending on their fermentation characteristics; either non-structural carbohydrate fermenters (NSC) or structural carbohydrate fermenters (SC) (Russell *et al.*, 1992). NSC ferment starch and sugars and require peptides and AA as their source of N for optimal growth, while SC ferment only cell wall and require only ammonia which can also be produced by the NSC microbes (Russell *et al.*, 1992).

Certain AA are crucial to the growth of several bacteria (Wang *et al.*, 2008), as was confirmed in an earlier study by Kajikawa *et al.* (2002). Looking at the effects of several AA on the growth rates and efficiency of mixed ruminal bacteria, Kajikawa *et al.* (2002) found that by adding a mixture of 20 AA to an *in vitro* incubation, both growth rates and efficiency were significantly improved by 46% and 15%, respectively. They found that seven specific AA were crucial for bacterial growth as when each one of the seven AA were removed from the original 20 AA supplement, bacterial growth declined. These seven AA included Leu, Trp, Tyr, Gln, Met, Phe and Val (Kajikawa *et al.*, 2002) which partially agreed with the findings by Wang *et al.* (2008) who concluded in their study that branch chain amino acids (BCAA) (Leu, Ile and Val) as well as Met are essential for microbial growth. Wang *et al.* (2008), interestingly, also looked at the effects of AA removal on the protozoa protein to bacteria protein ratio in a culture medium and found that Lys-removal had a significantly negative effect on the protozoa. They found that the ratio was the highest when BCAA were removed and the lowest when Lys was removed, therefore concluding that BCAA could be a limiting growth factor for bacteria while Lys could be a limiting growth factor for protozoa.

Belanche *et al.* (2012) investigated the effects of feeding 110 vs. 80% (0.44 kg/d vs. 0.34 kg/d) N requirement to cows as well as the type of carbohydrate (fibre rich or starch rich) on the changes in the rumen microbial population. They found that the biodiversity and concentrations of microorganisms in the rumen, i.e. bacteria, anaerobic fungi, methanogens and protozoa, were higher for cows being fed a fibre rich diet versus the starch-rich diet. They postulated that this was because of the wider range of carbohydrate sources supplied by the fibre rich diet. When looking at high or low protein diets, the concentrations of bacteria, anaerobic fungi, protozoa and methanogens show a noticeable decline in the concentrations with the lower

protein diet being fed. This decline also leads to a decrease in the biodiversity of fungi and bacteria. Overall, microorganism concentrations were higher in the high protein, fibre rich diets with increased ruminal pH, ammonia concentrations and acetate to propionate ratio.

When looking at vitamins and minerals, deficiencies in minerals within ruminant diets can often lead to a decrease in feed intake which is believed to be partially due to the reduced or hindered activity of rumen microbes. This lead to the concept that microbial requirements should not be evaluated according to the total dry matter content but rather in terms of fermentable energy as mineral requirements of microbes should be equal to the amount of energy available for fermentation and digestion (Durand and Komisarczuk, 1988).

In earlier research, Hubbert et $al.$ (1958) looked at the optimal and toxic level of certain minerals on cellulose digestion in $vitro$. In two of their trials looking at the addition of sulphur (S) to fermentation liquid, they found that when adding 10 to 500 and 10 to 600 µg of S per ml fluid for trial one and two, respectively, S had the potential of increasing in $vitro$ cellulose digestion by 7.8%. The addition of S above 1000 µg/ml tended to have a negative effect but, it was noted that cellulose digestion still took place which could indicate the microorganisms' tolerance to high S levels. This could not be said for the addition of magnesium (Mg) added at a level of 1000 µg/ml which prohibited cellulose digestion completely, indicating a toxic effect on rumen microorganisms. However, Mg added to fermentation fluid between 20 to 80 µg/ml in one trial and between 20 to 160 µg/ml in another trial showed 15-25% increased cellulose digestion when being compared to a control. Although Hubbert et $al.$ (1958) found a positive response in cellulose digestion of around 10% when calcium (Ca) was added in $vitro$, in a later study it was described that microbes do not have a skeletal structure, therefore do not require Ca and phosphorus (P) for bone formation, although they are needed for cellular metabolism (Van Soest, 1994)

Ruminants have a specific requirement for vitamin B_{12}, which is converted from cobalt by microorganisms in the rumen. Therefore, microbes have a high requirement for cobalt from the ruminant diet (Hubbert et $al.,$ 1958; Van Soest, 1994). Although cobalt is needed by microorganisms for the synthesis of vitamin $B_{12,}$ it does not seem to have an apparent effect on cellulose digestion as found by Hubbert et $al.$ (1958).

As little as 5 µg/ml fermentation fluid was shown to decrease cellulose digestion by 27.8%, when compared to a control receiving no cobalt.

2.4 Fibre degradation

Ruminants themselves are unable to break down and degrade fibre as they do not produce fibrolytic enzymes. Thus, they rely on the microorganisms in their rumen which attach themselves to the feed particles and secrete the necessary enzymes which are capable of fermenting the cell-wall polysaccharides (Dado and Allen, 1996). There are three dominant cellulolytic bacteria in the rumen, namely *Ruminococcus flavefaciens, Fibrobacter succinogenes,* and *Ruminococcus albus* (Miron *et al.*, 2001; Weimer, 1996).

The initial phases of fibre digestion by cellulolytic bacteria involve the transport to the feed particles (Miron *et al.*, 2001) followed by bacterial attachment to the outer surface (McAllister *et al.*, 1994a). The bacterial glycocalyx found on the outer surface of the dominant cellulolytic bacteria (mentioned above) is essential for the initial attachment to feed particles (Demeyer, 1981) and protects the bacteria from protozoa predation and cellulolytic enzymes (Weimer, 1996). Most plants have a protective surface layer which makes them resistant to bacterial attachment and therefore most bacteria gain attachment through damaged sites which could be caused by mastication or mechanical processes such as milling or grinding (Beauchemin *et al.*, 1994; McAllister *et al.*, 1994b), or through natural openings (e.g. stomata). Competition for binding sites is reduced between bacteria as it has been found that different bacterial species have different preferences for binding and colonization (Dinsdale *et al.*, 1978) and they bind to feed particles which they are most capable of digesting (McAllister *et al.*, 1994b). Dinsdale *et al.* (1978) found that ruminococci had a preference for digesting grasses while rod-like organisms such as *F.succinogenes* preferred cell walls and cotton fibres. The time it takes for the bacteria to ultimately bind to the feed particles and the time it takes them to digest the feed is linked to the extent and rate of digestion as well as lag time, and is determined by the fibres chemical characteristics (McAllister *et al.*, 1994b). Not only are there substrate related issues to bacterial adhesion but there are also environmental factors within the rumen that can alter or inhibit the attachment to the

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surface of feed particles. These factors include temperature, pH and the presence of cations (Miron *et al.,* 2001).

The rumen pH is regarded as one of the most significant factors affecting fibre digestion as most cellulolytic bacteria are known to be sensitive to changes in pH (Sung *et al.*, 2007). When observing the attachment of the main cellulolytic bacteria to rice straw under an initial low (5.7), middle (6.2) and high (6.7) pH, Sung *et al.* (2007) found that the lowest number of attached bacteria, for all three species, was observed at the low pH. There were no significant differences (P > 0.05) seen between the middle and high pH values. The lower number of attached bacteria at the low pH also corresponded to the low dry matter digestibility (DMd). There was a significant increase in DMd as the initial incubation pH was increased from 5.7 to 6.2 and 6.7 along with an increase in DMd, gas production as well as VFA production also increased and Sung *et al.* (2007) attributed this to the increased number of attached bacteria with an increased initial pH.

Roger *et al.* (1990) studied the effects of pH on cellulose attachment by *R. flavefaciens 007* and *F. succinogenes S85. R. flavefaciens 007* was studied in a pH range of 3.5 to 9.0 while *F. succinogenes S85* in a range of 4.5 to 7.0. Unlike Sung *et al.* (2007), they found that *R. flavefaciens* remained stable at pH values as low as 3.5 and remained stable as pH increased to 7.5. Only when pH increased to 8.0 and above, did they see a drop in the number of attached cells to 40%. Similar trends for *F. succinogenes* were seen between the two studies. Roger *et al.* (1990) found that *F. succinogenes* became more stable as pH increased from 4.5 to 6.0 with 80% of attached cells been seen at a pH of 6.0. The cells remained relatively constant between pH 6.0 and 7.0 but decreased in attachment when pH reached 7.5. The few discrepancies between the two studies may be attributed to the differences in cultures with Sung *et al.* (2007) using a mixed culture while Roger *et al.* (1990) studied pure cultures.

2.5 Techniques to measure rumen fibre degradation

The nutrient availability of various feedstuff is essential for the formulation of diets to ensure diets are formulated to fulfil the animal's nutritional requirements for production, maintenance etc. The nutrients available from different feeds are thus

determined through various techniques such as *in vitro*, *in situ* and *in vivo* (Ali *et al.*, 2014) and the results analysed. An estimate of the nutritional values of feedstuff for livestock, such as roughages, is routinely determined more often through *in vitro* or *in situ* fermentations. These (Hall, 2015)can be used to determine the neutral detergent fibre digestibility (NDFd), amongst other parameters, of forage and non-forage fibre sources.

2.5.1 *In situ* technique

The *in situ* technique involves the use of material bags made of varying material, namely either nylon or polyester, commonly containing between 1 to 2 g of sample and being incubated within the rumen via a rumen cannula (Krizsan *et al.*, 2013; Lindberg and Knutsson, 1981), although discrepancies do occur between various authors as the method has not been standardized (Huhtanen *et al.*, 1998).

The *in situ* method has both strengths and weaknesses. One weakness being that the results can be negatively affected by the rumen environment of the host animal, resulting from the diet for example (Cherney *et al.*, 1993) as well as by the procedure such as bag material, pore size, sample size, etc. (Vanzant *et al.*, 1998; Ørskov *et al.*, 1980), whereby hindering the method's ability to estimate potential NDF degradation for forages and feedstuff. On the other hand, feed within rumen is undergoing typical and natural degradation which allows one to estimate actual NDFd (Bossen *et al.*, 2008), however, this could be argued by an earlier study which believes that particles do not undergo complete degradation as material is simply broken down to a particle size small enough to leave the nylon bag and that caution should be taken when analysing the results (Ørskov *et al.*, 1980).

Huhtanen *et al.* (1998) also agreed with the statement that caution should be taken when analysing results for feed evaluation when using the *in situ* technique, but their remark was based on the lower microbial activity and enzymes within the nylon bag. They concluded that enzymatic activity from microbes within the bag does not correspond with the enzymatic activity of the rumen ingesta. Carboxymethylcellulase (CMCase) and xylanase activities were measured in the rumen ingesta and showed an activity of 12.6 and 39.6 µmol/g DM per min, respectively. However, when compared to the activity within the *in situ* bag, the highest activity achieved was only

7.4 and 29.14 µmol/g DM per min for a bag with a pore size:free surface area (µm:%) of 200:45 and 7.87 and 28.86 µmol/g DM per min for the bag with a ratio of 41:33. When a modelling approach was applied to attempt cancelling out any discrepancies with regards to feed particles originating from different meals in the rumen ingesta compared to the feed sample within the nylon bag, it was still very clear that enzymatic activity within the bag was considerably lower. These findings agreed with findings on lower enzymatic activity and bacterial count by Meyer and Makie (1986).

In a recent study by Krizsan *et al.* (2015), they compared the in situ indigestible neutral detergent fibre (iNDF) values of samples with different grind sizes (0.8 mm vs. 2 mm), using in situ polyester bags with different pore sizes (6 µm, 11 µm, 12 µm and 17 µm) as well as different characteristics such as manufacturer. The bags were incubated for 288 h in three different cows using ten different samples. The bags with pore size 12 µm were obtained from Saatitech S.p.A., (Veniano, Como, Italy) and made from Saatifil PES 12/6 cloth whereas all other bags were 07-6/5, 07-11/5 and 07-17/9 Sefar Petex cloths (Sefar AG, Heiden, Switzerland).

When comparing the samples and bag type, bags with the pore size of 12 µm had significantly higher ($P < 0.01$) values for iNDF for all but three of the samples analysed. When looking within the Sefar Petex bags, barley straw had a significantly lower ($P < 0.01$) iNDF values for the 17 µm when compared to the bags with a pore size of 6 µm and 11 µm (263 g/kg DM vs. 291 g/kg DM and 274 g/kg DM for sample size 0.8mm; 275 g/kg DM vs. 284 g/kg DM and 291 g/kg DM for sample sizes 2 mm). In the experiment, only faecal samples, lucerne and oat hay showed significant differences ($P < 0.01$) in iNDF values between the two different grind sizes within each sample, however, only the lucerne sample was significant when looking at all feed samples and grinding size when incubated in the 6 µm polyester bags. In all significant samples concerned, samples milled to 2 mm resulted in a higher concentration of iNDF compared to samples at 0.8 mm. It was recommended that bags with the smaller pore sizes, namely 6 µm and 11 µm, should be used with samples ground at 2 mm to avoid any particle losses or reduced microbial activity (Krizsan *et al.*, 2015).

Damiran *et al.* (2008) compared the sample grind size (1 mm vs. 2 mm) and sample size (0.25 g vs. 0.5 g) on the effects of DMd and NDFd in different techniques including *in situ*. They found higher digestibility values ($P < 0.01$) for both the grass

hay and grass straw samples ground to 1 mm compared to 2 mm for DM and NDF. Interestingly, they also found higher digestibility values when sample size was reduced from 0.5 g to 0.25 g. They concluded by stating that the *in vivo* digestibility values obtained via the *in situ* technique were more accurate than the *in vitro* techniques and DAISY[II] (Ankom[®] Technology Corp. Fairport, NY, USA) technique (Damiran *et al.*, 2008). Differences in digestibility values could be attributed to bag pore size as the second study did not analyse this.

Looking at a study by Valente *et al.* (2011) whose main objective was to compare DMd and NDFd *in situ* using three different bag textiles, found that using nylon material (50 µm) resulted in higher lag and degradation estimates for DM and NDF for a variety of forages (*P*<0.05), compared to non-woven textile (NWT – 100 g/m^2) followed by the commonly used F57 bags (Ankom[®]). Although higher estimates were observed for nylon when looking at the potentially degradable fraction of NDF, nylon presented the lower estimates for the undegradable fraction of NDF while no differences were observed for either fraction of NDF for NWT and F57 (*P* > 0.05). The lower potentially degradable fraction of NDF but the higher estimates for DM were attributed to the possibility of nylon having a broader porosity resulting in a greater loss of particles from the bag within the rumen (Valente *et al.*, 2011a), thus affecting the estimates. The porosity of NWT and F57, however, is irregular being due to the fact the fibres of the material are not woven resulting in a more narrow porosity (Valente *et al.*, 2011b). When the porosity of bags becomes too thin, this can prevent the gases from within the bag from escaping which can inhibit the microorganisms from reaching the forage or substrate (Uden and Van Soest, 1984).

2.5.2 *In vitro* technique

The *in vitro* digestibility technique is a common technique used to determine forage digestibility, as well as factors that may affect digestibility (Ferreira and Mertens, 2005), without having to make use of live animals. The technique serves as a way to mimic the rumen, making the *in vitro* digestibility technique more cost effective, less time-consuming and less laborious. Continuous culture is an alternative to batch culture *in vitros*. It is more representative of the rumen as it is able to perform many of the typical functions as seen in the rumen such as the removal of end-products,

the continuous flow of buffer medium as well as the addition of substrate (Stewart *et al.*, 1961). They allow for the opportunity to change substrate content as well as physical characteristics of the substrate such as particle size without affecting other variables (e.g. pH, dilution rates, etc.) (Rodríguez-Prado *et al.*, 2004). Continuous culture systems are, however, mainly used in research labs and are less likely to be found in commercial laboratories as they are expensive as well as difficult to maintain.

During the *in vitro* fermentation technique, the presence of an incubation medium is required. The purpose of this medium is to ensure suitable conditions for the fermentation process and to maintain these conditions within the environment (Mould *et al.*, 2005). The incubation medium supplies the necessary nutrients and has a buffering capability. The presence of a buffer is required to subsequently perform the same buffering capability as that of saliva that would be found in a ruminant. Three incubation media used include the McDougall (McDougall, 1948), Goering and Van Soest (Goering and Van Soest, 1970) as well as the Kansas State (Marten and Barnes, 1980). All three media make use of a phosphate:carbonate buffer but quantities vary between media, as with their microminerals, Mg and Ca. Interestingly, only Goering and Van Soest (1970) supplemented with additional N in the form of ammonium carbonate. One possible reason being for the exclusion of additional N from the media is that an adequate supply of N would be present in the rumen liquor and incubated substrate (Mould *et al.*, 2005). Sulphur is another mineral that is present in most incubation media often in the form of magnesium sulphate or sodium sulphide, however, to avoid excess inclusions of S, Na_2S (sodium sulphide) can be omitted or magnesium sulphate replaced by magnesium chloride as seen with McDougall (1948). The omission of Na_2S has been recommended as it can be detrimental to microbial growth through either toxic intermediaries or precipitation of metal ions (Fukushima *et al.*, 2003).

McDougall (1948) based the composition of his incubation media on the composition and output of sheep saliva (Table 2.3). He was able to determine that a large amount of carbon dioxide, bicarbonate and phosphate were present in the saliva, making the combination of ions an ideal buffer. Using the information gained from the study, McDougall (1948) was able to suggest a formula for synthetic saliva that could be used as a buffer to better simulate the conditions within the rumen.

Table 2.3. Suggested composition of synthetic saliva[1]

Salt	mmol/L	g/L
$NaHCO_3$	117.0	9.80
$Na_2HPO_4.12H_2O$	26.0	9.30
NaCl	8.0	0.47
KCl	8.0	0.57
$CaCl_2$ anhydrous	0.2	0.04
$MgCl_2$ anhydrous	0.3	0.06

[1]Adapted from McDougall (1948).

2.5.2.1 Gas production vs. residual measurement

The procedure described by Tilley and Terry (1963) has been widely used to determine organic matter digestibility (OMd) (Cone *et al.*, 1999; Jančík *et al.*, 2011) as well as the nutritional quality of various feeds (Getachew *et al.*, 2004), and uses the residual matter at each end-point of fermentation (Cone *et al.*, 1999) to calculate dry matter loss. This technique, however, is not well suited to determine fermentation kinetics (Cone *et al.*, 1999) which is why the gas production procedure described by Menke *et al.* (1979) has become a popular technique to obtain information with regards to the extent and rate of digestion (k_d) (Jančík *et al.*, 2011) of both the soluble and insoluble fraction of forages and feeds (Cone *et al.*, 1999; Menke and Steingass, 1988). Knowledge about the digestibility of both the soluble and insoluble fractions of feed is of importance when formulating diets for high producing animals (Calabrò *et al.*, 2002).

Doane *et al.* (1997) investigated the relationship that exists between NDF disappearance, gas production as well as VFA production *in vitro* using six forages. They incubated forage samples that were unfractionated as well as the isolated NDF from each forage. They compared the rate of digestion between the three variables using a one-pool logistic model. Interestingly, they found similar rates of digestion for

NDF, gas and VFA production. This was surprising because both gas and VFA production measure the combined digestion of NDF as well as neutral detergent solubles (NDS) and it would therefore be expected for them to have a higher rate of digestion compared to NDF, which only measured the rate of NDF disappearance (Doane *et al.*, 1997). Immature lucerne had a consistently higher rate of digestion between all forages while wheat straw had the lowest rate of digestion. When compared against a linear regression, the mean slope for gas produced (ml) per mg NDF digested was 0.350 which was similar to the results later found by Calabrò *et al.* (2002). They too compared the rate of digestion between gas and VFA production and NDF disappearance using three forages and obtained a mean slope of 0.41 for gas produced (ml) per mg NDF digested. Unfractionated lucerne always had the highest rate of digestion when looking at gas production, followed by maize silage and ryegrass + oat hay. This order changed however when looking at the NDF of samples and the NDF digestibility rates and gas production. Maize silage had the highest rate followed by lucerne and ryegrass + oat hay. Unlike Doane *et al.* (1997) who attributed the similar rates of digestion for NDF and NDS to the low lignin content of the forages, Calabrò *et al.* (2002) found that lucerne had a slower rate of digestion when looking at the isolated NDF disappearance rate because of the higher levels of lignin content compared to the two other forages.

Both authors found the rate of digestion for NDF disappearance and gas production comparable and that the assumptions around using the gas production technique to determine the rate of digestion to be correct.

2.5.2.2 Rumen inoculum vs. faecal inoculum

The use of rumen fluid as an inoculum for *in vitro* digestibility techniques requires the use of cannulated animals which present a few problems including maintenance expenses and availability (El-Meadaway *et al.*, 1998) as well as ethical issues (Mauricio *et al.*, 2001; Posada *et al.*, 2012). Therefore, the idea of using fresh faeces as an alternative inoculum for *in vitro* techniques would be advantageous as there would no longer be a need for cannulated animals (El-Meadaway *et al.*, 1998; Hughes *et al.*, 2012; Ramin *et al.*, 2015). Limitations for the use of faecal inoculum have, however, already been identified by several authors such as lower enzymatic

activity (Mauricio *et al.*, 2001). To increase the number of microorganisms present in the faeces and to therefore increase the enzymatic activity and forage digestibility, it has been suggested that the ratio of liquor to sample be increased as well as the incubation time (Omed *et al.*, 2000).

Mauricio *et al.* (2001) compared bovine rumen fluid and bovine faeces as an inoculum for an *in vitro* gas production technique for evaluating different forages. They found a high correlation between both the OMd results using rumen fluid and faeces (r^2 = 0.89 and r^2= 0.77, respectively) as the inoculum when compared to the known *in vivo* OMd values of the forages used. Although the use of rumen fluid resulted in slightly higher OMd, when statistical analysis was applied, the digestibility of the forages used were ranked similarly for both rumen fluid and faeces. The lag phase, however, was somewhat longer for the faecal inoculum compared to the rumen inoculum and this was attributed to microorganisms present as was also found in the study by *Posada et al.* (2012). Both teams of authors attributed this to the fact that fermentation in the caecum/colon is lower than in the rumen and high energy feeds are mainly digested within the rumen, therefore energy available for microbial growth in the caecum and colon is limited (Mauricio *et al.*, 2001).

Although similar rankings of forages were observed for Mauricio *et al.* (2001), Hughes *et al.* (1998) found that fibrous feeds were digested better by faecal microorganisms compared to rumen microorganisms, therefore incorrectly ranking feedstuff as an overestimation of *in vitro* OMd can be made when using faecal inoculum. The concept of using different ratios of liquor to sample was previously tested by Hughes *et al.* (1998) who found that using 450 g bovine faeces per one litre buffer increased OMd significantly (P < 0.05) for most forages when compared to 250, 300, 350 and 400 g. Using 450 g faeces showed comparable potential potency to rumen fluid inoculum for digesting forage substrates, with even higher digestibility values for sorghum seed and African star grass (4.5% and 9.2%, respectively). Unfortunately, this was not seen in the study by El-Meadaway *et al.* (1998) who also tested different inclusion percentages of faeces as inoculum. They found non-significant differences (P > 0.05) between rumen fluid and faeces inoculum containing 3% fresh faeces but significantly lower *in vitro* DMd values for faeces inoculum containing 6% and 9% fresh faeces compared to rumen fluid.

Overall, all studies concluded that faecal inoculum does have the potential to replace rumen fluid in *in vitro* experiments but further research is required to address certain downfalls (Cutrignelli *et al.*, 2007; El-Meadaway *et al.*, 1998; Mauricio *et al.*, 2001).

2.5.3 Use of fibre degradation measurements

Values obtained from in vitro or in situ techniques are used to describe the truly digestible nutrient and metabolisable energy (ME) content of feed (NRC, 2001) or to rank feeds in terms of nutrients availability.

The fibre degradation measurements are often used to determine the rate and extent of digestion (Sampaio *et al.*, 2011; Meyer and Mackie, 1986) with the *in situ* technique often being used and referred to as the reference technique for degradation parameters of feed stuff within the rumen (De Boever *et al.*, 2002). Long term fermentation estimates (216 h) have also been used to determine the rate of digestion (kd) for various feedstuffs as well as for the estimation of potentially digestible NDF (pdNDF) (Raffrenato *et al.*, 2009).

Neutral detergent fibre values are a good indicator of forage quality as well as the maturity of the forage (Oba and Allen, 1999). However, they should always be accompanied by NDFd values to better describe the quality characteristics of various feeds (Getachew *et al.*, 2004), because when comparing forage across species NDF amount does not represent a good quality predictor alone anymore (Van Soest, 1994). Furthermore, fibre has often been associated with rumen fill (Ruiz *et al.*, 1995) therefore resulting in a decrease in dry matter intake which is not always ideal in a production system (Oba and Allen, 1999). It is therefore important to put more emphasis on the digestible fraction of NDF when formulating a diet, as with increased digestibility there may be an increase in the clearance of fibre from the rumen resulting in less rumen fill and allowing for more DMI (Dado and Allen, 1996).

2.6 Factors affecting *in vitro* fibre degradation

2.6.1 pH and starch

Carbohydrates such as starch are fermented to yield VFA as end products which subsequently results in a decrease in rumen pH. Sveinbjörnsson *et al.* (2006) looked at the effects of different proportions of NDF and starch on *in vitro* fermentation. Two

types and levels of starch were used, namely raw and cooked potato starch at 300 or 600 g/kg DM as well as two types of forages, timothy hay and meadow hay. They found that only starch ratio had an effect on NDFd and not starch type, with a greater effect upon NDFd being seen with meadow hay (decrease from 0.69 to 0.45) than with timothy hay (decrease from 0.39 to 0.25).

Unlike with NDFd, pH was only affected by starch type with an average pH of 6.07 being measured for cooked potato starch and 6.19 for raw potato starch, across all treatments (P < 0.05). When looking at the effect of starch level upon pH, opposite effects were seen when increasing the starch level from 300 to 600 g/kg DM. Cooked starch showed a lowered average pH from 6.19 to 5.95 while raw starch increased the pH from 6.15 to 6.23, however, the starch level (300 vs. 600 g/kg DM) was non-significant.

It is known that fibre fermentation is reduced with a decrease in pH, mainly due to the hindered ability of cellulolytic bacteria to ferment fibre at low pH levels (Niwińska, 2012). However, even though pH was not affected by the starch level, there was a strong negative effect of starch level on NDFd (Sveinbjörnsson *et al.*, 2006). This has been previously described as a "carbohydrate effect" as opposed to a pH effect (Mould *et al.*, 1983), probably due to a higher use of limiting nutrients by amylolytic bacteria.

2.6.2 Particle size

Dehority and Johnson (1961) looked into the possibility of increasing cellulose digestion *in vitro* for mature forages by ball-milling for different lengths of time to reduce the particle size. They found a positive relationship between cellulose digestion and the length of ball-milling and therefore reduced particle size. Cellulose digestion increased until 30 hrs, after which digestion plateaued with the height of the plateau increasing with the increased duration of ball-milling. The study did not, however, mention the exact size of the particles used neither the exact duration of ball-milling in minutes.

Similar results with regards to reduced particle size were found in studies by both Damiran *et al.* (2008) and Ferreira and Mertens (2005). Damiran *et al.* (2008) compared the Wiley mill grind size (1 mm and 2 mm) and different digestibility

techniques using various forage species. Grass straw and grass hay milled at 1 mm resulted in higher DM digestibility values for all techniques used, namely; *in vitro*, DAISY (Ankom® Technology Corp. Fairport, NY, USA) and *in situ*, when compared to samples milled at 2 mm. The 1 mm particle size also resulted in slightly higher *in vitro* DM and NDF disappearance when compared to 4 mm particle size for Ferreira and Mertens (2005). However, both the 1 and 4 mm particle sizes resulted in significantly higher *in vitro* DM disappearance when compared to using whole maize silage samples.

2.6.3 Ratio sample:medium and sample size

Church and Petersen (1960) analysed the effects of using different combinations of varying concentrations of rumen liquor, mineral, and substrate on *in vitro* rumen fermentation of DM and cellulose. They observed a decrease in DM and cellulose digestibility as the substrate amount was increased from 2 to 18 g, with the minimum point of digestibility reached being dependent on the volume of rumen liquor present but having no effect with changes in mineral concentration. The increase in volume of rumen liquor from 20 to 120 ml showed a linear increase in the percentage of DMd as well as cellulose digestibility, with the rate of increase for DMd being dependent on both substrate and mineral concentration. Volatile fatty acid production was dependent on both the amount of substrate and rumen liquor present, with mineral concentration only having an indirect effect. Furthermore, VFA production increased considerably as substrate concentration increased while there was a tendency for VFA to increase as rumen liquor was increased from 20 to 120 ml (Church and Petersen, 1960).

Sayre and Van Soest (1972) also tested the effect of using different sample sizes while following the adapted procedure described by Goering and Van Soest (1970). They, however, also adapted the amount of rumen fluid and buffer proportionally to the amount of sample used, 0.5, 0.375 or 0.275 g. Their findings proved to be non-significant ($P > 0.05$) for all 3 samples sizes and 4 forages used, namely orchard grass, lucerne, bromegrass and timothy hay, when looking at the forage species x sample size interaction.

2.6.4 Fermentation vessel and technique

Different techniques when performing *in vitro* studies can often lead to varying results when looking at digestion kinetics (Grant and Mertens, 1992). Along with varying techniques, fermentation vessels may also differ. The most commonly used fermentation vessels during *in vitro* experiments include Erlenmeyer flasks, polyethylene centrifuge tubes and serum vials (Hall and Mertens, 2008). Different fermentation vessels are often used due to their differences in shape, size and ability to efficiently use space, as inefficiency can result in a decreased number of samples being analysed at a time (Sayre and Van Soest, 1972).

Different vessels, same technique

Sayre and Van Soest (1972) compared three different fermentation vessels using the same technique with different forages. They found a significant interaction between forage species and fermentation vessel with the higher NDFd values being for timothy hay seen in the Erlenmeyer flasks versus the centrifuge tubes and screw capped tubes, and the lower NDFd values for bromegrass using the centrifuge tubes. Overall, it was noted that the centrifuge tubes provided consistently lower NDFd values in comparison to the other two fermentation vessels.

Different techniques, same vessels

Hall and Mertens (2008) studied the interaction between both fermentation vessel as well as technique. Fermentation vessels were shown to have differences amongst techniques for both NDFd as well as pH. Although non-significant differences ($P = 1.0$) were seen for NDFd between the serum vials (with or without gas release), Erlenmeyer flasks with continuous CO_2 and sealed tubes, there was a difference ($P < 0.035$) in NDFd when looking at the tubes with the gas release valves and tubes receiving continuous CO2 ($P < 0.01$). The lowest NDFd value for all treatments was seen for the tubes with the gas release valve. Grant and Mertens (1992) also obtained the slowest rate of NDFd in tubes only being purged with CO_2 and the highest rate for tubes and flasks with a continuous supply of CO_2. Overall, in all three studies, Erlenmeyer flasks resulted in consistently higher values for NDFd when being compared with other fermentation vessels as well as techniques.

Different techniques, different vessels

Hall (2015) looked at two different *in vitro* techniques using two different fermentation vessels to determine fibre digestibility of four fibrous feeds (lucerne hay, maize silage, soy hulls and ryegrass hay). The one technique is commonly used and makes use of Erlenmeyer flasks placed under continuous CO_2 pressure (Goering and Van Soest, 1970; GV) and the other technique makes use of glass tubes that were purged with CO_2, sealed and placed in a shaking incubator (TU). The overall efficiency of NDFd for the two techniques showed variable results. Neutral detergent fibre digestibility was greater for TU at 24 h but greater for GV at 48 h while there was no difference at 30 h. With regards to the substrate used, differences were seen with soy hulls and maize silage. However, Hall (2015) attributed the differences in NDFd values at the different hours to the technique itself and substrate used, claiming that the greater NDFd values seen for TU at 24 h were as a result of continuous mixing of the sample to improve microbial access to the substrate while the decrease in NDFd at 48 h for maize silage may be due to a decrease in pH in the sealed tubes. Ultimately, Hall (2015) suggested that factors that could have affected GV in the earlier stages of fermentation could resolve themselves as a decline in across run variability was seen for GV as fermentation hour increased whereas similar values were observed for TU.

2.7 CONCLUSIONS

There is no standardized procedure for the *in vitro* or *in situ* technique as seen in the literature where sample - and particle size, medium, fluid:sample ratios, etc. have differed. The *in vitro* system maintains a static and controlled environment where the pH is buffered, medium reduced, temperature controlled and feed supply limited which is unlike the dynamic system found in the rumen. The *in situ* technique is thought to replicate the processes of the rumen more closely compared to the *in vitro* system; however, the use of the bag too has its flaws because of differences found in its micro-environment. Both techniques have been used for decades and bot are used with varying parameters and thus do lack a standardized procedure. We do believe, however, that even if difficult to accomplish, many factors that can be controlled should be defined and standardized within the specific procedure.

Although the *in vitro* and *in situ* systems cannot completely replace or replicate studies done *in vivo,* they do have the advantage of being more cost effective, less labour intensive and giving animal nutritionists a better understanding of the chemical composition and characteristics of feeds when formulating diets. They, however, very often result in different outcomes, because of the mentioned reasons, and the values should be used accordingly and compared only if they are the result of an identical procedure. Furthermore, while the *in vitro* procedure will result in a value that is affected mainly by the intrinsic characteristics of the feed analysed, the *in situ* procedure will also be affected by the dynamic environment of the rumen and therefore the two values may not be comparable.

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CHAPTER 3

Interactions between *Saccharomyces cerevisiae*, medium and forage type and their effects on *in vitro* ruminal fermentation

3.1 ABSTRACT

The objective of this study was to investigate the effects of a live yeast, *Saccharomyces cerevisiae* CNCM I-1077, at four doses (0, 1×10^5, 1×10^6 and 1×10^7 cfu/ml) according to the reducing medium used [Goering-Van Soest (GV), McDougall (MD) or Kansas State (KS)] on *in vitro* neutral detergent fibre digestibility (NDFd), rate of digestion of NDF (kd), organic matter digestibility (OMd), dry matter digestibility (DMd), pH as well as volatile fatty acids (VFA) concentration and using two forages (oat hay and wheat straw) with differing chemical composition. The maximum *in vitro* NDFd, DMd, OMd as well as kd were obtained with dose 1×10^6 cfu/ml, although differences between doses were not always significant. The pH estimates of the samples decreased as dose increased from 0 to 1×10^7 cfu/ml. The differences were not all significant; however, 1×10^7 cfu/ml corresponded to significantly lower estimates compared to the control and 1×10^5 (6.53 vs. 6.61 and 6.61, respectively). The decrease in pH was accompanied by an increase in VFA concentrations as the yeast dose increased. The KS medium resulted in the lowest digestibility estimates, pH estimates as well as kd, regardless of yeast dose. The 1×10^6 cfu/ml was the better performing yeast dose *in vitro* resulting in higher digestibility estimates which indicates the yeasts ability to stimulate the microorganisms within the rumen through providing vitamins, nutrients and dicarboxylic acids as well as through the removal of O_2 which enters the rumen via feed or water, etc. from the rumen fluid which may hinder the growth of strict anaerobic bacteria. The McDougall and Goering-Van Soest media provide a better environment for fermentation than the KS medium, resulting in higher *in vitro* NDFd, DMd, OMd, pH estimates as well as rate of NDF digestion.

3.2 INTRODUCTION

In recent years, more emphasis is being placed on substitutes to antibiotics in the dairy industry with the main focus being on direct fed microbials (DFM) as they are

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recognized as being a safer alternative to both animal and consumer (Thrune *et al.*, 2009). Yeast additives or direct-fed microbials (DFM) have been reported to increase animal performance and health (Chaucheyras-Durand *et al.*, 2008), however, the responses have been somewhat inconsistent (Carro *et al.*, 1992a; Doreau and Jouany, 1998) or dismissive (Raeth-Knight *et al.*, 2007). Many studies attribute the variable effects to the differences in experimental diets, feeding systems, different doses and strains of yeast being used (Chaucheyras-Durand *et al.*, 2008; Patra, 2012; Wang *et al.*, 2016).

Chaucheyras-Durand *et al.* (2008) previously stated that not all yeast strains should be classified as the same, and neither should different species of yeast, as seen in the study by Wang *et al.* (2016). Three different species of yeast were compared *in vitro* with regards to the fermentation of cereal straws. When looking at the *in vitro* fermentation of cereal grains, digestibility values [dry matter digestibility (DMd) and neutral detergent fibre digestibility (NDFd)] are regarded as the most important parameters (Wang *et al.*, 2016). Wang *et al.* (2016) found noticeable differences in both *in vitro* DMd and NDFd between three different yeast species incubated in different doses. *Candida tropicalis* improved both DMd and NDFd for maize stover and rice straw, while *Candida utilis* decreased both DMd and NDFd compared to the control. *Saccharomyces cerevisiae* showed non-significant differences when compared to the control, but the differences were dependant on the dose (Wang *et al.*, 2016). It should be noted that the highest dose of yeast did not always result in the highest digestibility values as seen in *in vivo* studies as well (Nocek *et al.*, 2002). Furthermore, when the objective is to quantify the effect of a DFM (yeast) using an *in vitro* fermentation, the stimulated rumen environment itself could interact with the yeast, the specific dose and the sample(s) used. Currently, *Saccharomyces cerevisiae* is the most common yeast species being used as a supplement for high producing dairy cows (Chaucheyras-Durand *et al.*, 2008; Opsi *et al.*, 2012; Thrune *et al.*, 2009).

Many *in vitro* studies make use of the Goering-Van Soest (Bossen *et al.*, 2008; Elghandour *et al.*, 2014; Goering and Van Soest, 1970) or the McDougall incubation medium (Carro *et al.*, 1992b; McDougall, 1948; Kung *et al.*, 1997), with some studies also making use of the Kansas State medium (Holden, 1999) but not many studies have been conducted to compare the three media. Leo Penu *et al.* (2012) compared

the Goering-Van Soest (GV) and Kansas State (KS) incubation media on gas production *in vitro* and found significant differences between the two ($P < 0.05$). They found the GV medium to allow for better gas production at 24, 48 and 72 h as well as having a better buffering capacity than that KS medium (Leo Penu *et al.*, 2012).

The objective of this study were to therefore investigate the effects of a live yeast, *Saccharomyces cerevisiae* CNCM I-1077, at four doses, 0, 1×10^5 (10^5), 1×10^6 (10^6) and 1×10^7 (10^7) cfu/ml, according to the reducing medium used (GV, MD or KS) on *in vitro* NDFd, kd, OMd, DMd, pH and volatile fatty acids (VFA) concentration using two forages (oat hay and wheat straw) with differing chemical compositions.

3.3 MATERIALS AND METHODS

3.3.1 Forages and chemical analysis

Two forages, oat hay (OH) and wheat straw (WS), were dried at 60°C for 48 hours and then ground through a 1-mm screen using a Wiley Mill (Thomas Scientific, Swedesboro, NJ). Samples were analysed for moisture and ash (AOAC, 2002; method 934.01 and 942.05), neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), ether extract (EE), crude protein (CP) and starch. Neutral detergent fibre was analysed as described by Mertens (2002) using Gooch crucibles with porosity 2. Acid detergent fibre and lignin were analysed as described by Raffrenato and Van Amburgh (2011). All fibre fractions were analysed with Gooch crucibles fitted with glass fibre filter with porosity 1.5 μm (934-AH™ by Whatman®, Whatman Limited – GE Healthcare, Maidstone, UK; Raffrenato and Van Amburgh, 2011). Ether extract was determined using Tecator Soxtec System HT 1043 Extraction Unit (AOAC, 2002; Method 920.39). Crude protein was measured with a Leco N analyser ("FP-528" Leco Africa (Pty), Ltd, Kempton Park, South Africa), while starch was determined as described by Hall (2008). The chemical composition of the two forages used in this study can be seen in Table 3.1.

Table 3.1. Chemical composition of the forages used in the study on a DM basis (%).

Forages	Item							
	NDF	ADF	ADL	EE	CP	Starch	Moisture	Ash
Oat hay	60.61	34.00	5.27	3.06	3.12	16.14	5.50	6.62
Wheat straw	83.11	54.15	6.71	0.82	7.85	0.12	10.89	4.13

3.3.2 *In vitro* fermentations

Treatments for this experiment consisted of three media, three doses of yeast and two forages. Combinations of feed sample, medium and yeast dose were incubated in quadruplet for 0, 12, 24 and 48 hours, following the procedure described by Goering and Van Soest (1970) with adjustments to the medium used. Samples were also incubated for 120 and 240 h to estimate indigestible NDF and calculate the rate of NDF digestion (kd) according to Raffrenato and Van Amburgh (2010). Samples were weighed (0.5 ± 0.05 g) into 125-ml Erlenmeyer flasks and 40 ml of medium was added to each flask. Three different media were used for this trial, namely KS, MD and GV. All three media were prepared as described by Marten and Barnes (1980), McDougall (1948) and Goering and Van Soest (1970), respectively.

An active dry yeast, *S.cerevisiae* CNCM I-1077, was prepared using peptone water (FDA and US Food and Drug Administration, 2001) and was injected at 0 h into each flask to make up a final dose of 10^5, 10^6 or 10^7 cfu/ml yeast within the flasks. Flasks with no yeast were present to serve as controls for each time point and blank flasks were also used to rectify for any particles present in the rumen fluid. Flasks were incubated with 10 ml rumen fluid as described by Goering and Van Soest (1970). Rumen fluid was collected in the afternoon from two lactating Holstein cows receiving a total mixed ration (TMR; Table 3.2) and being housed at the Stellenbosch University research farm, Western Cape, South Africa. The trial was approved by the Stellenbosch University Research Ethics Committee (Animal Care and Use; Approval SU-ACUD15-00060). Rumen fluid was transported, in thermos flasks, to the laboratory where it was mixed and filtered through four layers of cheese cloth into a

pre-warmed flask kept at 39°C. Once all fluid had been filtered, the air space above the fluid in the flask was purged with CO_2, before it was injected into the flasks.

Table 3.2. Total mixed ration fed to the donor cows.

Ingredient	% DM
Ground maize	38.30
Lucerne hay	28.31
Maize gluten	7.25
Wheat straw	6.60
Sugarcane molasses	5.62
Soybean meal	3.07
Barley malt	3.03
Potato by-product meal	2.17
Dry molasses	1.84
Feather meal with blood	1.54
Limestone	0.85
Blood meal	0.65
Salt	0.44
Urea	0.15
Monocalcium phosphate	0.13

Duplicates of the fermentations' residuals were analysed for NDF (Mertens, 2002) and pH was measured at the mentioned time points. Volatile fatty acids (VFA) were also determined via gas-liquid chromatography according to Siegfried (1984). The VFA analysed included acetate (A), propionate (P), butyrate (B), isobutyrate (IB), valerate (V), isovalerate (IV) and total VFA (TVFA), although more emphasis was placed on A, P, B, TVFA and A:P ratio. Extra flasks with each treatment combination

were added and removed at 0 h to measure initial pH and VFA as initial reference points. After ruminal *in vitro* fermentation, the other flasks were subjected to acid-pepsin digestion and analysed for dry and organic matter digestibility values (DMd and OMd) as described by Tilley and Terry (1963). All fermentations and digestions were run three times with each forage run separately

3.3.3 Statistical analyses

Rates of NDF digestion (kd) were computed using a first order decay model according to the following equation:

$$NDF(t) = pdNDF(0) * e^{-kd(t-L)} + iNDF$$

Where pdNDF(0) is the potentially digestible NDF at time 0; kd is the fractional rate of digestion of NDF; L is the lag and iNDF is the indigestible NDF. Simultaneous estimations of the parameters pdNDF, kd, iNDF and L were initially obtained using PROC NLIN of SAS (SAS Institute, Inc., Cary, NC) and the Marquardt algorithm. The Marquardt algorithm was selected to improve the efficiency of providing least-squares estimation for the non-linear curve fitting approach. Non-linear regression was chosen as the standard procedure because the method assumes equal error at each observation and by simultaneously fitting all parameters to the data, the result provides the smallest residual sums of squared deviations. The necessity of establishing initial parameters values for the non-linear estimations was solved using a linear approach to seed the non-linear estimation as done by Grant and Mertens (1992). We used the log-linear approach of Van Soest *et al.* (2005) to generate the initial values for each sample to parameterize the decay model, including an indigestible pool for the model using 240 h residual NDF to estimate the pdNDF. *In vitro* NDF digestibility values, the estimated rates, DMd, OMd, pH and VFA were analysed as response variables by the GLIMMIX procedure of SAS using a factorial arrangement of forage, medium, yeast and all interactions. Run was added as random factor. The highest order interaction (dose x medium x forage x time) was removed from the model because non-significant. The control parameters for NDF were the digestibility and rates of the forages, when fermented alone. Differences between means and the control were declared significant at $P \le 0.05$ using the least squares means and the Tukey adjustment. Statistical differences resulting in $0.05 < P$

≤ 0.10 were considered tendencies. Treatments are reported as least squares means.

3.4 RESULTS AND DISCUSSION

3.4.1 Neutral detergent fibre digestibility

As yeast dose increased from 0 up to 10^6, NDF digestibility increased. However, differences were not always significant. Dose of yeast also significantly interacted with medium and time ($P < 0.0001$), and tended to interact with forage ($P = 0.0799$), resulting in different responses when considering these variables (Figure 3.1). The highest NDFd was obtained when using 10^6 for the GV and KS media (0.2558 and 0.3410 NDF^{-1}) and 10^7 for the MD medium (0.3467 NDF^{-1}). When analysing the interaction dose x time, there was no difference among doses at 12 h, while 10^6 resulted in higher NDFd at both 24 and 48 h (Table 3.3). It appeared that neither the lowest dose nor the highest dose resulted in the highest digestibility estimates, but rather the middle dose of 10^6, which presented similar results also seen in work by Nocek *et al.* (2002) and Wang *et al.* (2016). This also differs from the dose often suggested (1×10^5 cfu/ml) by yeasts companies (personal communication, E. Chevaux). The increase in the digestibility estimates are assumed to be due to some of the proposed modes of action of the yeast supplement which include the ability to provide vitamins, nutrients and dicarboxylic acids (Newbold *et al.*, 1998) as well as through the removal of O_2 which enters the rumen via feed or water, etc. from the rumen fluid which may hinder the growth of strict anaerobic bacteria (Auclair, 2001).
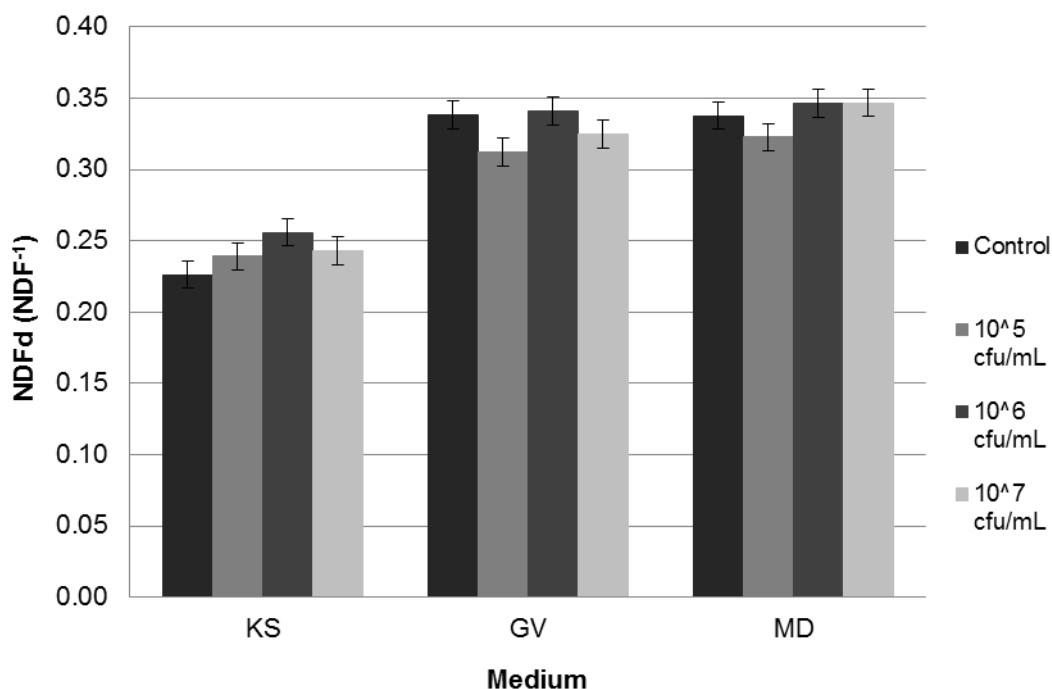
Figure 3.1. The effect of medium and dose on NDFd.

Table 3.3. The effect of dose on NDFd over time.

Time (hours)	Dose (cfu/ml)				
	Control	1×10^5	1×10^6	1×10^7	SEM[1]
12	0.1459	0.1284	0.1405	0.1290	0.0096
24	0.3009[ab]	0.2927[a]	0.3185[b]	0.3046[ab]	0.0096
48	0.4554[a]	0.4529[a]	0.4842[b]	0.4809[b]	0.0096

[ab]Means within a row with different superscripts differ ($P < 0.05$).
[1]Standard error of the means.

Dose tended to interact with forage, resulting in the largest numerical NDFd at 10^5 and 10^6 for OH and WS, respectively ($P = 0.0799$). Although not significant ($P = 0.0936$), there was a tendency for WS to have higher NDFd estimates compared to OH. This was not expected as OH had lower NDF and lignin contents (60.72 %DM and 5.22 %DM) compared to WS (83.41 and 6.78 %DM for NDF and lignin,

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respectively). This trend was seen when comparing both forages with different media and yeast doses as well as over time. When pooling doses and media, wheat straw appeared to be more digestible than OH as fermentation time increased (Figure 3.2). This clearly demonstrates that the amount of NDF present in a forage is not necessarily a measurement of forage quality, especially when comparing different species, but *in vitro* NDF digestibility estimates are necessary to fully evaluate a forage. However, the unexpected tendency of the wheat straw to be a better forage when compared to oat hay could have been supported by other chemical analyses for antimicrobial components that may have been present in the oat hay. Unfortunately these analyses were not performed during the study.
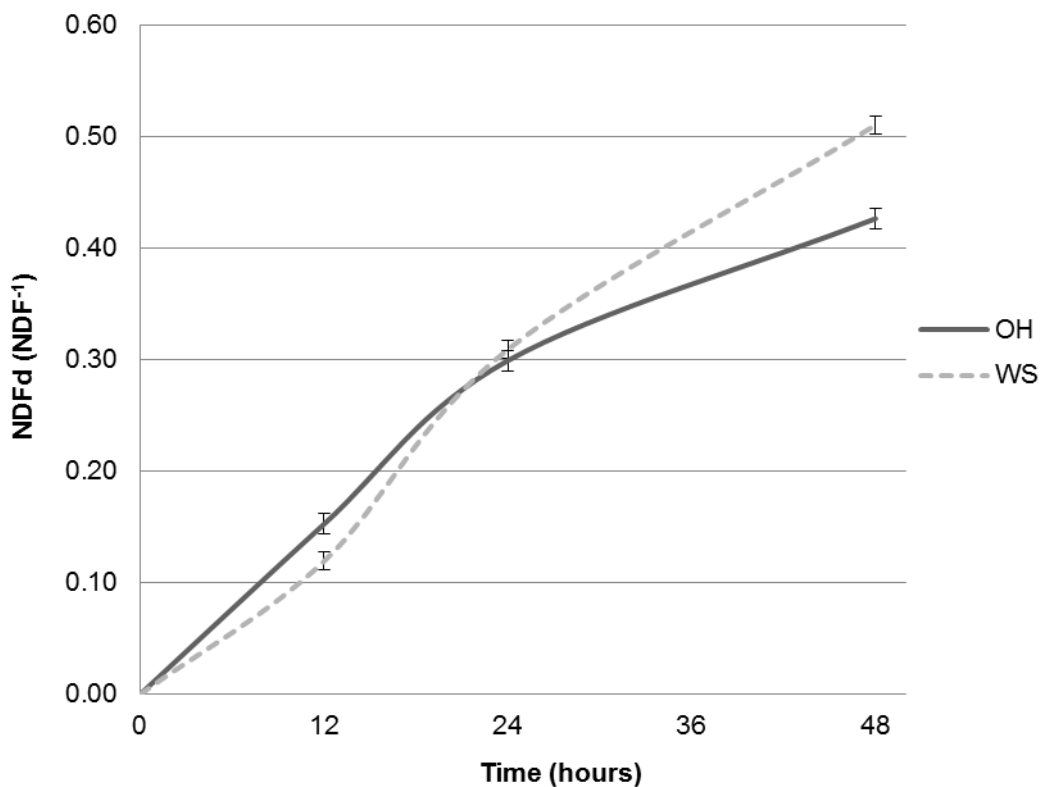

Figure 3.2. The effect of forage on NDFd over time.

There was a significant ($P < 0.0003$) interaction between forage and medium. The KS medium had lower estimates compared to MD and GV for both forages. This could be as a result of the micro-environment provided for by the medium, with the KS not

providing a suitable environment for fibrolytic bacteria, such as having a lower pH compared to GV and MD.

Medium and forage both interacted significantly with time ($P < 0.0001$). All NDFd differences were highly significant ($P < 0.01$) when looking at the combinations resulting from the interaction medium x time, except at 12 h where MD and GV media had a non-significant difference ($P = 0.2188$; Figure 3.3). The KS medium presented the lowest NDFd up to 48 h, with 120 and 240 h showing similar values (not shown).
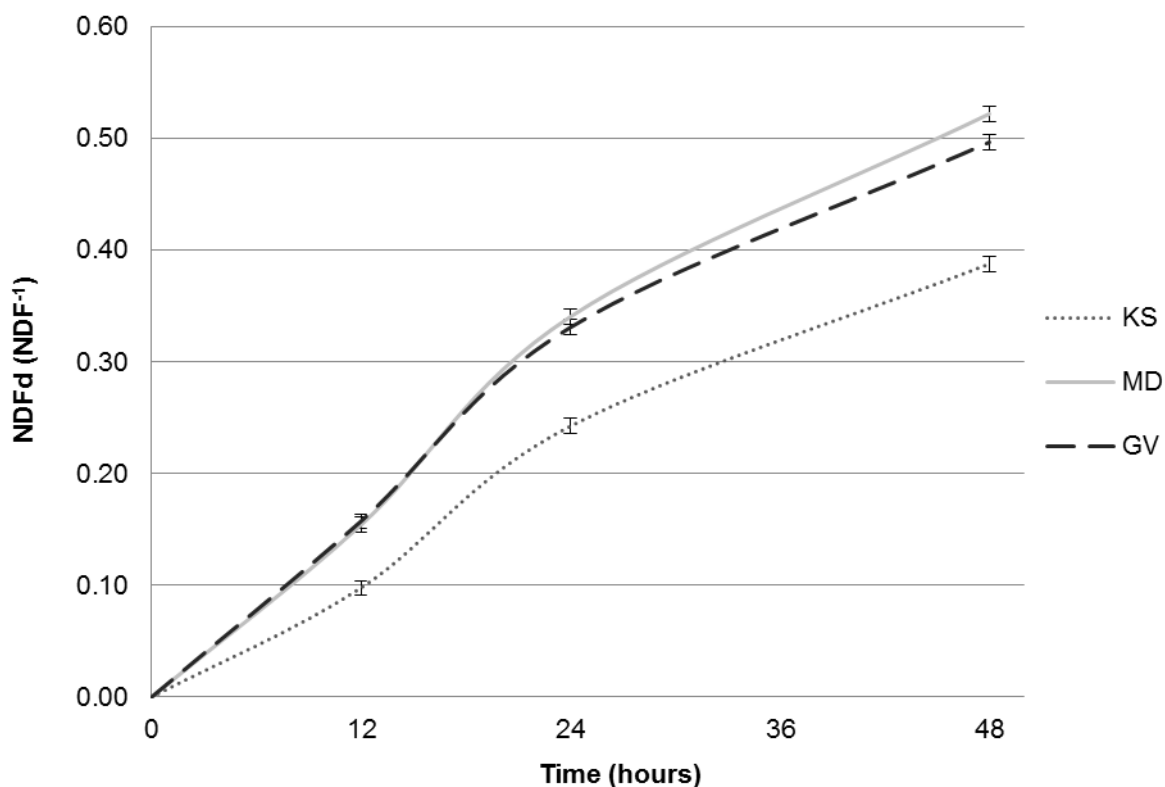
Figure 3.3. The effect of medium on NDFd over time.

3.4.2 Rate of NDF digestion

Yeast did not have a significant effect on the rate of NDF digestion, probably because the yeast effect on NDFd was not consistent and not always significant. However, variable estimates were seen between the control and yeast doses with the numerically highest rate being observed for the 10^6 dose. The yeast effect was however variable across media ($P = 0.0004$), while there was a tendency to interact with forage ($P = 0.0534$). In fact, wheat straw responded more to the yeast and at a

different dose (10^6 vs. 10^5) when compared to OH, resulting in higher kd estimates. The higher NDF content of the straw may suggest an increase in the yeasts efficacy with forages higher in fibre.

The KS resulted in the lowest estimates ($P < 0.0001$) for all three doses as well as the control compared to GV and MD (Figure 3.4), reflecting the results from the NDFd estimates. The only significant difference ($P = 0.0060$) seen between MD and GV was for the highest dose resulting in 0.0367 vs. 0.0337 h^{-1} for MD and GV, respectively. Dose tended to interact with forage, with WS having higher kd estimates for the control, 10^6 and 10^7 (Figure 3.5).

The interaction medium x forage was also significant ($P = 0.0065$). The KS medium had lower estimates compared to GV and MD for both forages (Table 3.4), confirming the NDFd results.
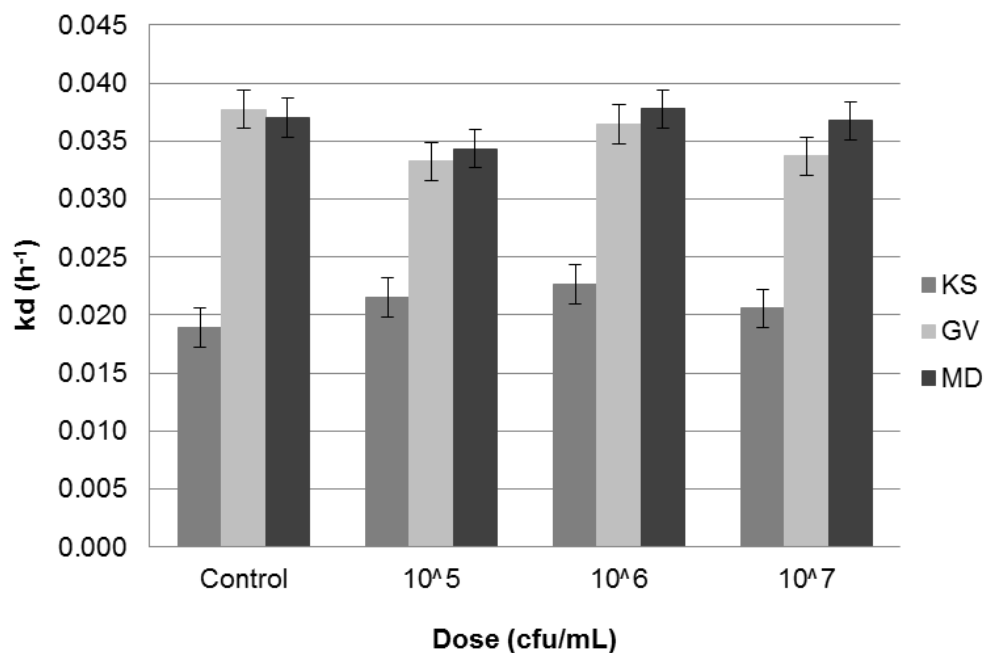
Figure 3.4. The interaction between medium and dose on kd.
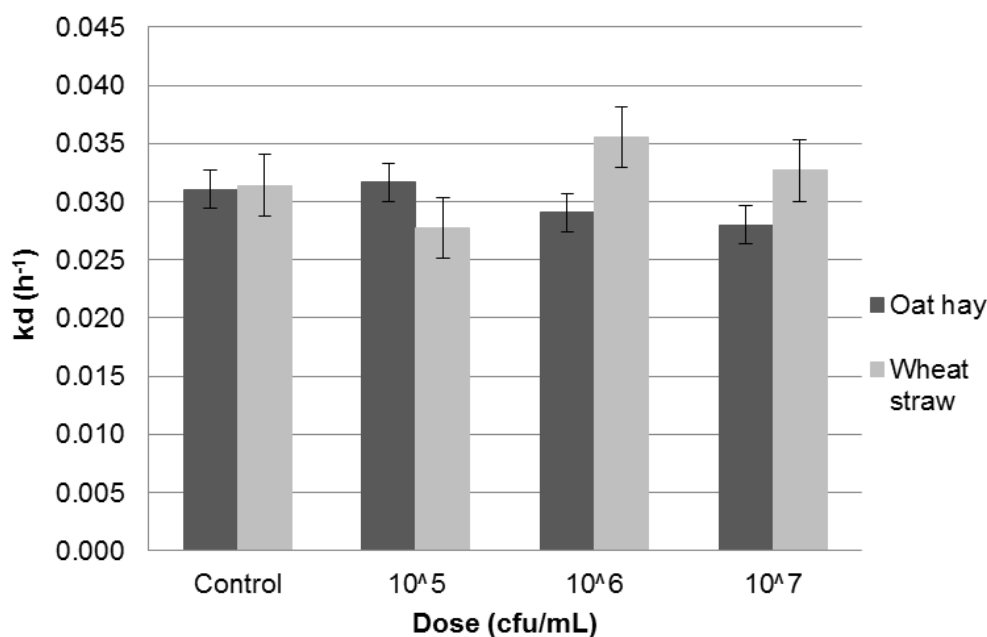
Figure 3.5. The interaction between forage and dose on kd.

Table 3.4. The effect of medium and forage on kd.

Forage	Medium			
	KS	GV	MD	SEM[1]
OH	0.0198[a]	0.0353[b]	0.0347[b]	0.0016
WS	0.0220[a]	0.0352[b]	0.0383[c]	0.0014

[a-c]Means within a row with different superscripts ($P < 0.05$).
[1]Standard error of the means.

3.4.3 Organic matter and dry matter digestibility

Yeast had a significant effect on both OMd and DMd ($P = 0.0014$ and 0.0007, respectively). Organic and dry matter digestibility increased as yeast dose increased from 0 up to 10^6. This was not seen in the study by O'connor *et al.* (2002) who found that yeast had little effect on the extent of *in vitro* DMd for different yeast cultures as well as two varying doses. Yeast dose interacted significantly with time for both DMd and OMd, while only showing a tendency to interact with forage for OMd. Dose 10^6

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also resulted in the highest estimates for OMd and DMd across all time points. Oat hay had significantly higher ($P < 0.0001$) DMd and OMd estimates compared to WS, with dose 10^6 resulting in the highest digestibility for both forages (Figure 3.6 and 3.7). This was unlike the results seen for NDFd, where WS had higher digestibility values than OH, which was unexpected and reflected the better quality NDF present in the WS compared to OH. A possible hypothesis for the higher DMd and OMd estimates for OH could be due to the acid-pepsin digestion, where a large part of the matter digested is non-fibre and therefore being higher in OH than WS.
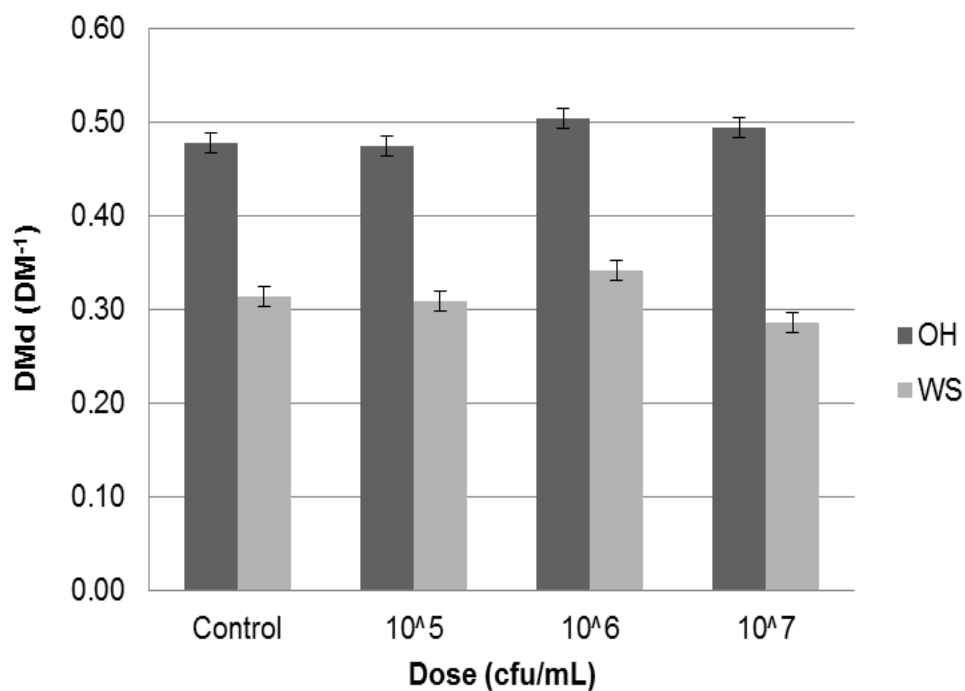
Figure 3.6. The effect of dose and forage on DMd.
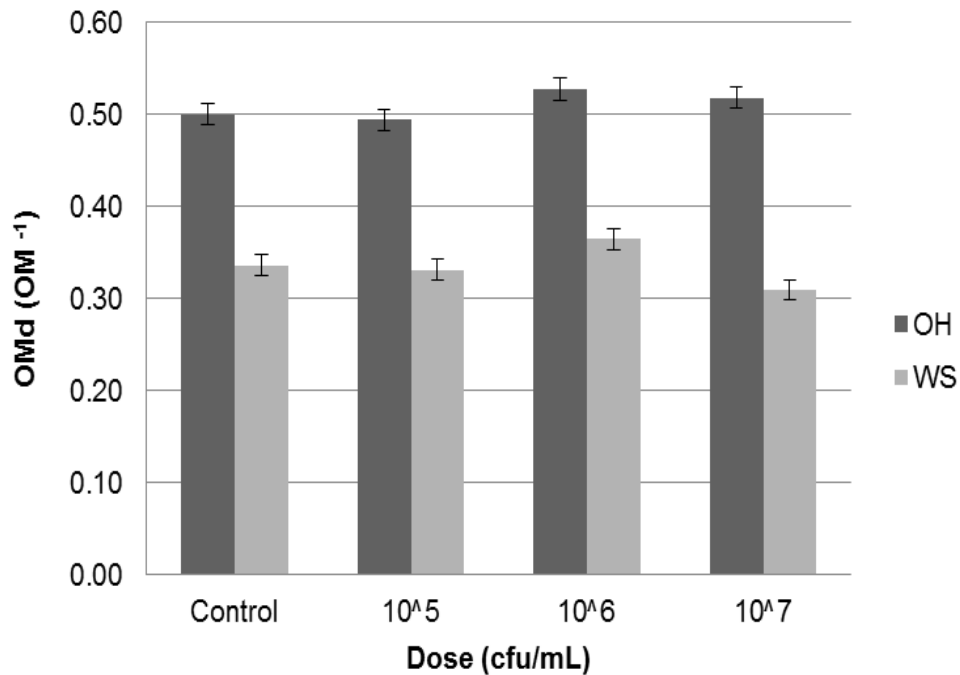
Figure 3.7. The effect of dose and forage on OMd.

When looking at the interaction medium x time, at 12 h none of the media were different ($P > 0.05$) from one another, while at 24 h all the media resulted in different OMd and DMd ($P < 0.01$). The McDougall medium resulted in the highest DMd and OMd estimates (0.4256 DM^{-1} and 0.4472 OM^{-1}) followed by GV and KS. The same results were seen at 48 h with all media being different ($P < 0.01$) from one another, with MD resulting in the highest DMd and OMd estimates followed by the GV and KS media. The MD medium proved to provide a better environment for the microorganisms compared to GV and KS, during the initial rumen *in vitro* stage. It is assumed that during the second stage of the Tilley and Terry (1963) procedure, all the digestible matter is digested by the acid-pepsin treatment and therefore the differences between the media mainly originate from the first stage when fibre is degraded as well. The medium that resulted in extra fibre degraded during the ruminal fermentation can also free more digestible matter trapped by the cell wall. In other words, the more fibre was degraded in the first stage of the Tilley and Terry procedure, the more OMd and DMd in the second stage.

Figure 3.8. The effect of forage on DMd over time.

Figure 3.9. The effect of forage on OMd over time.

3.4.4 Volatile fatty acids

The concentration of VFA increased significantly ($P < 0.01$) as the dose increased from 10^5 to 10^7. Dose interact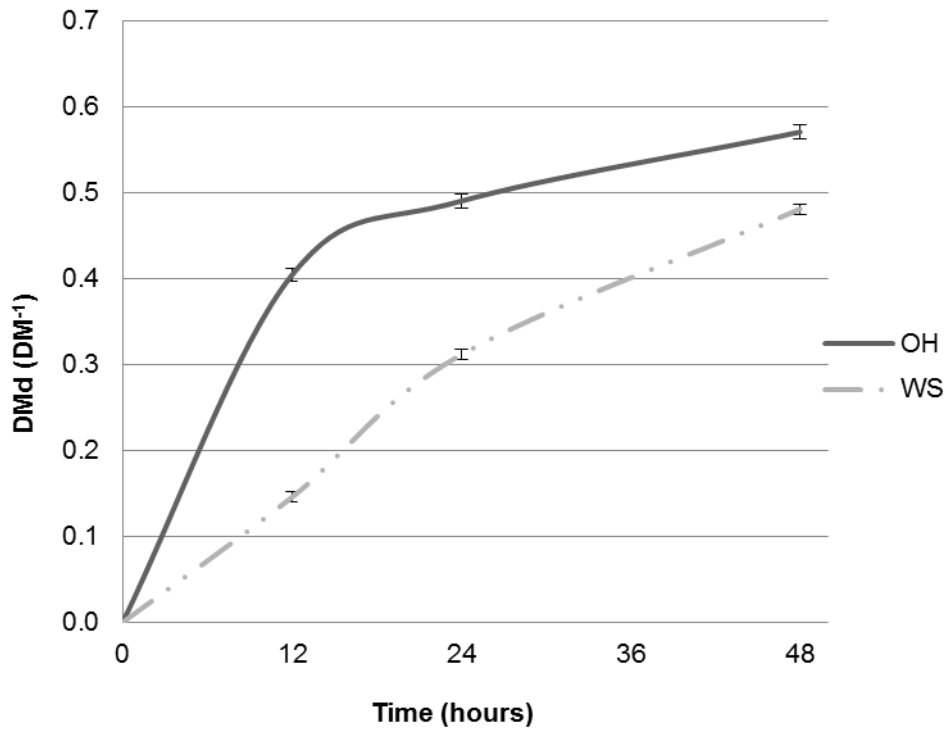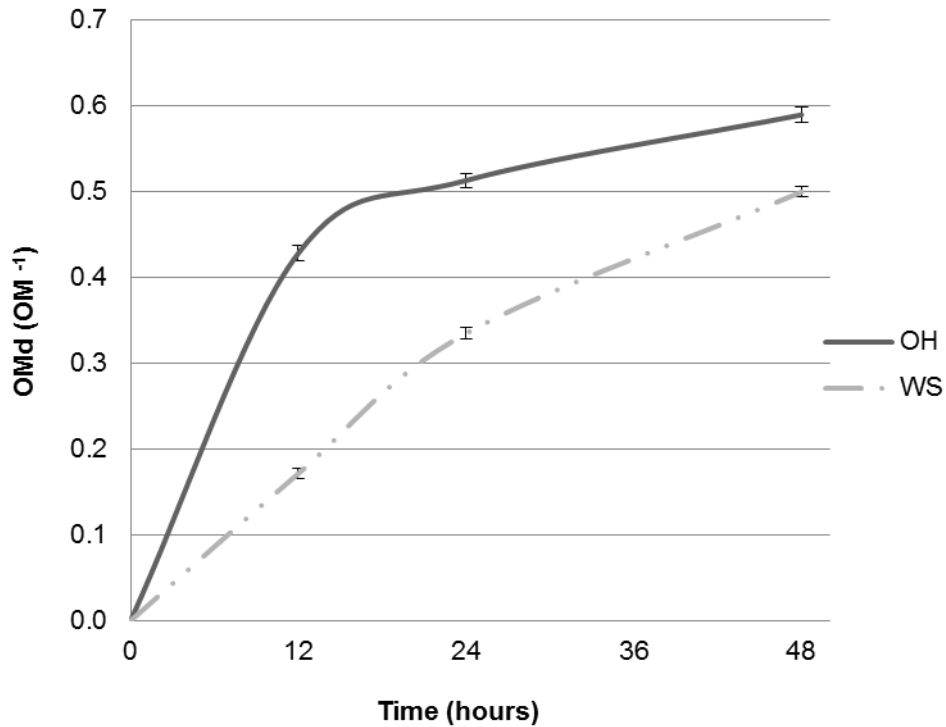ed significantly with medium for A, P and TVFA, while there was a non-significant ($P > 0.05$) interaction between dose and forage for all VFA (Table 3.5).

The GV medium resulted in the highest concentrations for all VFA followed by MD and KS. Even though significant, the difference between the GV and the MD medium was opposite than the results for NDFd. However, the differences for both NDFd and VFA for the two media were relatively small and probably not biologically important. It is also possible that the medium itself may interact with the VFA analysis, biasing the results or degrading the VFA at different rates for the two media. According to our knowledge, there is no work looking at the effect of medium on VFA analysis and degradation. Dose 10^7 resulted in the highest VFA concentrations when compared to the other doses, this demonstrates how yeast promotes bacterial activity. The highest ratio (i.e. 2.53; $P < 0.0001$) between acetic and propionic acid corresponded to the 10^6 dose, confirming however the results from NDFd.

The concentrations of VFA did increase over time, with dose 10^7 resulting in the highest concentrations across all times, although the interaction was not significant. The difference in concentration between forages was only significant for P and TVFA, while the interaction between forage and medium being significant for A, P, B and TVFA. Oat hay resulted in higher concentrations compared to WS (Figure 3.10), although not always significantly. While the TVFA produced by the OH corresponds to the larger portion of non-fibre organic matter of the OH, the results of the two forages for acetic acid followed a different trend when compared to the NDFd results. This might be due to a difference in NDF degradation but not digestion and absorption by the bacteria between the two forages which is not clear to interpret. The highest dose (10^7) resulted in highest TVFA concentrations for both forages, indicating the increase in rumen microbial fermentation.
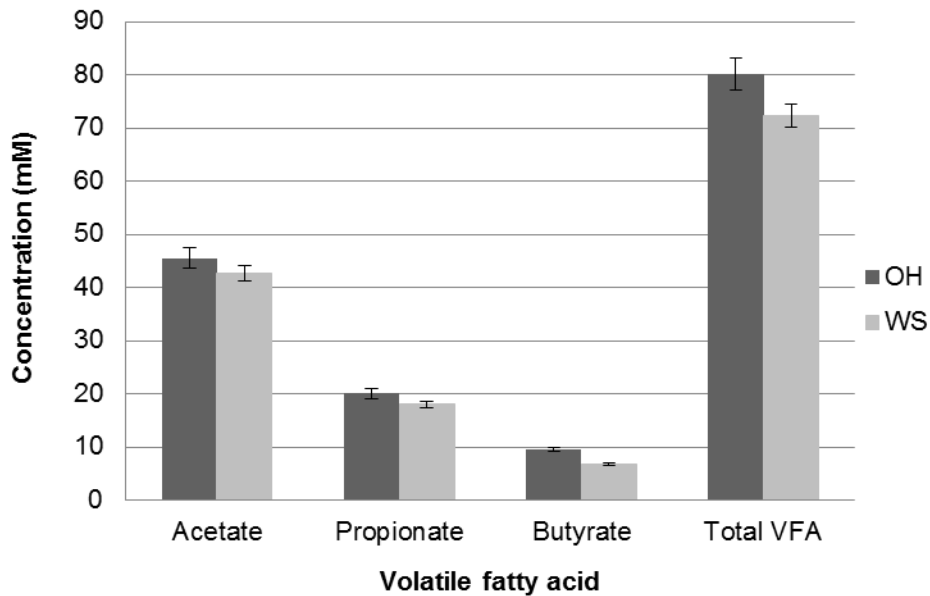
Figure 3.10. The effect of forage on VFA concentration.

Although the interaction between dose and time was non-significant for VFA, the interaction between dose and time for the A:P ratio was significant (P = 0.0009; Table 3.5). The A:P ratio for dose 10^7 remained significantly higher compared to the control and 10^5 at 12 and 24 h, however, at 48 h dose 10^7 was able to reduce the ratio to result in non-significant results between the control and 10^5. The highest A:P ratios were observed for 10^6 which followed the trend seen for NDFd. The higher A;P values indicate increased degradation of fibre resulting in increased amounts of A compared to P. Regardless, A:P for all doses remained higher compared to the control across time, although not always significantly. These finding agreed with findings by O'connor et al. (2002) but not with Wang *et al.* (2016) who found a significant decrease in the A:P ratio compared to the control with an increase in yeast dose. The A:P ratio gives the indication as to which direction fermentation is favouring, either towards acetic acid which is fermented from fibre or towards propionic acid which is starch derived.

Table 3.5. The effect of dose on VFA over time.

Time (hours)	Dose (cfu/ml)				
	Control	1×10^5	1×10^6	1×10^7	SEM[1]
Acetate (mM)					
12	34.40	31.94	33.38	37.19	2.3065
24	46.25[a]	40.96[bc]	44.19[ac]	48.30[a]	2.2912
48	55.79[a]	49.56[b]	50.89[b]	55.99[a]	2.2912
Propionate (mM)					
12	15.87[a]	14.97[abc]	12.87[c]	15.61[ab]	0.9844
24	21.38[a]	18.56[bc]	17.28[b]	20.68[ac]	0.9811
48	25.07[a]	21.30[b]	20.57[b]	24.60[a]	0.9811
A:P ratio					
12	2.21[ac]	2.17[a]	2.58[b]	2.40[c]	0.0776
24	2.18[a]	2.21[a]	2.55[a]	2.34[b]	0.0773
48	2.22[a]	2.32[b]	2.45[b]	2.27[a]	0.0773
Butyrate (mM)					
12	6.59[a]	5.89[a]	7.41[b]	8.55[c]	0.4250
24	8.29[ab]	7.35[a]	8.57[b]	9.82[c]	0.4226
48	9.06[a]	7.72[b]	8.74[a]	10.51[c]	0.4226
Total VFA (mM)					
12	59.49[ab]	56.00[a]	57.17[a]	65.85[b]	3.4874
24	79.71[ab]	72.02[a]	75.31[a]	85.35[b]	3.4682
48	94.54[a]	84.24[bc]	86.09[ac]	98.75[a]	3.4682

[a-d]Means within a row with different superscripts differ ($P < 0.05$).
[1]Standard error of the means.

3.4.5 pH

The pH estimates decreased as dose increased from 0 to 10^7. The differences were not all significant; however, 10^7 had significantly lower ($P < 0.01$) estimates compared to the control and 10^5. These findings were similar to those found by Wang *et al.* (2016). In that case, the pH for maize stover and rice straw also decreased when the dose of *S. cerevisiae* increased, although not always significantly. In the present study, the decrease in pH estimates with an increase in yeast dose can be as a result of the increase in VFA as 10^7 resulted in significantly higher VFA concentrations across all interactions. Yeast interacted significantly with medium ($P < 0.0001$) and time ($P = 0.0016$) but not with forage ($P = 0.6604$). The KS medium resulted in the overall lowest ($P < 0.0001$) pH estimates followed by MD and GV. From 24 h, the pH of the KS medium dropped below pH 6 which could explain why greater digestibility differences were observed at 24 and 48 h compared to MD and GV, whose pH values remained above 6 for all time points. Previously, it had been reported that fibre digestibility is hindered when pH drops below 6 (Rode, 2000) as was also seen by Shriver *et al.* (1986) who observed a decrease in *in vitro* NDFd as pH decreased from 6.2 to 5.8.

For the MD and GV media, the highest pH was observed for the control and decreased as the yeast dose increased (Table 3.6) and this might be related to a more active fermentation in the presence of yeast, even though yeast is known to mitigate pH decrease in the rumen (Chaucheyras-Durand *et al.*, 2008). The pH decreased for all doses as time increased. At 0 h there were no significant differences between the pH estimates for either the control or yeast treatments (Table 3.7).

Table 3.6. The effect of medium and dose on pH.

Medium	Dose (cfu/ml)				
	Control	1×10^5	1×10^6	1×10^7	SEM[1]
KS	6.10[a]	6.18[b]	6.17[b]	6.06[a]	0.0241
MD	6.81[a]	6.77[ab]	6.73[bc]	6.70[c]	0.0241
GV	6.91[a]	6.88[abc]	6.85[bd]	6.84[cd]	0.0241

[a-d]Means within a row with different superscripts differ ($P < 0.05$).
[1]Standard error of the means.

Table 3.7. The effect of dose on pH over time.

Time (hours)	Dose (cfu/ml)				
	Control	1×10^5	1×10^6	1×10^7	SEM[1]
0	6.88[a]	6.86[a]	6.84[a]	6.85[a]	0.0276
12	6.63[a]	6.62[a]	6.57[a]	6.51[b]	0.0243
24	6.50[a]	6.50[a]	6.48[ab]	6.43[b]	0.0243
48	6.42[a]	6.45[a]	6.44[a]	6.34[b]	0.0243

[ab]Means within a row with different superscripts differ ($P < 0.05$).
[1]Standard error of the means.

Medium and forage interacted significantly ($P < 0.0001$) with time. For both medium and forage, pH estimates deceased as time increased. The KS medium had significantly ($P < 0.0001$) lower pH estimates compared to GV and MD across all time points. Wheat Straw resulted in significantly higher ($P < 0.01$) pH estimates compared to OH at 0, 12 and 24 h (Figure 3.11). At 48 h, there were no significant differences between the pH estimates ($P > 0.05$). The lower pH levels observed for OH could be attributed to the increased concentrations for acetate (A) and propionate (P) compared to WS. Higher concentrations of A and P were observed for OH

compared to WS (62.95 vs. 49.97 mM and 26.80 vs. 20.82 mM, respectively). There was no significant difference seen for butyrate between the two forages.

The interactions medium x forage x dose and medium x forage x time were also both highly significant ($P < 0.001$).
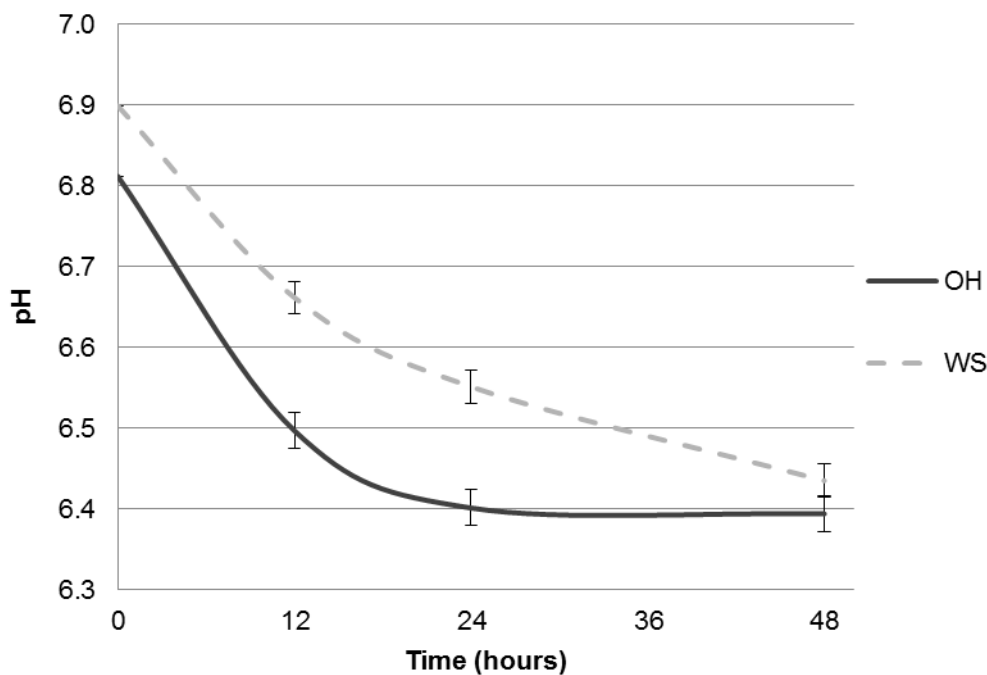
Figure 3.11. The effect of forage on pH over time.

3.5 CONCLUSIONS

The 10^6 cfu/ml was the better performing yeast dose *in vitro* resulting in higher digestibility estimates. The highest dose (10^7) instead resulted in significantly higher VFA concentrations demonstrating the yeast's ability to facilitate fibre digestion *in vitro* and by supplying the microorganisms with a better micro-environment. Somewhat dismissive effects by the yeast were seen on the pH due to the control having higher pH estimates compared to the all three yeast treatments. The significantly higher concentrations of VFA as yeast dose increased, corresponds with the decrease in pH. It should, however, be emphasized that even small differences in pH of 0.1 could result in significant differences between treatments. Taking that into account, yeast was capable of stimulating fermentation while maintaining a relatively

stable pH. However, as seen previously, all pH estimates remained within the normal ranges for fermentation to occur, therefore it cannot be determined as to whether the yeast had the ability to stabilize rumen pH. The repeatability of our *in vitro* fermentations were high enough to detect small differences even if they were not biologically important.

The MD & GV media provide a better environment for fermentation than the KS medium, resulting in higher NDFd, DMd, OMd, pH estimates as well as rate of digestion and VFA. This study gives important insight regarding different media used *in vitro* and how they can result in variable digestibility values as well as their interaction with *Saccharomyces cerevisiae.* It is important that care be taken when effects of feed additives on *in vitro* fermentations parameters are evaluated, especially when comparing studies with different forages and media as interactions between variables may result in different outcomes.

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CHAPTER 4

Interactions between *Saccharomyces cerevisiae* and forage type in an oxygen challenged *in vitro* fermentation system

4.1 ABSTRACT

Yeasts, such as the species *Saccharomyces cerevisiae,* are aerobic organisms that are thought to be able to stabilize the anaerobic environment of the rumen by utilizing oxygen that enters the rumen and by stimulating the facultative anaerobic bacteria by supplying nutrients and vitamins. The objective of this study was to investigate the effects of a live yeast, *Saccharomyces cerevisiae* CNCM I-1077, on *in vitro* neutral detergent fibre digestibility (NDFd), and rate of NDF digestion (kd), organic matter digestibility (OMd), dry matter digestibility (DMd), pH as well as volatile fatty acids (VFA) concentration using two forages (lucerne and maize silage) while simulating the effect of oxygen entering the rumen. Yeast did not have any significant effect on NDFd, kd, OMd and DMd, nor did it interacted with forage, oxygen or time. Two-doses of yeast caused a more stable and controlled pH environment over time as there was no significant interaction between pH and two-doses at 12, 24 or 48 h (6.80 vs. 6.82 vs. 7.02, respectively). Oxygen stress resulted in about 5% reduced NDFd, rate of digestion of NDF as well as VFA concentrations while no differences were observed with pH. Maize silage resulted in higher digestibilites which were accompanied by higher VFA concentrations and lower pH values as expected. This study confirms the strict anaerobic environment that is found within the rumen and the negative effects O_2 has on the fermentation efficiency.

4.2 INTRODUCTION

The rumen can be considered as one of the largest naturally occurring fermentation chambers with a large and diverse microbial population (Kamra, 2005; Krause *et al.*, 2003; Rode, 2000). The anaerobic environment of the rumen is accompanied by a negative redox potential which reflects the absence of oxygen (O_2) and strong power of reduction (Marden *et al.*, 2005). Oxygen does, however, enter the rumen through the feed and water that is consumed by the animal as well as mastication and thus, the rumen microorganisms are also exposed to aerobic conditions (Marden *et al.*,

2005). Strict anaerobia bacteria within the rumen cannot grow or attach themselves to fibre particles in the presence of O_2 (Jouany, 2001; Roger *et al.*, 1990). However, microorganisms within the rumen have the ability to maintain a constant environment and that the facultative anaerobes are capable of utilising highly oxidative compounds whereby maintaining a relatively constant anaerobic environment with a low redox potential (Baldwin and Emery, 1960). This was confirmed in a study by Ellis *et al.* (1989) who investigated the effects of O_2 on the respiratory activities of mixed ruminal organisms, protozoa and bacteria. During the experiment, dissolved O_2 concentrations were increased from 0 to 51 µM in 5% intervals lasting 15 min. They found that both bacterial and protozoal populations were able to utilize O_2 at similar rates when the O_2 concentration was at the same concentration as that of the lower detected level of O_2 in the rumen (0.25 µM) as well as the higher concentration reached directly prior to feeding (1 to 1.5 µM).

Baldwin and Emery (1960) who observed neither an effect on volatile fatty acid nor on ammonia production, nor any differences in the redox potential or any changes caused to the end products after fermentation when O_2 was added to the *in vitro* environment. Yeast products have been said to utilize O_2 that may enter the rumen and therefore create a more optimal environment for the microbial population especially cellulolytic bacteria (Chaucheyras-Durand *et al.*, 2008; Jouany, 2001; Kutasi *et al.*, 2004). Kutasi *et al.* (2004) showed that O_2 can be removed from the rumen within 50 min using commercially available yeast (Live-Sacc®) and that different strains can adapt differently to their anaerobic environment.

As it has been reported that yeast can have a positive effect on cellulolytic bacteria by utilizing O_2 within the rumen, the objective of this study was to investigate the effect of yeast on *in vitro* on NDFd, kd, OMd, DMd, pH and VFA concentration using two forages with differing chemical under oxygen stress.

4.3 MATERIALS AND METHODS

4.3.1 Forages and chemical analysis

Two forages, lucerne and maize silage (MS), were dried at 60°C for 48 hours and then ground through a 1-mm screen using a Wiley Mill (Thomas Scientific, Swedesboro, NJ). Samples were analysed for moisture and ash (AOAC, 2002;

method 934.01 and 942.05), neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), ether extract (EE), crude protein (CP) and starch. Neutral detergent fibre was analysed as described by Mertens (2002) using Gooch crucibles with porosity 2. Acid detergent fibre and lignin were analysed as described by Raffrenato and Van Amburgh (2011). All fibre fractions were analysed with Gooch crucibles fitted with glass fibre filter with porosity 1.5 µm (934-AH^{TM} by Whatman®, Whatman Limited – GE Healthcare, Maidstone, UK; Raffrenato and Van Amburgh, 2011). Ether extract was determined using Tecator Soxtec System HT 1043 Extraction Unit (AOAC, 2002; Method 920.39). Crude protein was measured with a Leco N analyser ("FP-528" Leco, Africa (Pty), Ltd, Kempton Park, South Africa), while starch was determined as described by Hall (2008). The chemical composition of the two forages used in this study can be seen in Table 4.1.

Table 4.1. Chemical composition of the forages used in the study on a DM basis (%).

Forages	Item							
	NDF	ADF	ADL	EE	CP	Starch	Moisture	Ash
Lucerne	43.26	38.55	8.72	1.49	17.29	2.28	6.40	9.14
Maize silage	49.40	26.76	3.02	3.02	6.53	25.50	9.21	4.72

4.3.2 *In vitro* fermentations

A 2x3 factorial design was set-up for this experiment. Six treatment combinations were tested: no yeast and no oxygen stress; no yeast and oxygen stress; one-dose yeast and no oxygen stress; one-dose yeast and oxygen stress; two-doses of yeast and no oxygen stress and lastly; two-doses of yeast and oxygen stress.

Combinations of feed sample, yeast and oxygen stress were incubated in quadruplet for 0, 12, 24 and 48 h, following the procedure described by Goering and Van Soest (1970). Samples were also incubated for 120 and 240 h, to calculate rate of NDF disappearance according to Raffrenato and Van Amburgh (2010). Each sample was

weighed (0.5 ± 0.05 g) into 250-ml centrifuge bottles (Nalgene, model 2189-0008, Thermo Fisher Scientific, Waltham, USA) and 40 ml of medium was added to each bottle as described by Goering and Van Soest (1970). Centrifuge bottles were used instead of 125-ml Erlenmeyer flasks to accommodate for the extra volume added by the water injected into the bottles. Rumen fluid was collected in the afternoon from two lactating Holstein cows receiving a maize and lucerne based total mixed ration (TMR; Table 4.2) and being housed at the Stellenbosch University research farm, Western Cape, South Africa. The rumen fluid microflora was thus already adapted to the feeds used in the study. The trial was approved by the Stellenbosch University Research Ethics Committee (Animal Care and Use; Approval SU-ACUD15-00060). Rumen fluid was transported, in thermos flaks, to the laboratory where it was mixed and filtered through four layers of cheese cloth into a pre-warmed flask kept at 39°C. Once all the fluid had been filtered, the air space above the fluid in the bottles was purged with CO_2, before 10 ml of the fluid was injected into each bottle. Blanks bottles were also used to rectify for any particles that may have been present in the rumen fluid.

An active dry yeast, *Saccharomyces cerevisiae* CNCM I-1077, was prepared using peptone water (FDA and US Food and Drug Administration, 2001) and was injected at 0 h into the specific bottles receiving yeast to obtain a final concentration of $1x10^6$ cfu/ml within the flasks. At 24 h, a second dose of yeast was injected only into the bottles receiving two doses. These bottles were further incubated for another 24 h.

The oxygen stress consisted of uncapping the specific bottles for few seconds every 3 h after the start of the incubation. Once uncapped, the bottles were injected with 5 ml of air and 5 ml of 20 ± 2 °C distilled water. Frequency of stress and amounts of water and air had the objective of simulating meal frequency in a dairy cow, adjusted to *in vitro* conditions.

Duplicates of the fermentations' residuals were analysed for NDF (Mertens, 2002) and pH was measured at the mentioned time points. Volatile fatty acids (VFA) were also determined via gas-liquid chromatography according to Siegfried (1984). The VFA analysed included acetate (A), propionate (P), butyrate (B), isobutyrate (IB), valerate (V), isovalerate (IV) and total VFA (TVFA), although more emphasis was placed on A, P, B, TVFA and A:P ratio. Extra flasks with each treatment combination were added and removed at 0 h to measure initial pH and VFA as initial reference

points. After ruminal *in vitro* fermentation, the other flasks were subjected to acid-pepsin digestion and analysed for dry and organic matter digestibility values (DMd and OMd) as described by Tilley and Terry (1963). All fermentations and digestions were run three times with each forage run separately.

Table 4.2. Total mixed ration fed to the donor cows.

Ingredient	% DM
Ground maize	38.30
Lucerne hay	28.31
Maize gluten	7.25
Wheat straw	6.60
Sugarcane molasses	5.62
Soybean meal	3.07
Barley malt	3.03
Potato by-product meal	2.17
Dry molasses	1.84
Feather meal with blood	1.54
Limestone	0.85
Blood meal	0.65
Salt	0.44
Urea	0.15
Monocalcium phosphate	0.13

4.3.3 Statistical analyses

Rates of NDF digestion were computed using a first order decay model according to the following equation:

NDF(t) = pdNDF(0) * e$^{-kd(t-L)}$ + iNDF

Where pdNDF(0) is the potentially digestible NDF at time 0; kd is the fractional rate of digestion of NDF; L is the lag and iNDF is the indigestible NDF. Simultaneous estimations of the parameters pdNDF, kd, iNDF and L were initially obtained using PROC NLIN of SAS (SAS Institute, Inc., Cary, NC) and the Marquardt algorithm. The Marquardt algorithm was selected to improve the efficiency of providing least-squares estimation for the non-linear curve fitting approach. Non-linear regression was chosen as the standard procedure because the method assumes equal error at each observation and by simultaneously fitting all parameters to the data, the result provides the smallest residual sums of squared deviations. The necessity of establishing initial parameters values for the non-linear estimations was solved using a linear approach to seed the non-linear estimation as done by Grant and Mertens (1992). We used the log-linear approach of Van Soest et al. (2005) to generate the initial values for each sample to parameterize the decay model, including an indigestible pool for the model using 240 h residual NDF to estimate the pdNDF. *In vitro* NDF digestibility values and the rates estimated by nonlinear regression were analysed as response variables by the GLIMMIX procedure of SAS using a factorial arrangement of forage, oxygen stress, yeast, yeast frequency and all interactions. Run was added as random factor. The control parameters for NDF were the digestibility and rates of the forages, when fermented alone. Differences between means and the control were declared significant at $P \leq 0.05$ using the least squares means and the Tukey adjustment. Statistical differences resulting in $0.05 < P \leq 0.10$ were considered tendencies. Treatments are reported as least squares means.

4.4 RESULTS AND DISCUSSION

4.4.1 Neutral detergent fibre digestibility

Neutral detergent fibre digestibility decreased with the addition of yeast, although the differences were not significant ($P = 0.2505$). There were no significant interactions between dose with forage ($P = 0.8937$), air ($P = 0.9837$), or time ($P = 0.1312$). The control resulted in numerically higher NDFd estimates for both forages compared to the yeast treatments, although the one-dose presented higher estimates compared to the two-doses (Table 4.3).

Table 4.3. The effect of dose and forage NDFd.

Forage	Dose			
	Control	One-dose	Two-doses	SEM[1]
MS	0.5295	0.5280	0.5201	0.0137
Lucerne	0.3513	0.3503	0.3460	0.0137

[1]Standard error of the means.

The highest NDFd estimates were obtained with the absence of oxygen stress for the control and both yeast doses, with NDFd decreasing numerically with the addition of yeast regardless of oxygen stress (Figure 4.1). Digestibility was hindered significantly ($P < 0.0001$) with the presence of additional O_2 within the *in vitro* environment, which could be an indicator of the inability of anaerobic bacteria to tolerate increased levels of O_2, and causing a reduction in the adhesion of bacteria to cellulose (Roger *et al.*, 1990). It was previously suggested by Baldwin and Emery (1960) that facultative anaerobes are capable of utilising highly oxidative compounds such as O_2, whereby maintaining a relatively constant anaerobic environment with a low redox potential. They found no noticeable effects on *in vitro* fermentation with the addition of O_2, which was not the case in the present study. It has also been suggested that yeast products have the ability to utilize O_2 that may enter the rumen and therefore create a more optimal environment for the microbial population especially cellulolytic bacteria (Chaucheyras-Durand *et al.*, 2008; Jouany, 2001; Kutasi *et al.*, 2004). This too was not the case in the present study which showed dismissive effects of yeast on NDF digestion in the presence of O_2. This, however, could be attributed to the strain of *S.cerevisiae* being used as seen by Newbold *et al.* (1996). They found that *S.cerevisiae* strains NCYN 240, NCYN 1026 and a commercial product (Yea-Sacc®) had the ability to stimulate O_2 uptake, while strains NCYC 694 and NCYC 1088 failed to have any noticeable effects.

Although NDFd increased with an increase in time, there were no significant interactions between dose and time (P = 0.1312).
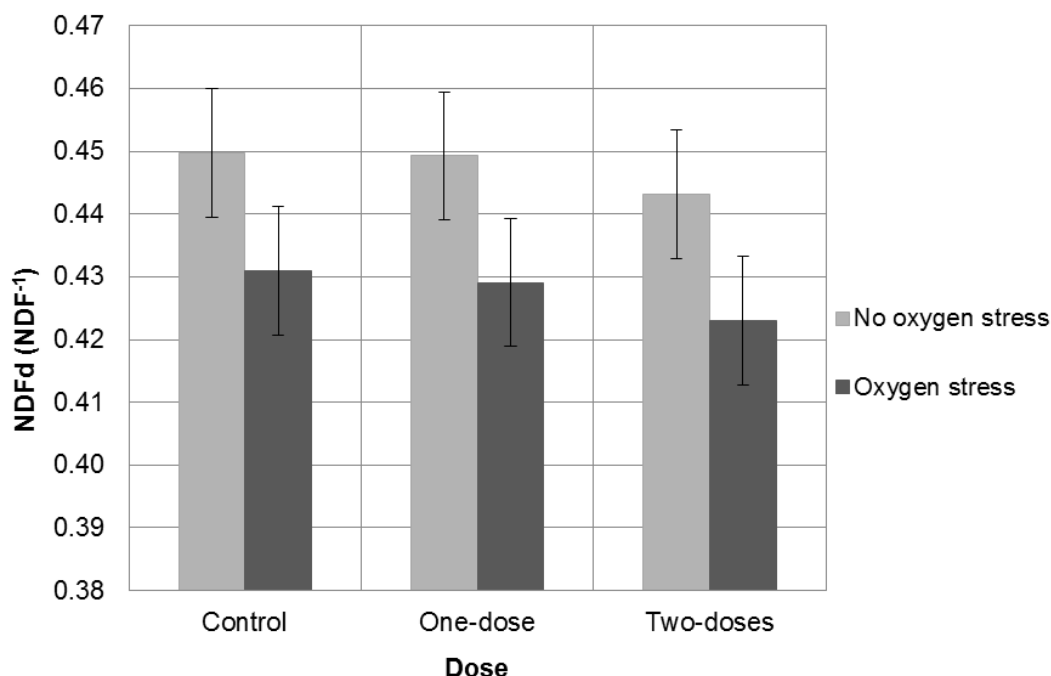

Figure 4.1. The effect of oxygen stress and dose on NDFd.

Maize silage had a higher quality NDF fraction resulting in higher digestibility (P < 0.0001), when compared to lucerne. However, NDF digestion of MS was also more sensitive to oxygen compared to lucerne that was not affected by the air and water injections (P = 0.6714; Table 4.4). Lucerne has previously been shown to have a stronger buffering capacity when compared to other forages (Jasaitis *et al.*, 1987). In our study, lucerne was also able to create a more stable environment when challenged by oxygen. However, if a different lucerne source had been used with a more digestible cell wall, it might have resulted in a significant difference, as seen with MS. The microflora may have also been more adapted to lucerne since it was also part of the donor cows' diet, unlike maize silage. Therefore, the oxygen stress did not affect the bacteria connected to the lucerne forage particles as much as it did the bacteria connected to maize silage.

**Table 4.4.** The effect of oxygen stress and forage on NDFd.

Forage	Oxygen stress		
	No	Yes	SEM[1]
Maize silage	0.5445[Aa]	0.5073[Ab]	0.0134
Lucerne	0.3503[B]	0.3480[B]	0.0134

[AB]Means within a column with different upper case superscripts differ ($P < 0.05$).
[ab]Means within a row with different lower case superscripts differ ($P < 0.05$).
[1]Standard error of the means.

Oxygen stress had no significant effect ($P = 0.1704$) on NDFd at 12 h while at 24 and 48 h NDFd values were different, with absence of oxygen stress resulting in higher ($P < 0.0001$) estimates compared to the oxygen stress treatment (Figure 4.2). This demonstrated how bacteria were more sensitive to repeated air and water injections from 12 h.
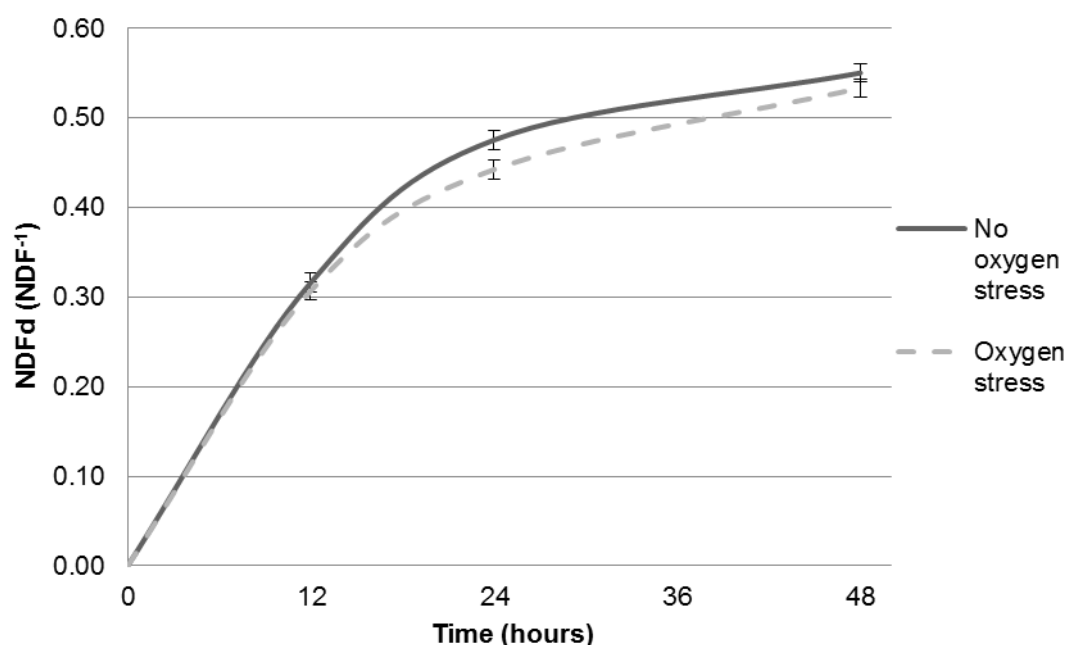
Figure 4.2. The effect of oxygen stress on NDFd over time.

4.4.2 Rate of NDF digestion

The rate of NDF digestion increased as the number of yeast doses increased, although not significantly ($P = 0.8659$), confirming the NDFd results. There were no significant interactions between dose and forage ($P = 0.9306$) or between dose and air ($P = 0.8094$). Lucerne had higher ($P < 0.0001$) rate estimates compared to MS within the same yeast treatment group (Figure 4.3). This could be due to the fact that lucerne usually has a smaller amount of potentially digestible NDF (pdNDF) due to a higher lignin content which results in a higher rate of digestion.
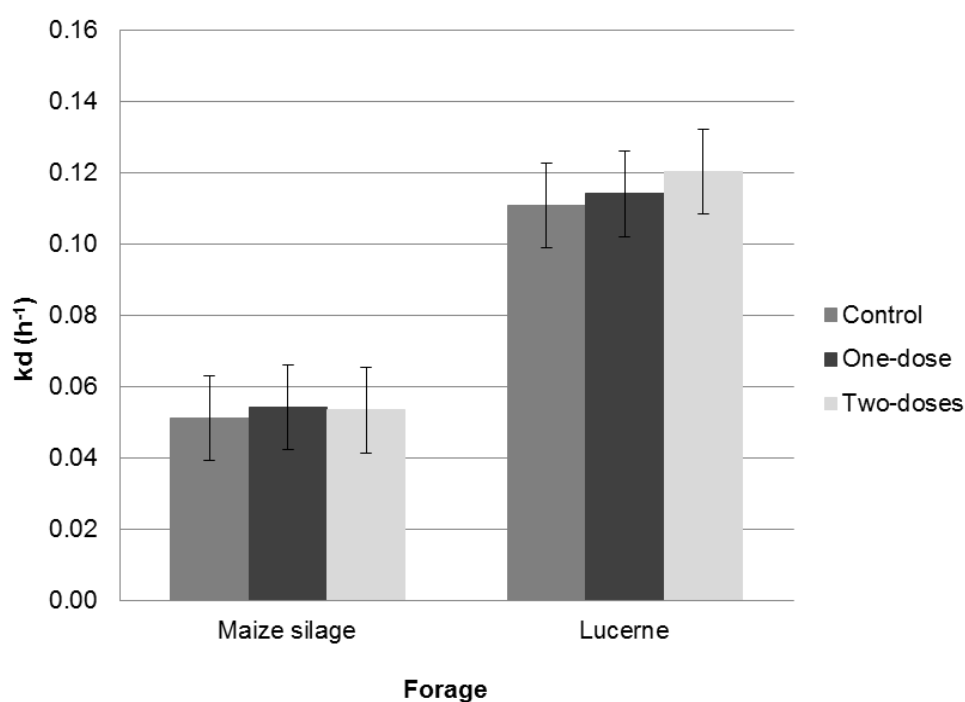

Figure 4.3. The effect of dose and forage on the kd.

Treatments without oxygen stress had a higher rate of digestion of NDF for both forages compared to treatments with oxygen stress (Figure 4.4) confirming the NDFd results and the negative effects of oxygen on fibrolytic bacteria.
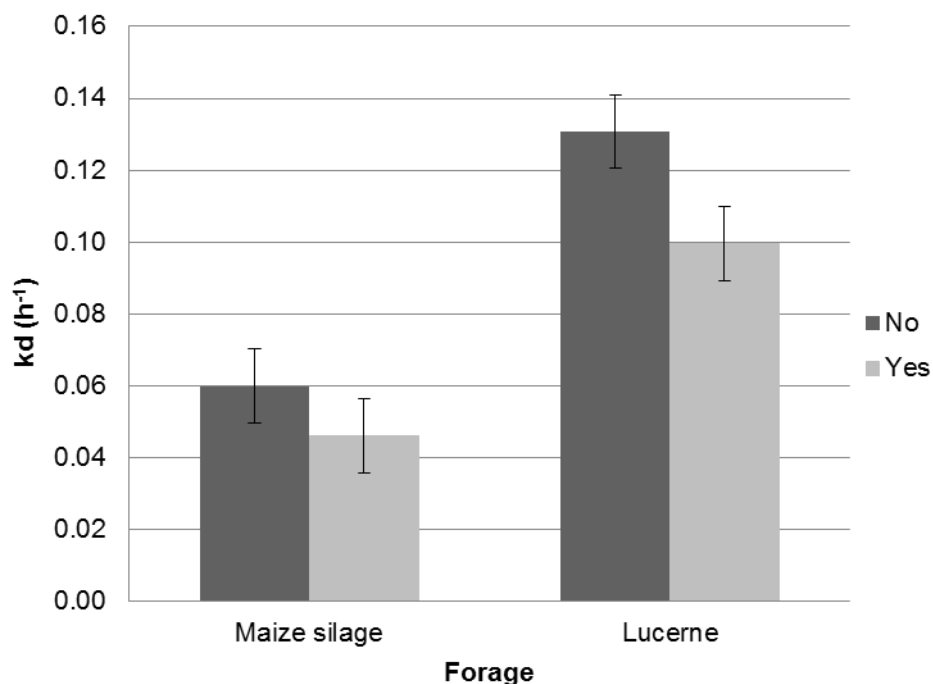
Figure 4.4. The effect of forage and oxygen stress on the kd.

4.4.3 Organic matter and dry matter digestibility

Yeast did not have any effect on DMd or OMd (P = 0.2851 and 0.2667, respectively). There were no significant interactions between dose with forage, air or time. Maize silage had significant differences (P < 0.05) between digestibility estimates resulting in higher values compared to lucerne. For MS, digestibility estimates decreased with an increase in the number of yeast doses while for lucerne the highest digestibility estimate was achieved with one-dose (Tables 4.5 and 4.6). However, we don't believe the differences to be biologically important since the difference of DMd for maize between no yeast and two doses of yeast was of only 0.0082 DM^{-1}.

Table 4.5. The effect of dose and forage on DMd.

Forage	Dose			
	Control	One-dose	Two-dose	SEM[1]
MS	0.7121[a]	0.7074[ab]	0.7039[b]	0.008524
Lucerne	0.6617[a]	0.6643[a]	0.6613[a]	0.008524

[ab]Estimate values within a row with different superscripts differ ($P < 0.05$).
[1]Standard error of the means.

Table 4.6. The effect of dose and forage on OMd.

Forage	Dose			
	Control	One-dose	Two-dose	SEM[1]
MS	0.7206[a]	0.7162[ab]	0.7119[b]	0.0091
Lucerne	0.6735[a]	0.6767[a]	0.6743[a]	0.0091

[ab]Means within a row with different superscripts differ ($P < 0.05$).
[1]Standard error of the means.

Oxygen stress had a significant effect on MS OMd and DMd with higher estimates being observed for samples not receiving oxygen stress, while there were no significant differences observed for lucerne (Figures 4.5 and 4.6).
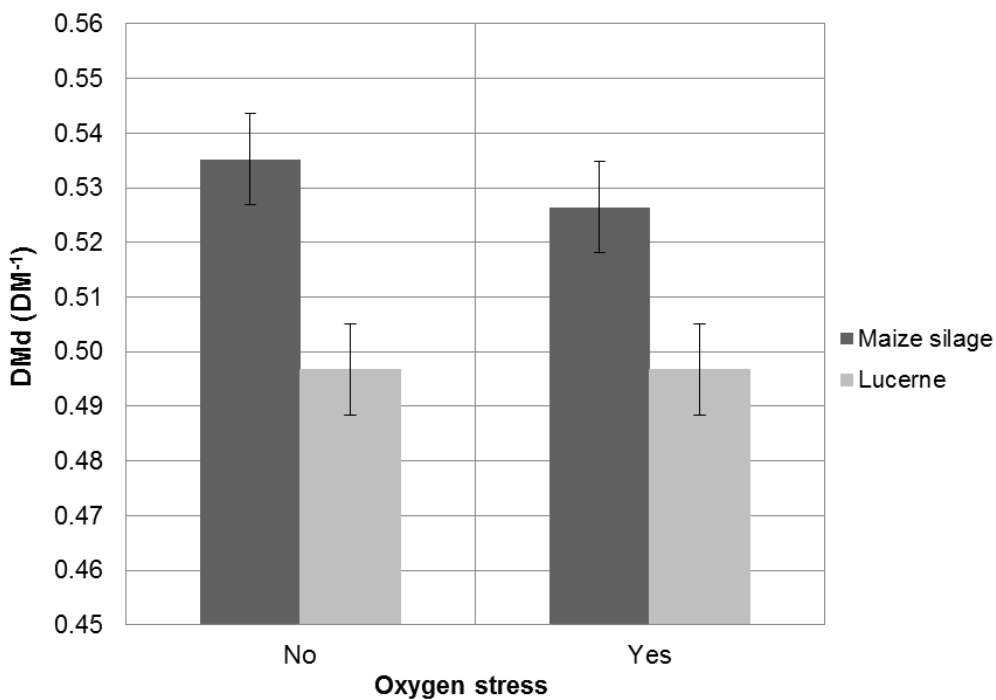
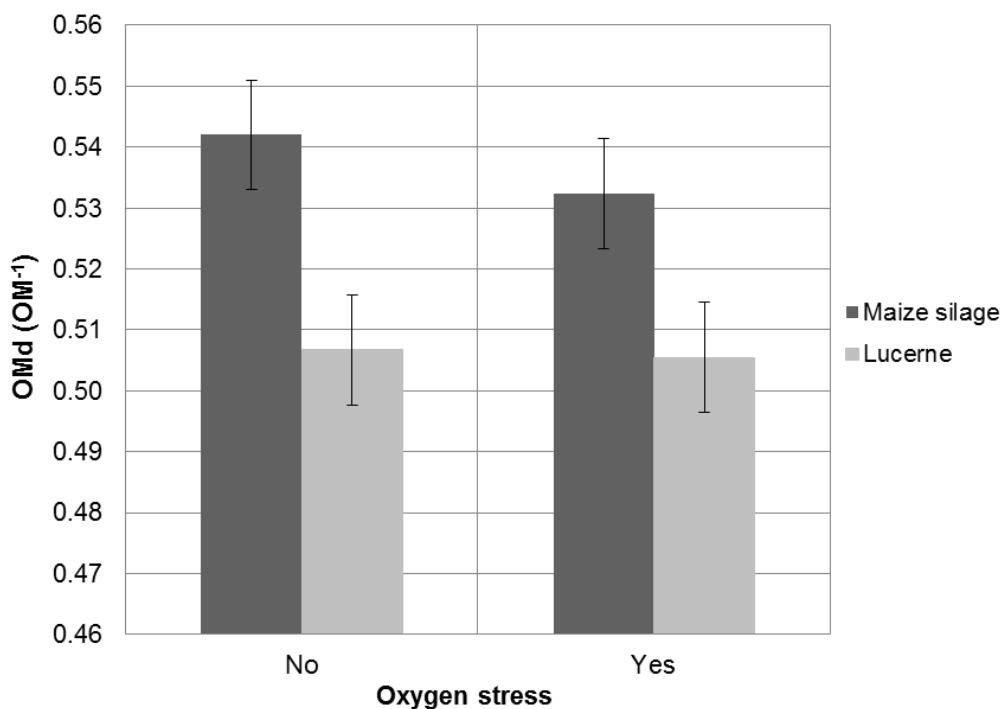**Figure 4.5.** The effect of forage and oxygen stress on DMd.

Figure 4.6. The effect of forage and oxygen stress on OMd.

Yeast had no significant effect on digestibility whether oxygen stress was applied or not (P = 0.8602 and 0.7466 for DMd and OMd, respectively). There were no significant differences (P > 0.05) between either yeast treatments and the control within the 12 and 24 h time points whereas at 48 h, the two-dose yeast treatment had significant differences (P < 0.05) for DMd and OMd resulting in lower estimates.

The two forages had variable results over the three time points. At 12 h, lucerne had significantly higher (P = 0.0128 and 0.0133 for DMd and OMd, respectively) estimates for both DMd and OMd compared to MS. However, MS had estimates for both DMd and OMd (Table 4.7) at 24 and 48 h, showing how the digestible pool for lucerne was exhausted by 24 h.

Table 4.7. The effect of forage on DMd and OMd over time.

Time (hours)	Forage		
	Maize silage	Lucerne	SEM[1]
DMd (DM^{-1})			
12	0.6092	0.6399	0.0085
24	0.7209[a]	0.6682[b]	0.0085
48	0.7933[a]	0.6791[b]	0.0085
OMd (OM^{-1})			
12	0.6210	0.6537	0.0091
24	0.7288[a]	0.6804[b]	0.0091
48	0.7990[a]	0.6904[b]	0.0091

[a,b]Means within a row with different superscripts differ (P < 0.05).
[1]Standard error of the means.

4.4.4 Volatile fatty acids

The yeast dose significantly affected the concentrations of P (P = 0.0448) and B (P < 0.0001), with the yeast doses resulting in lower estimates compared to the control.

There was no significant effects of dose ($P > 0.05$) on A, TVFA or A:P ratio. There was a significant interaction between dose and forage for B ($P < 0.0001$), while showing a tendency for TVFA ($P = 0.0581$; Table 4.8). The control resulted in higher B concentrations compared to one or two doses of yeast (10.45 vs. 9.12 and 8.95 mM, respectively). There were no significant interactions between dose and time ($P > 0.05$) for any of the VFA, while dose and air interacted significantly for P ($P = 0.0467$) and B ($P = 0.0002$) with a tendency for TVFA ($P = 0.0659$; Table 4.9). Oxygen stress was able to cancel out the yeast effect, as it resulted in significant differences only without the oxygen stress with lower VFA concentrations in presence of yeast.

Table 4.8. The effect of dose and forage on VFA concentration.

Forage	Dose			
	Control	One-dose	Two-doses	SEM[1]
Acetate (mM)				
Lucerne	40.13	40.01	38.96	4.7760
Maize silage	51.15[a]	45.60[b]	45.68[b]	4.7760
Propionate (mM)				
Lucerne	13.74	13.61	13.42	0.6712
Maize silage	17.73[a]	16.39[b]	16.32[b]	0.6712
A:P				
Lucerne	2.96	2.99	2.92	0.3572
Maize silage	2.82	2.75	2.78	0.3572
Butyrate (mM)				
Lucerne	4.99	5.11	5.10	1.0477
Maize silage	10.45[a]	9.12[b]	8.95[b]	1.0477
Total VFA (mM)				
Lucerne	63.33	64.40	63.54	5.7700
Maize silage	83.48[a]	75.91[b]	75.98[b]	5.7700

[ab]Means within a row with different superscripts differ ($P < 0.05$).
[1]Standard error of the means.

Table 4.9. The effect of dose and oxygen on VFA concentration.

Oxygen stress	Dose			
	Control	One-dose	Two-doses	SEM[1]
Acetate (mM)				
No	60.72[a]	56.14[b]	54.20[b]	3.5510
Yes	30.56	29.47	30.44	3.5510
Propionate (mM)				
No	20.75[a]	19.58[b]	18.95[b]	0.5385
Yes	10.72	10.42	10.78	0.5385
A:P				
No	2.95	2.87	2.86	0.2562
Yes	2.82	2.87	2.85	0.2562
Butyrate (mM)				
No	10.21[a]	9.20[b]	8.84[b]	0.7480
Yes	5.23	5.03	5.20	0.7480
Total VFA (mM)				
No	97.55[a]	91.90[b]	89.27[b]	4.2995
Yes	49.26	48.41	50.25	4.2995

[a,b]Means within a row with different superscripts differ ($P < 0.05$).
[1]Standard error of the means.

Forage was significant for P (P =0.0005), B (P = 0.0046) and showed a tendency for TVFA (P = 0.0690), with MS resulting in higher concentrations (Figure 4.7). While the higher P concentration for MS is associated with the higher starch content (Ishler *et al.*, 1996), the other increased VFA are due to a general larger digestible pool for MS, compared to lucerne, resulting in higher concentrations. There were significant interactions between forage and air for all VFA and the A:P ratio. In the absence of

oxygen stress, concentrations were near double the amount for both MS (Figure 4.8) and lucerne (Figure 4.9). This is indicative of the negative effect that oxygen has on all anaerobic bacteria within the rumen, not subjective to having an effect on a specific groups of bacteria such as cellulolytic or starch-degrading bacteria. However, the acetic acid reduction was apparently the largest compared to the other VFA.
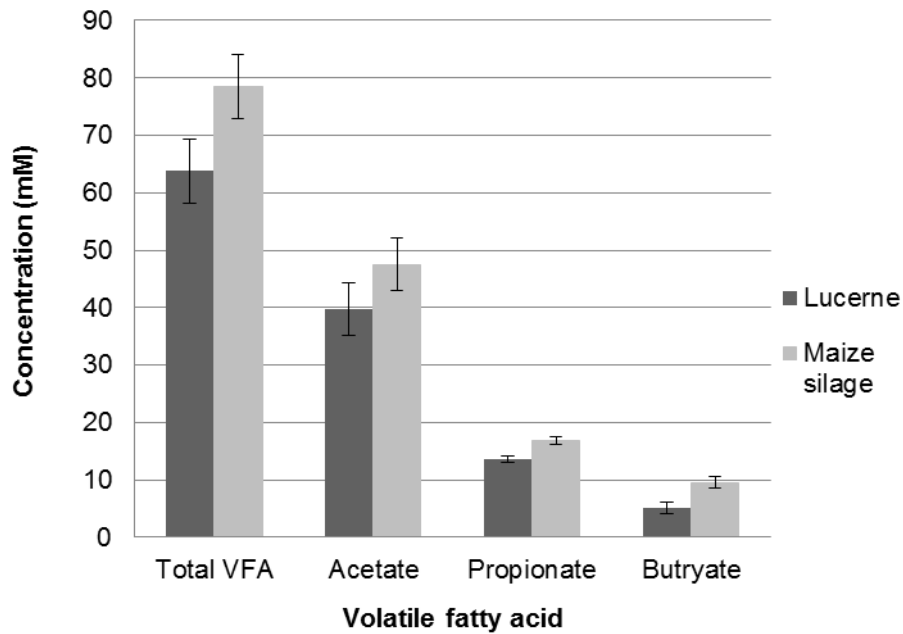

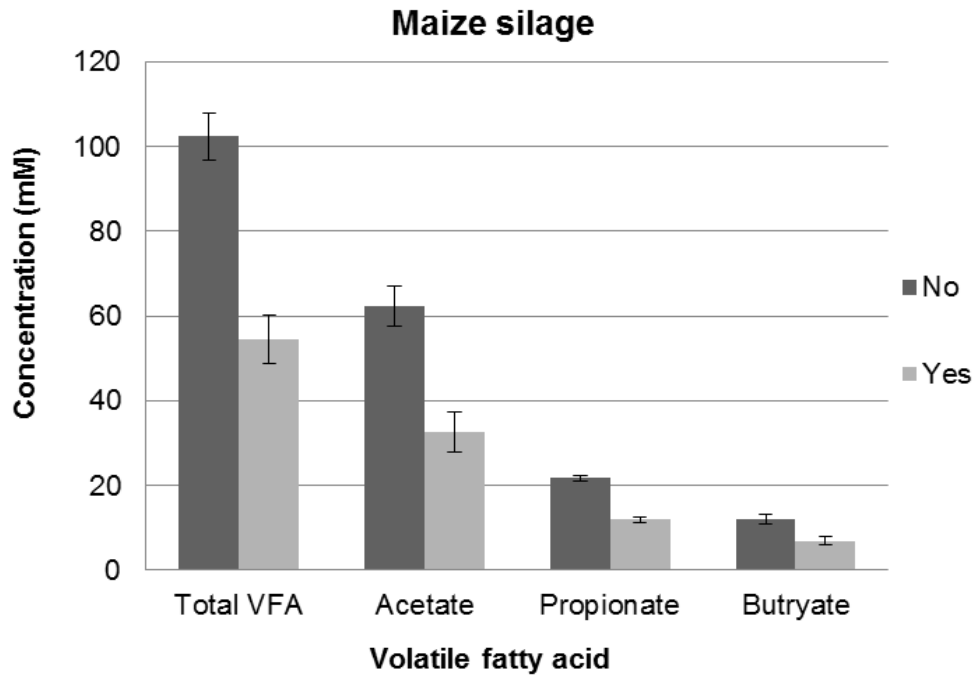**Figure 4.7.** The effect of forage on the concentration of VFA.

Figure 4.8. The effect of oxygen stress on the concentration of VFA for maize silage.
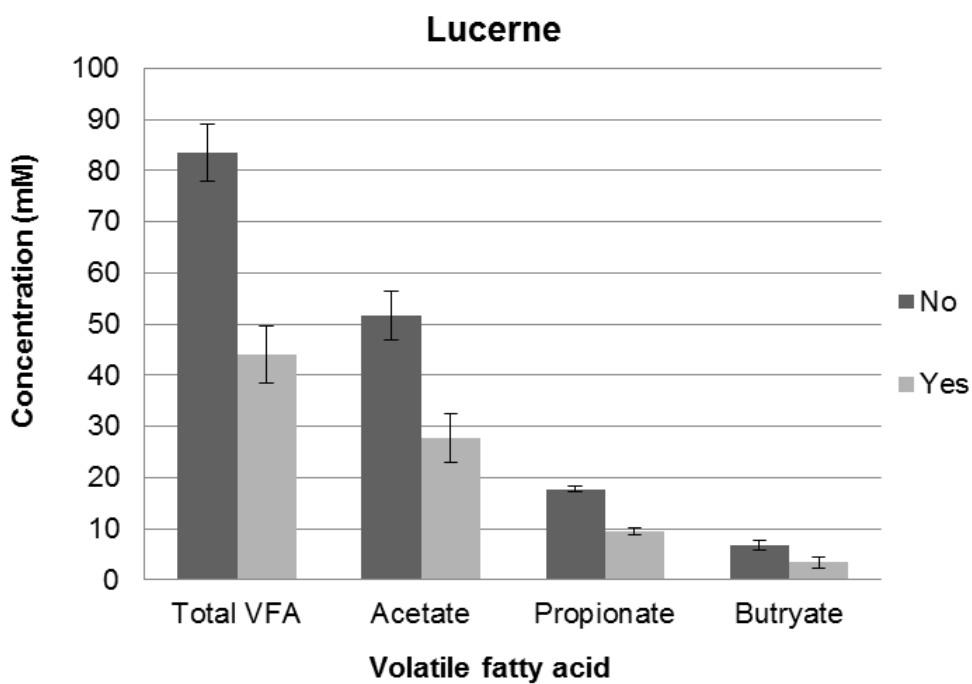

Figure 4.9. The effect of oxygen stress on the concentration of VFA for lucerne.

4.4.5 pH

Dose had a significant effect on pH ($P < 0.0001$) resulting in decreasing pH measured *in vitro* as the number of yeast doses increased. This does not correspond with the VFA results for dose where TVFA concentrations did not differ significantly. Yeast dose interacted with time ($P < 0.0001$) and tended to interact with forage ($P = 0.0502$), while no significant interaction was observed with oxygen stress ($P = 0.2458$). Although the pH decreased from 0 h up to 24 h and then increased again up to 48 h (Figure 4.10) for all treatments, a more positively stable pH environment was observed for the two-dose treatment. This is because there were no significant interactions between pH and two-doses at 12, 24 or 48 h, therefore resulting in a more stable and controlled pH environment by the yeast over time. Regardless, at 12, 24 and 48 h both yeast treatments differed significantly ($P < 0.05$) from the control, resulting in lower pH estimates for both treatments. This was similar to the results found by Wang *et al.* (2016) who found significantly lower ($P < 0.05$) pH estimates for the yeast treatments compared to the control, while O'connor *et al.* (2002) and Cobos *et al.* (2010) found no significant differences between the control and yeast treatments used. Although both authors used *Saccharomyces cerevisiae,* Cobos *et al.* (2010) used the strain 1026 and Wang *et al.* (2016) used strain 1355.
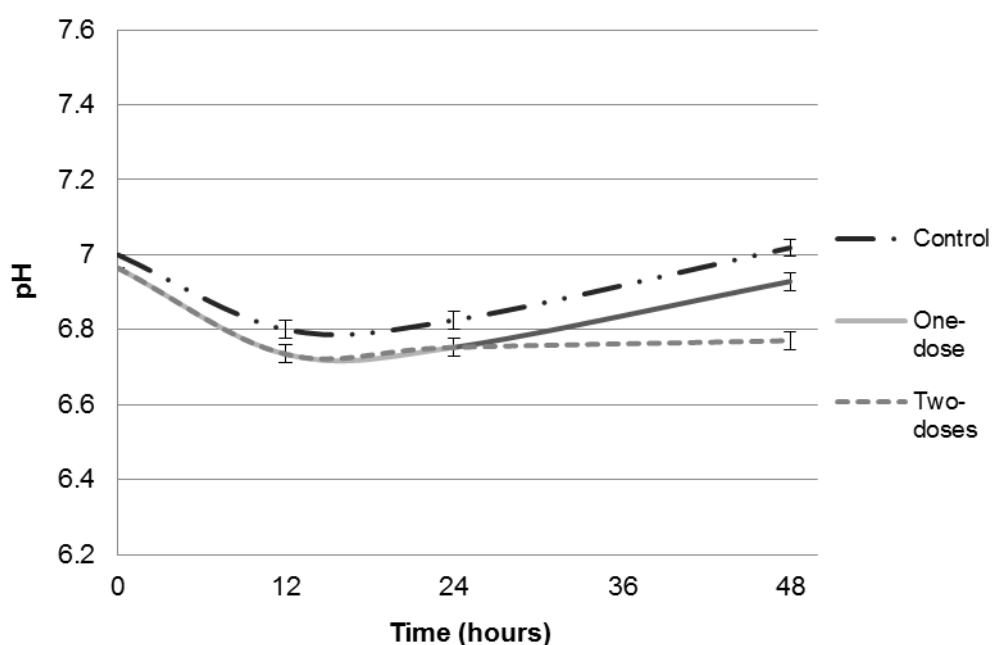

Figure 4.10. The effect of dose on pH over time.

Maize silage had significantly lower ($P < 0.0001$) estimates compared to lucerne with pH estimates decreasing as the number of yeast doses increased. Maize silage only resulted in a significantly higher ($P < 0.05$) pH compared to lucerne at 0 h after which lucerne had significantly higher estimates for 12, 24 and 48 h (Figure 4.11). The lower pH estimates observed for MS can be attributed to the higher level of both digestible NDF and starch, compared to lucerne, resulting in larger amount of volatile fatty acids *in vitro* which have a direct effect on pH. When looking at the effect of oxygen stress on pH, at 0 and 12 h there were no significant differences ($P > 0.05$; Table 4.10), while higher pH estimates were observed at 24 and 48 h. This goes against what we were expecting. In fact, O_2 entering on the environment would create a less negative redox potential which stimulates the growth of facultative anaerobes and lactic-acid producing bacteria and therefore a pH reduction is expected. On the other hand, most anaerobic rumen bacteria such as fibre digesting or lactic acid utilizers prefer a low redox potential. However, even if significant, the differences were very small and probably not biologically important (6.74 vs. 6.82 and 6.78 vs. 7.03 at 24 or 48 h, respectively).
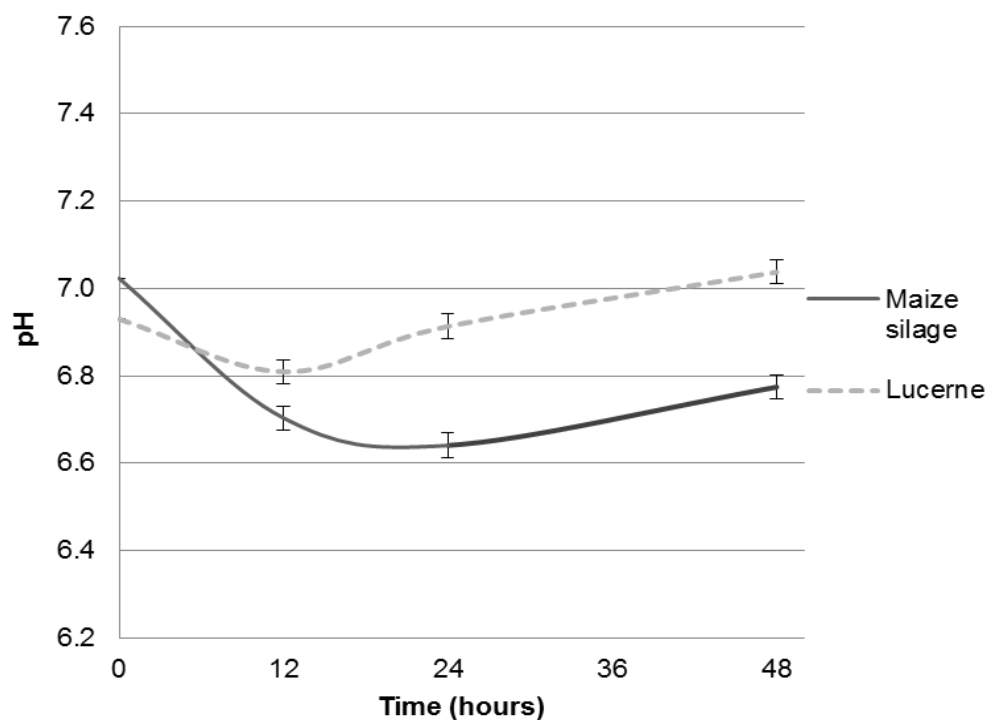

Figure 4.11. The effect of forage on pH over time.

Table 4.10. The effect of oxygen stress on pH over time.

Time (hours)	Oxygen stress		
	No	Yes	SEM[1]
0	6.98^A	6.98^A	0.0217
12	6.77^{BD}	6.74^B	0.0217
24	6.74^{Ca}	6.82^{Cb}	0.0217
48	6.78^{Da}	7.03^{Db}	0.0217

^{ABCD}Means within a column with different upper case superscripts differ ($P < 0.05$).
^{ab}Means within a row with different lower case superscripts differ ($P < 0.05$).
[1]Standard error of the means.

4.5 CONCLUSIONS

Yeast did not have significant effects on any of the response variables analysed and it did not result in significant interactions with either forage or oxygen stress for NDFd, kd, DMd and OMd. Yeast did interact with forage and oxygen stress for pH and VFA, however, the differences were not always significant.

Oxygen stress was instead consistent in causing decreases in digestibility estimates, VFA concentrations as well as rate of NDF digestion, while no differences were observed for pH. This indicates the negative effects that O_2 can cause upon the anaerobic bacteria within the rumen and, although facultative anaerobes are capable of utilizing O_2, the concentration of O_2 added in the current study may exceed their capability along with the fact that their presence in the rumen did not result in any compensation due to their lower numbers. Maize silage resulted in higher digestibilites which were accompanied by higher VFA concentrations and lower pH values as expected, however, lucerne had higher rates of digestion because of the smaller digestible pool.

This study confirms the strict anaerobic environment that is found within the rumen and the negative effects O_2 has on the fermentation efficiency. To our knowledge, there is not much research that has been done on the effects of O_2 *in vitro* on

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digestibility parameters with the use of a yeast supplement. We did not quantify molecular oxygen and therefore the amount injected through air and water, while simulating feeding and drinking behaviour, might have been higher than the oxygen that the yeast is able to cope with. Therefore further research and more precise measurements might be needed to determine O_2 concentrations at which bacteria can withstand and a concentration of O_2 which *S. cerevisiae* is shown to decrease effectively. Further research can also be done on the specific strains of yeast to identify which strains are able to utilize O_2 more efficiently.

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CHAPTER 5

Interactions between *Saccharomyces cerevisiae* and forage type in a starch- and pH-challenged *in vitro* fermentation system

5.1. ABSTRACT

The objective of this study was to investigate the effects of using a live yeast, *Saccharomyces cerevisiae*, in a starch- and pH-challenged *in vitro* environment on neutral detergent fibre digestibility (NDFd) and rate of NDF digestion (kd), organic matter digestibility (OMd), dry matter digestibility (DMd), pH and volatile fatty acids (VFA) concentration. Two forages (lucerne and maize silage) were used and wheat grain was added as a source of starch and initial pH was reduced to 6.2 using citric acid. Wheat grain was combined with the forages with the objective of having a 1:1 ratio between NDF and starch for lucerne and maize silage. Another combination with maize silage (MS) was created but excluding the starch from the silage (DMS) in the calculation. Dose did not result in significant differences for NDFd, kd, DMd or OMd, while there were significant differences for pH and acetate, butyrate and total VFA. There were no significant interactions between dose and forage, starch or time for NDFd, kd, DMd, OMd or VFA. Forage interacted significantly with starch resulting in lower NDFd, DMd, OMd and pH (6.53 vs 6.24 and 6.3 vs 6.06 and 6.28 vs. 5.90 for lucerne, MS and DMS, respectively) estimates when wheat-starch was added. Forage had a positive interaction with yeast, showing increased pH estimates for yeast compared to the control, although differences were small. This study confirms the drop in pH values with added starch. Yeast was also shown to have a stabilizing effect on the rumen environment showing a numerical increase in pH and digestibility when challenged by the presence of starch.

5.2. INTRODUCTION

The diet of ruminants plays a large role in determining the pH of the rumen or the maintenance thereof, with pH being among the major factors affecting fibre digestion (Hoover, 1986). Among carbohydrates, starch in the diet is one of the causes for a

decrease in rumen pH, as a result of accumulation of propionic and lactic acids and subsequent effects on the microorganisms' population (Ishler *et al.*, 1996).

Although it has been reported that the rumen can reach a pH of 5.7, when the pH drops below 6, the digestion of fibre by bacteria is severely impaired (Rode, 2000). Cellulolytic bacteria are unable to grow at a low pH (less than 6.0; Rode, 2000) and to attach to feed particles (Miron *et al.*, 2001), therefore hindering the bacteria's ability to digest fibre. Although Grant and Mertens (1992) found no significant effects of pH on DMd of lucerne, hay or maize silage, there was a significant difference in the lag times for all three substrates. A low pH resulted in longer lag compared to a higher one. Only maize silage resulted in a significant increase in the rate of digestion of NDFwhen pH was increased from 5.8 to 6.8.

Lynch and Martin (2002) found that the addition of *S. cerevisiae* live cells or *S. cerevisiae* culture had the ability to increase *in vitro* pH, although not always significant, when incubated with various substrates which were similar to findings by Thrune *et al.* (2009). They compared *in vivo* pH between cows supplemented with 0.5 g/d of *S. cerevisiae* to a control. There was a significant increase in the mean, minimum and maximum pH values with the supplementation of the yeast.

Based on the positive relationship established between *in vitro* and *in vivo* experiments for live yeast effects, the objective of this study was to investigate the effects of using a live yeast, *S. cerevisiae*, in a starch- and pH-challenged *in vitro* micro-environment, on NDFd, kd, OMd, DMd, pH and VFA concentration.

5.3. MATERIALS AND METHODS

5.3.1 Forages and chemical analysis

Two forages, maize silage (MS) and lucerne, were dried at 60°C for 48 hours and then ground through a 1-mm screen using a Wiley Mill (Thomas Scientific, Swedesboro, NJ). Wheat grain that was used as a means of introducing starch as a stressor, was dried and milled in the same way. Samples were analysed for moisture and ash (AOAC, 2002; method 934.01 and 942.05), neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), ether extract (EE), crude protein (CP) and starch. Neutral detergent fibre was analysed as described by Mertens (2002) using Gooch crucibles with porosity 2. Acid detergent fibre and lignin

were analysed as described by Raffrenato and Van Amburgh (2011). All fibre fractions were analysed with Gooch crucibles fitted with glass fibre filter with porosity 1.5 μm (934-AH[TM] by Whatman®, Whatman Limited – GE Healthcare, Maidstone, UK; Raffrenato and Van Amburgh, 2011). Ether extract was determined using Tecator Soxtec System HT 1043 Extraction Unit (AOAC, 2002; Method 920.39). Crude protein was measured with a Leco N analyser ("FP-528" Leco Africa (Pty), Ltd, Kempton Park, South Africa), while starch was determined as described by Hall (2008). The chemical composition of the two forages used in this study can be seen in Table 5.1.

Table 5.1. Chemical composition of the forages used in the study on a DM basis (%).

Forages	Item							
	NDF	ADF	ADL	EE	CP	Starch	Moisture	Ash
Lucerne	43.26	38.55	8.72	1.49	17.29	2.28	6.40	9.14
Maize silage	49.40	26.76	3.02	3.02	6.53	25.50	9.21	4.72

5.3.2 *In vitro* fermentations

Two different ratios of wheat to MS and one ratio of wheat to lucerne were used for this experiment, with the objective of obtaining a specific starch proportion. The wheat used, even if ground with a 1-mm screen, it was sieved to a particle size smaller than 500 μm, to decrease the contribution of NDF from wheat and to increase sample homogeneity. The wheat had a starch and NDF content of 64.79 and 9.6 %DM, respectively. Forages only were included to serve as controls. The amount of total starch for the combination wheat-MS was calculated by either including (MS) or not including (DMS) the maize-starch to determine if the different starch source and amount can have a different effect on the response variables measured. The total amount of starch was decided based on preliminary tests that revealed the minimum amount needed to affect the fermentation profile. The final ratio of NDF:starch was

theoretically equivalent to 1 with the final starch content for lucerne, MS and DMS being 26.49, 36.09 and 42.50 %DM, respectively.

Combinations of each forage with yeast and wheat were incubated in quadruplet for 0, 12, 24 and 48 h, following the procedure described by Goering and Van Soest (1970). Samples were also incubated for 120 and 240 h, to calculate rate of NDF disappearance according to Raffrenato and Van Amburgh (2010).

Each sample, in the correct ratio, was weighed into 125-ml Erlenmeyer flasks. Medium was made up according to Goering and Van Soest (1970) and was kept warm at 39°C and anaerobic under CO_2. Preliminary tests showed very little reduction of medium pH when wheat was fermented with forages at various proportions. To be able to further challenge the flask environment we lowered the initial medium pH to 6.2 using 1 M citric acid with an average of 70 ± 10 ml being added to 3.2 L of medium.

Rumen fluid was collected in the afternoon from two lactating Holstein cows receiving a total mixed ration (TMR; Table 5.2) and being housed at the Stellenbosch University research farm, Western Cape, South Africa. The trial was approved by the Stellenbosch University Research Ethics Committee (Animal Care and Use; Approval SU-ACUD15-00060). Rumen fluid was transported, in thermos flaks, to the laboratory where it was mixed and filtered through four layers of cheese cloth into a pre-warmed flask kept at 39°C. The correct volume of rumen fluid was measured and slowly added to the warm and anaerobic medium in a ratio of 1:4.The pH was measured again, and if needed, adjusted to a pH of 6.2. After obtaining a homogenous starting pH, medium and rumen fluid were properly mixed and 50 ml of the mixture was added to each flask. Blank flasks were also included to rectify for any particles that may have been present in the rumen fluid and all flasks were incubated for 0, 12, 24 or 48 h.

An active dry yeast, *Saccharomyces cerevisiae* CNCM I-1077, was prepared using peptone water (FDA and US Food and Drug Administration, 2001) and injected into the specific flasks receiving yeast to obtain a final concentration of $1x10^6$ cfu/ml within the flasks. At 24 h, a second dose of yeast (two-doses) was injected into the specific flasks and these flasks were further incubated for another 24 h.

Duplicates of the fermentations' residuals were analysed for NDF (Mertens, 2002) and pH was measured at the mentioned time points. Volatile fatty acids (VFA) were also determined via gas-liquid chromatography according to Siegfried (1984). The VFA analysed included acetate (A), propionate (P), butyrate (B), isobutyrate (IB), valerate (V), isovalerate (IV) and total VFA (TVFA), although more emphasis was placed on A, P, B and TVFA. Extra flasks with each treatment combination were added and removed at 0 h to measure initial pH and VFA as initial reference points. After ruminal *in vitro* fermentation, the other flasks were subjected to acid-pepsin digestion and analysed for dry and organic matter digestibility (DMd and OMd) as described by Tilley and Terry (1963). All fermentations and digestions were run three times with each forage run separately, leading to six replicates per forage and time point.

5.3.3. Statistical analyses

Rates of NDF digestion were computed using a first order decay model according to the following equation:

$$NDF(t) = pdNDF(0) * e^{-kd(t-L)} + iNDF$$

Where pdNDF(0) is the potentially digestible NDF at time 0; kd is the fractional rate of digestion of NDF; L is the lag and iNDF is the indigestible NDF. Simultaneous estimations of the parameters pdNDF, kd, iNDF and L were initially obtained using PROC NLIN of SAS (SAS Institute, Inc., Cary, NC) and the Marquardt algorithm. The Marquardt algorithm was selected to improve the efficiency of providing least-squares estimation for the non-linear curve fitting approach. Non-linear regression was chosen as the standard procedure because the method assumes equal error at each observation and by simultaneously fitting all parameters to the data, the result provides the smallest residual sums of squared deviations. The necessity of establishing initial parameters values for the non-linear estimations was solved using a linear approach to seed the non-linear estimation as done by Grant and Mertens (1992). We used the log-linear approach of Van Soest et al. (2005) to generate the initial values for each sample to parameterize the decay model, including an indigestible pool for the model using 240 h residual NDF to estimate the pdNDF. In vitro NDF digestibility values and the rates estimated by nonlinear regression were

analysed as response variables by the GLIMMIX procedure of SAS using a factorial arrangement of forage, starch ratio, yeast, yeast frequency and all interactions. Run was added as random factor. The control parameters for NDF were the digestibility and rates of the forages, when fermented alone. Differences between means and the control were declared significant at $P \leq 0.05$ using the least squares means and the Tukey adjustment. Statistical differences resulting in $0.05 < P \leq 0.10$ were considered tendencies. Treatments are reported as least squares means.

Table 5.2. Total mixed ration fed to the donor cows.

Ingredient	% DM
Ground maize	38.30
Lucerne hay	28.31
Maize gluten	7.25
Wheat straw	6.60
Sugarcane molasses	5.62
Soybean meal	3.07
Barley malt	3.03
Potato by-product meal	2.17
Dry molasses	1.84
Feather meal with blood	1.54
Limestone	0.85
Blood meal	0.65
Salt	0.44
Urea	0.15
Monocalcium phosphate	0.13

5.4. RESULTS AND DISCUSSION

5.4.1. Neutral detergent fibre digestibility

Neutral detergent fibre digestibility was highest for flasks receiving yeast compared to the control, although not significantly (P = 0.6483). There were no significant interactions between dose and forage (P = 0.6035), added starch (P = 0.3107) or time (P = 0.4478). The DMS combination resulted in the highest NDFd estimates and lucerne the lowest, with the difference between the two resulting in the only significance between forages (P = 0.0105). This indicates the higher quality NDF fraction of the maize compared to lucerne. The non-significant difference between the maize with and without added wheat grain in NDFd (0.3641 vs. 0.3294 NDF^{-1}) may have resulted from the addition of the extra highly digestible NDF from the grain, but this could not be determined after the fermentation. Digestibility increased numerically with the number of yeast doses for both treatments with maize forage; however the opposite was seen for lucerne with a decrease in digestibility (Table 5.3). The higher digestibilities seen with yeast for the maize silages could indicate the yeasts ability to interact better with a diet higher in starch, thus promoting the cellulolytic bacteria and increasing NDFd.

Table 5.3. The effect of dose and forage on NDFd.

Dose	Forage			
	Lucerne	MS	DMS	SEM[1]
Control	0.2735[b]	0.3208[ab]	0.3623[a]	0.0261
One-dose	0.2707[b]	0.3333[ab]	0.3648[a]	0.0261
Two-doses	0.2686[b]	0.3341[ab]	0.3651[a]	0.0261

[ab]Means within a row with different superscripts differ (P < 0.05).
[1]Standard error of the means.

When pooling combinations, samples with added starch resulted in lower digestibility values for all doses compared to samples with only forage ($P < 0.05$). Yeast increased digestibility over time but only from 24 h indicating yeasts ability to stabilize the rumen after 12 h and stimulating the cellulolytic bacteria. Forage interacted significantly ($P < 0.0001$) with starch resulting in digestibility estimates being lower (Figure 5.1). This could be due to the decrease in pH as a result of the higher starch content, hindering the growth and attachment to cell wall of the cellulolytic bacteria.
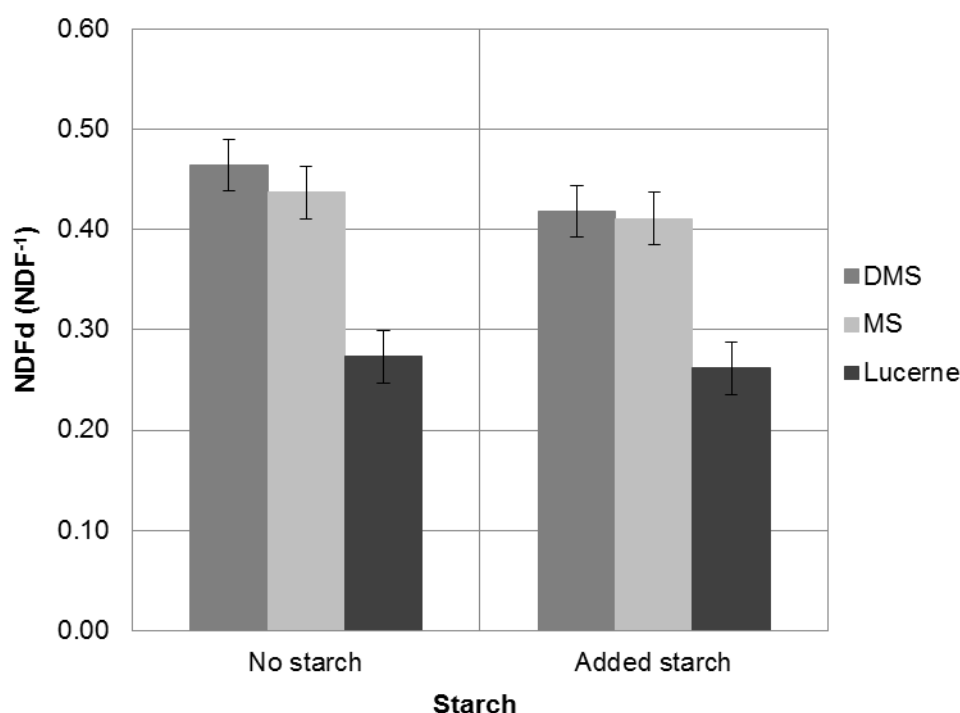
Figure 5.1. The effect of starch and forage on NDFd.

Digestibility increased for all forages over time ($P < 0.0001$), with both DMS and MS continuing to increase in digestibility after 24 h while lucerne beginning to plateau, indicating a smaller digestible NDF pool for lucerne (Figure 5.2). The interaction forage x starch x time was also highly significant ($P < 0.001$; Figures 5.3).
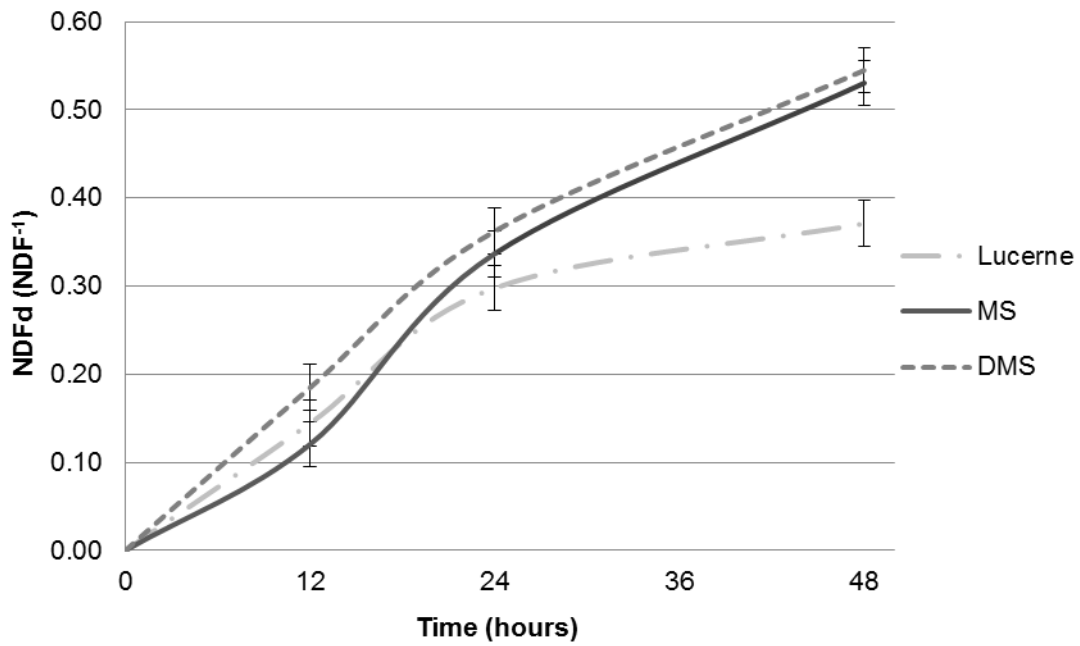
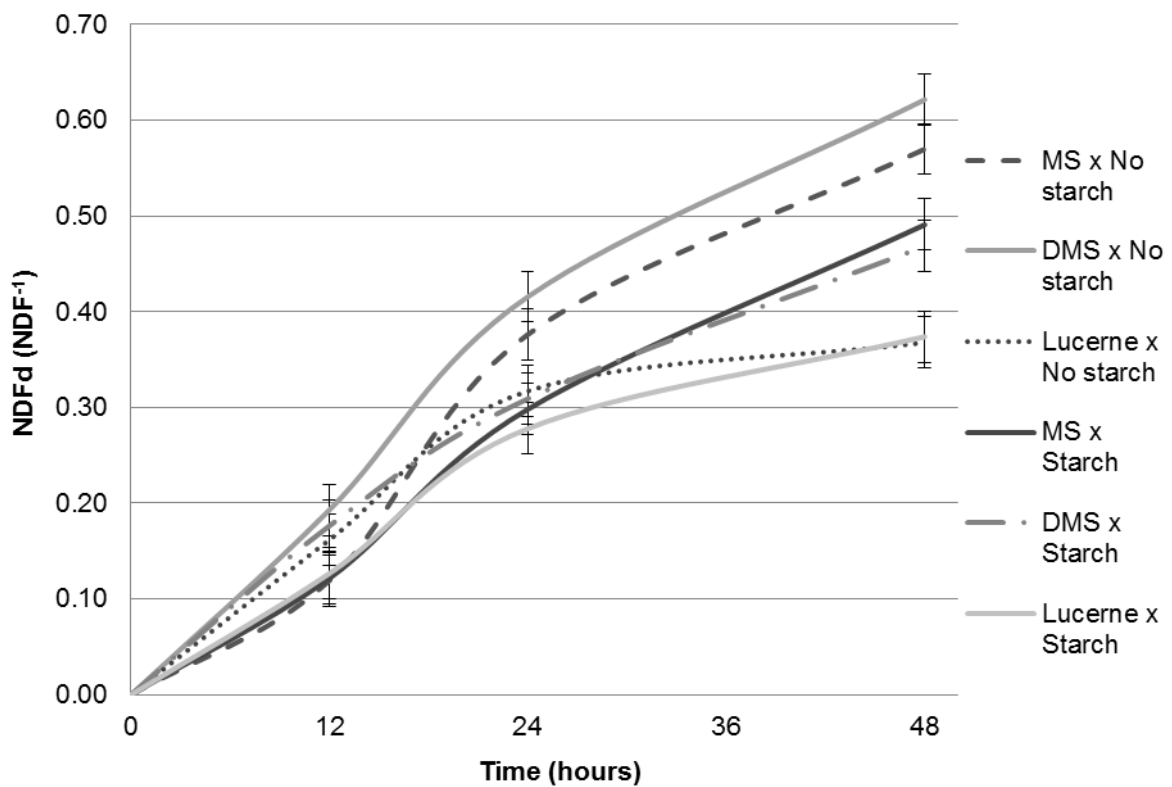**Figure 5.2.** The effect of forage on NDFd over time.

Figure 5.3. The effect of forage and starch on NDFd over time.

5.4.2. Rate of NDF digestion

The addition of yeast did not affect the rate of NDF digesion ($P = 0.9809$). There were no significant interactions between dose and forage ($P = 0.5865$) or between dose and starch ($P = 0.5398$). The rate of NDF digestion did, however, increase with the addition of yeast for both maize combinations while yeast decreased the estimates for lucerne (Figure 5.4). This again indicating yeasts ability to interact with a higher starch diet.
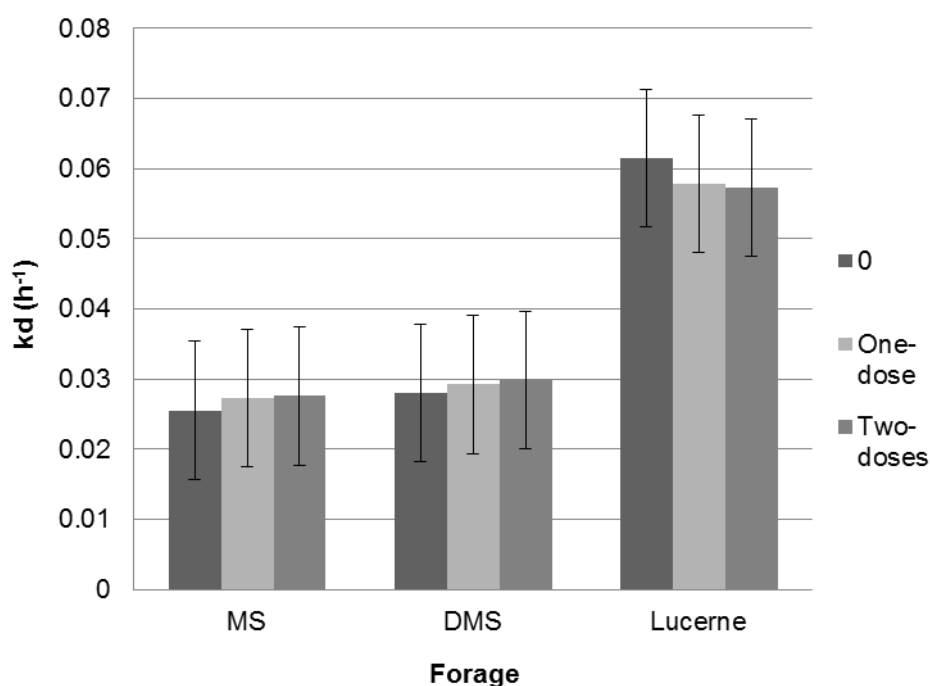

Figure 5.4. The effect of forage and dose on the kd.

The only significant differences were seen between the forages ($P = 0.0392$) and with starch ($P < 0.0001$). Lucerne had a higher rate of digestion of NDF compared to MS and DMS (Figure 5.5). Even though lucerne had a higher lignin content, the amount of potentially digestible NDF (pdNDF) of lucerne is usually smaller but of higher quality compared to maize or grasses.
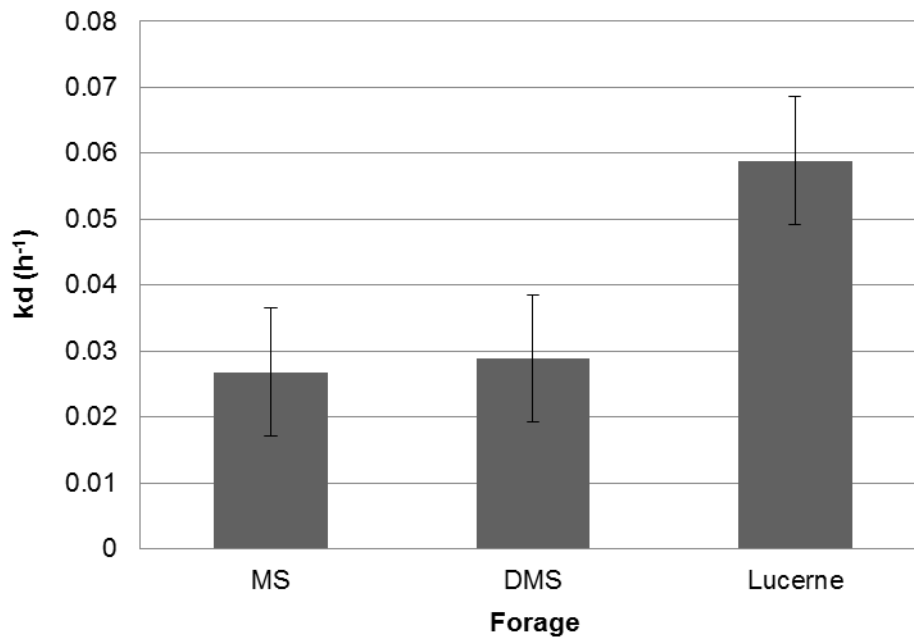
Figure 5.5. The effect of forage on the kd.

5.4.3. Dry matter and organic matter digestibility

Dry matter and organic matter digestibility increased with the addition of yeast, although the differences were not significant (P = 0.9510 and 0.9528). There were no significant interactions for DMd or OMd between dose and forage (P = 0.6442 and 0.5986), added starch (P = 0.2561 and 0.2798) or time (P = 0.8587 and 0.7857).

The highest DMd and OMd estimates were observed for DMS, while MS resulted in the lowest estimates across all doses, although not significantly. Estimates increased numerically with the addition of yeast for all forages, however, not significantly. There was a highly significant interaction between forage and starch for both DMd and OMd (P < 0.0001). None of the forages differed significantly from one another when no starch was added. However, with added starch, DMS and lucerne differed significantly from MS but did not differ from one another (Figures 5.6 and 5.7). The pooled DMd and OMd of the MS was in this case affected by the first stage of the digestion (i.e. ruminal) where the extra starch present, compared to the DMS, reduced NDFd, especially at 12 h (Figure 5.3).

The interaction between forage x starch x time was highly significant (P < 0.0001; Figures 5.8-5.1).
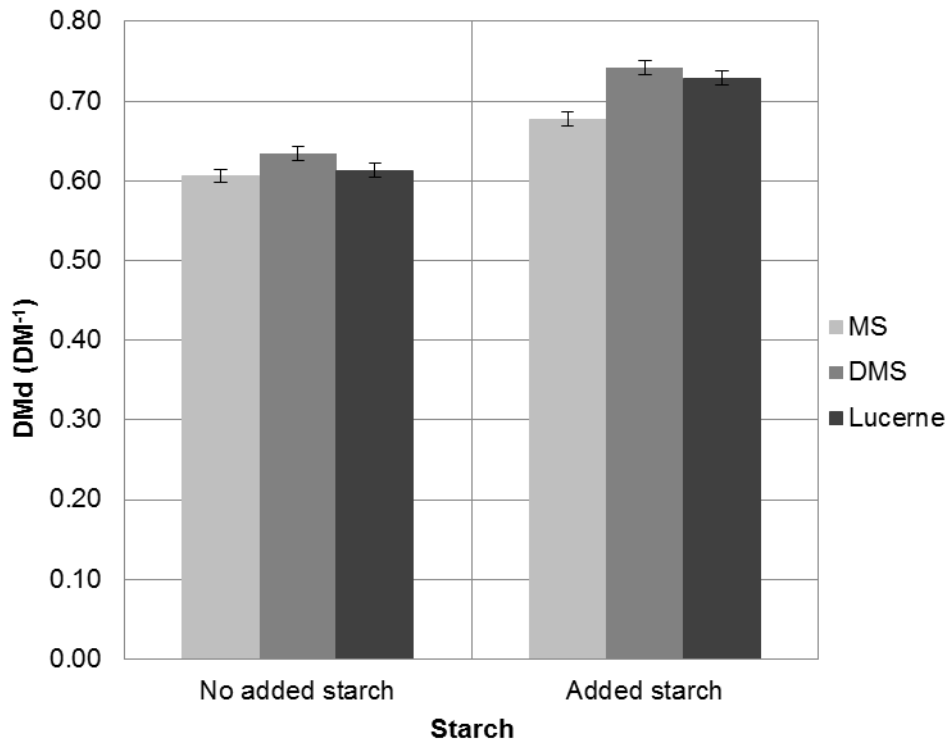
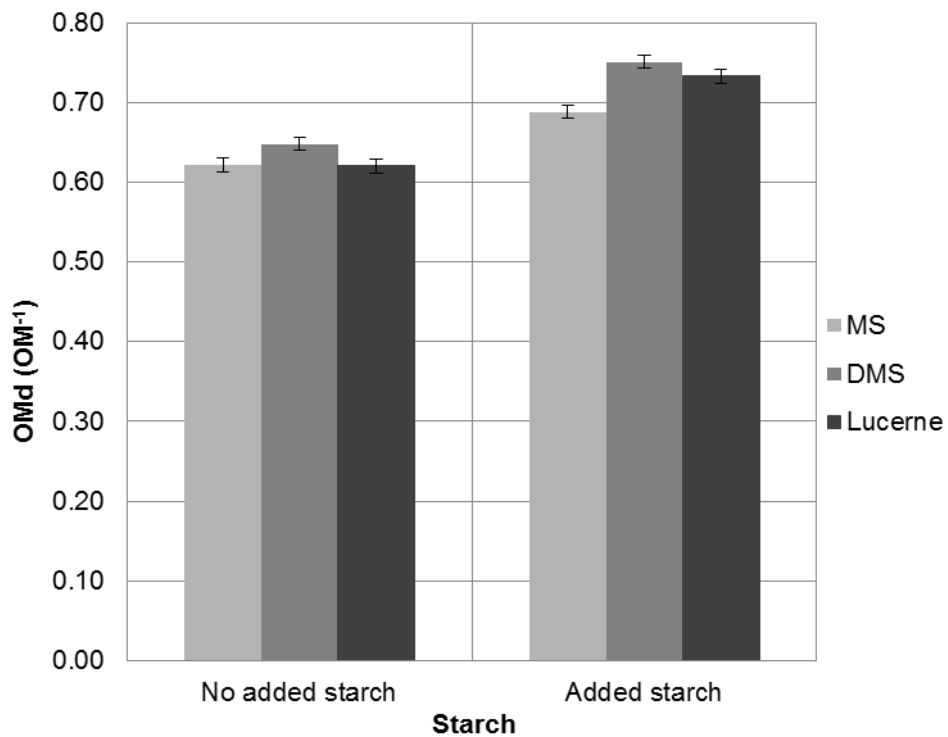**Figure 5.6.** The effect of forage and starch on DMd.

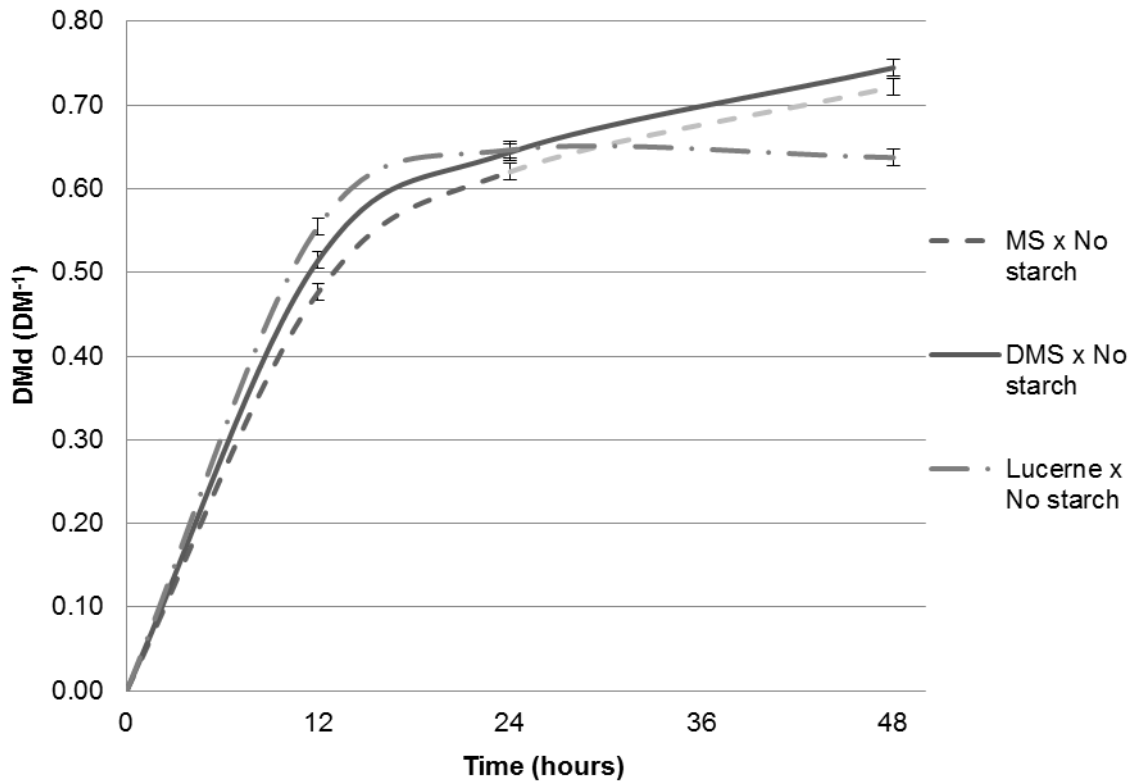**Figure 5.7.** The effect of forage and starch on OMd.

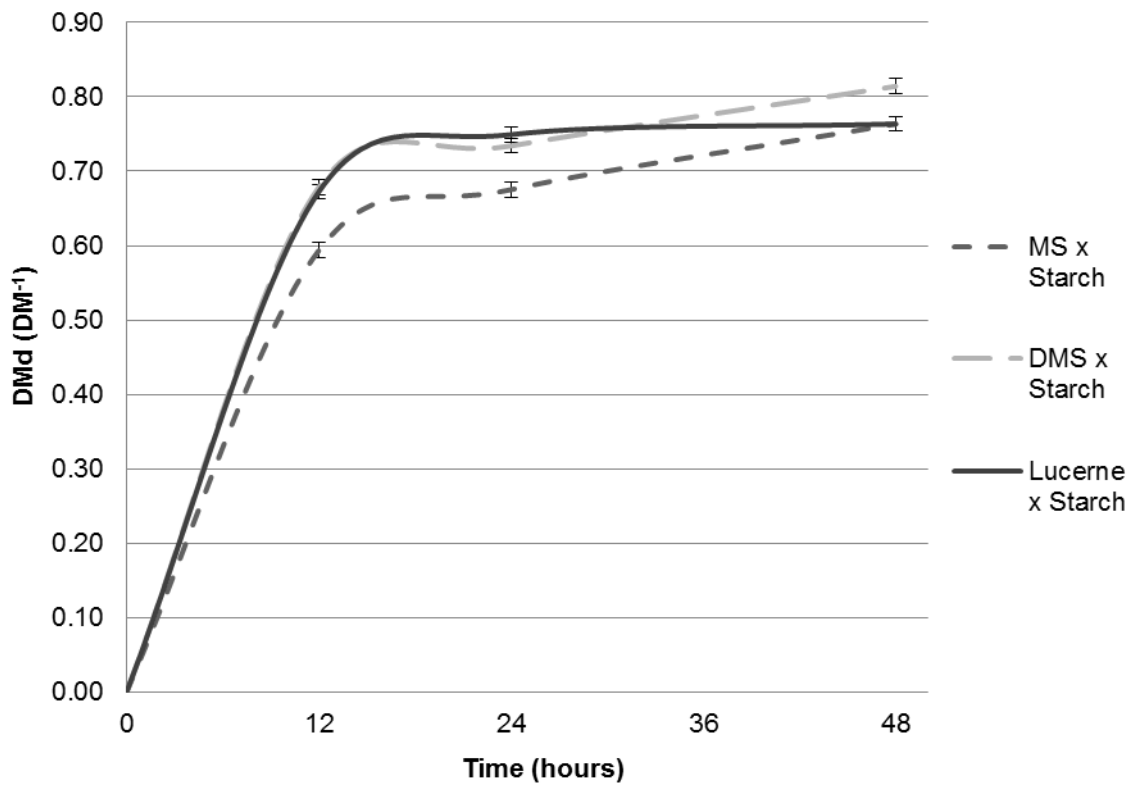**Figure 5.8.** The effect of forage and no added starch on DMd over time.

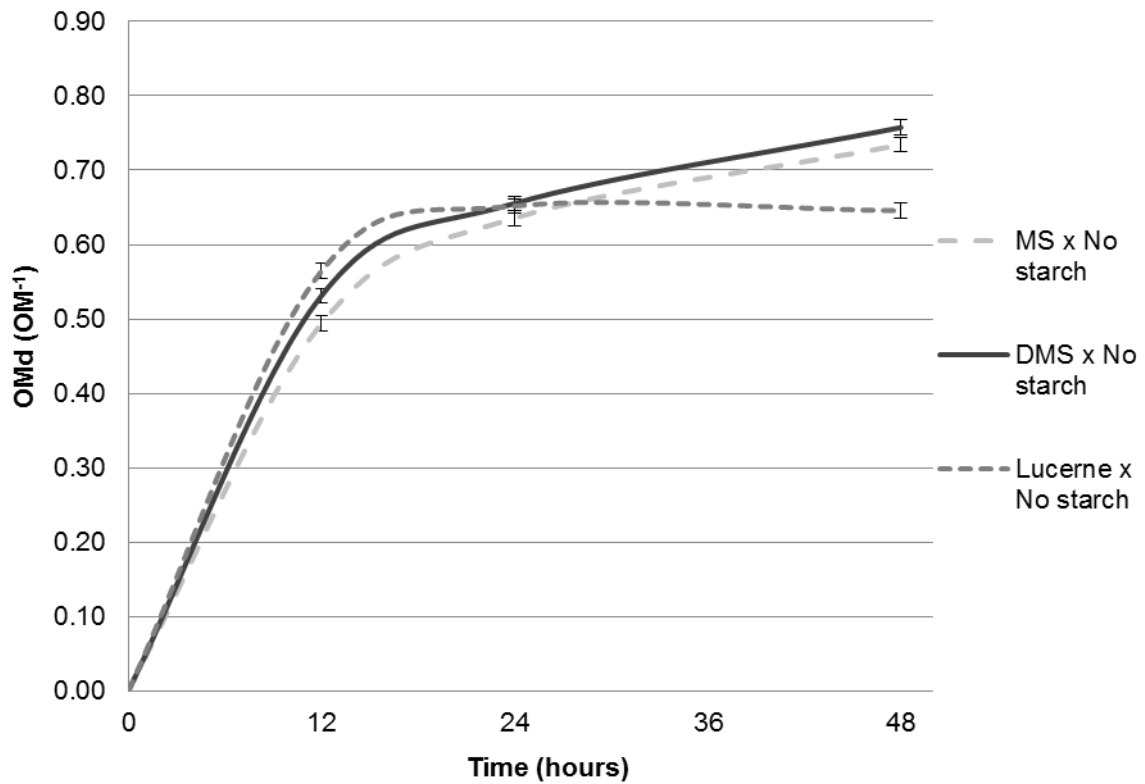**Figure 5.9.** The effect of forage and added starch on DMd over time.

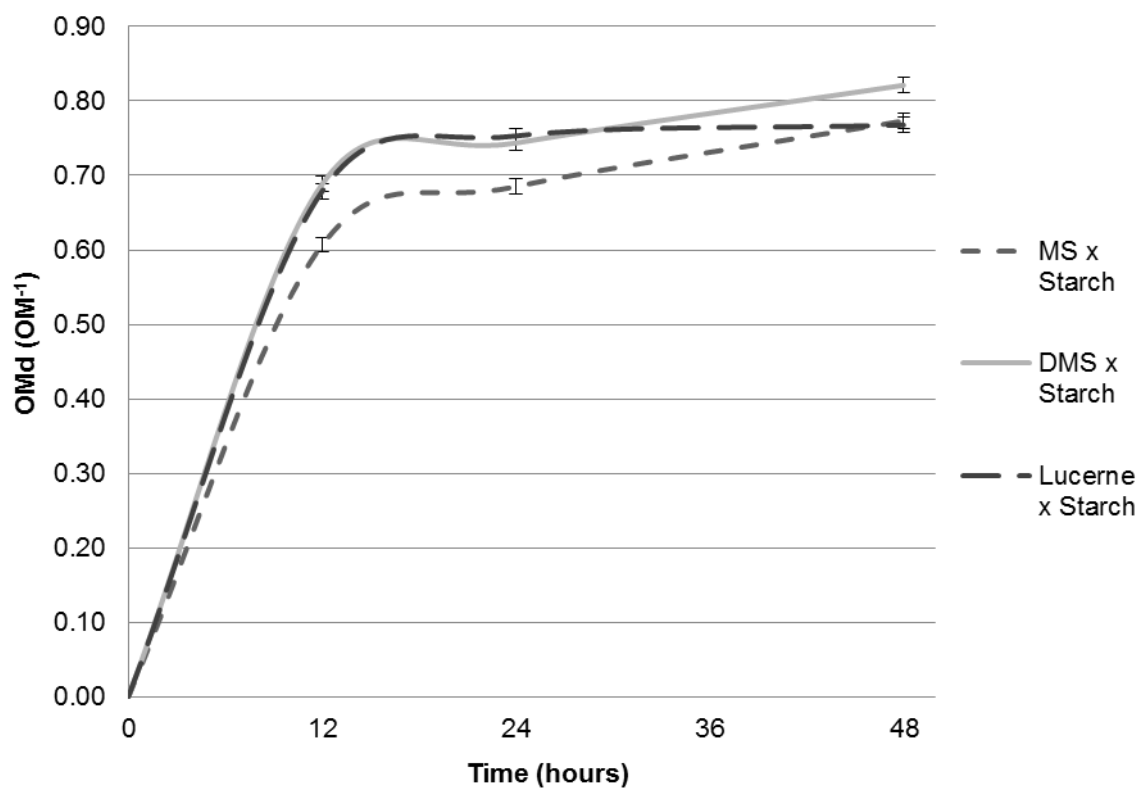**Figure 5.10.** The effect of forage and no added starch on OMd over time.

Figure 5.11. The effect of forage and added starch on OMd over time.

5.4.4. Volatile fatty acids

The concentration of A, B and TVFA decreased significantly ($P < 0.01$) when increasing yeast doses, while there was a tendency ($P = 0.0777$) for P concentration to decrease. There were no significant interactions between dose and forage, starch or time ($P > 0.05$) for any of the VFA concentrations. The maize forages resulted in higher concentrations compared to lucerne (Table 5.4). There were significant differences ($P < 0.01$) between forages with added starch compared to only forage. Added starch resulted in higher VFA concentrations compared to forages without added starch. This would be expected as the added starch would be digested to produce mainly propionic acid as an end-product.

Table 5.4. The effect of dose and forage on VFA concentration.

Forage	Dose (cfu/ml)			
	Control	One-dose	Two-doses	SEM[1]
Acetate (mM)				
Lucerne	89.25[a]	74.93[b]	73.61[b]	12.3665
MS	99.46[a]	87.12[b]	83.65[b]	12.3665
DMS	117.10	110.27	109.69	12.3665
Propionate (mM)				
Lucerne	27.88[a]	25.57[ab]	24.34[b]	3.2014
MS	26.33[a]	24.95[b]	23.84[b]	3.2014
DMS	34.12	33.54	33.34	3.2014
A:P				
Lucerne	3.36[a]	3.02[b]	3.11[b]	0.2119
MS	3.91[a]	3.55[b]	3.57[b]	0.2119
DMS	3.59[a]	3.41[b]	3.40[b]	0.2119
Butyrate (mM)				
Lucerne	10.73[a]	10.00[a]	9.65[b]	1.0046
MS	12.86[a]	11.09[b]	10.88[b]	1.0046
DMS	11.80	11.04	10.94	1.0046
Total VFA (mM)				
Lucerne	135.94[a]	119.68[ab]	116.80[b]	16.3664
MS	144.87	130.48	126.13	16.3664
DMS	168.31[a]	161.68[ab]	160.97[b]	16.3664

[ab]Means within a row with different superscripts differ ($P < 0.05$).
[1]Standard error of the means.

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Yeast dose decreased the A:P ratio significantly ($P < 0.0001$), although there was no significant interaction ($P = 0.5228$) between forage and dose. The decrease in ratio for all forages with added yeast was similar to the results found by Mao *et al.* (2013) and Wang et al (2016). Interestingly, Mao *et al.* (2013) obtained ratios in the range of 1.36 to 2.42 using MS with yeast, whereas the ratios obtained in this study were much higher for DMS at 3.55 to 3.57. The ratios were also higher for MS and DMS compared to lucerne (Table 5.6) which was not expected as both maize forages have a higher starch content which should result in an increased level of P as seen for samples with added starch.

Starch resulted in significant differences in the A:P ratio (Figure 5.12) with added starch resulting in lower ratios which was expected as there would be an increase in starch fermentation resulting in increased P and thus lowering the A:P ratio.

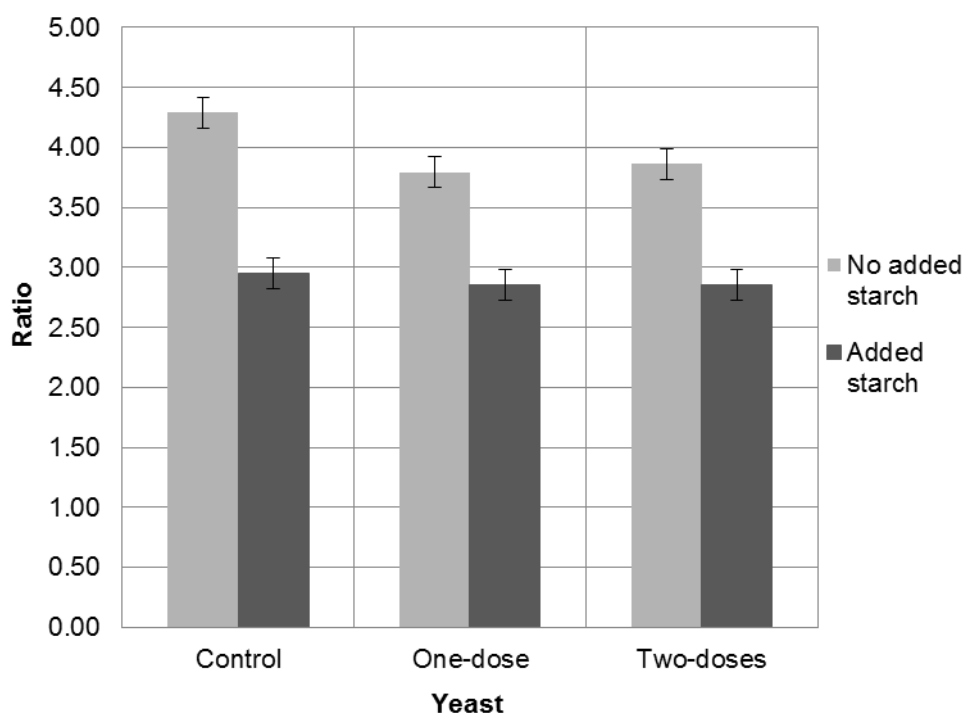**Figure 5.12.** The effect of dose and starch on the A:P ratio.

5.4.5. pH

As previously mentioned, we reduced initial pH as compared to the normal procedure to challenge the micro-environment and to reduce the buffering capacity of the buffer

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used. However, it was difficult to have a stable and lower initial pH because of the high buffer response. There were significant differences ($P = 0.0010$) in pH between the control and yeast treatments with pH increasing with yeast dose. The differences were, however, very small (< 0.05), and not necessarily biologically important, as seen by Lynch and Martin (2002), who found the differences to not always be significant. Yeast interacted significantly with starch ($P < 0.0001$) and time ($P = 0.0023$), but there was no interaction with forage ($P = 0.1254$).

Added starch resulted in lower pH with values increasing with the addition of yeast. This was confirmed by the higher VFA concentrations for added starch. The opposite was seen without the addition of starch, with estimates decreasing as the number of yeast doses increased. This could indicate the yeast ability to stabilize pH in the presence of increased starch levels, however, the differences between the control and yeast with added starch were very small (< 0.05) and not necessarily biologically important, within the flask system.

All pH estimates decreased over time with the highest estimates being observed when yeast was added. There was a significant interaction ($P < 0.0001$) between forage and starch with the two maize silages resulting in the lowest pH compared to lucerne whether there was added starch or not (Table 5.5). The lower pH values could have a direct effect on the lower NDFd observed for all forages with added starch. However, the NDFd differences were not significant between added starch and no added starch which comes as a surprise as the pH values for MS and DMS were very low for cellulolytic bacteria as they are unable to attach to the feed particles at low pH values (Miron *et al.*, 2001), therefore hindering the bacteria's ability to digest fibre.

Table 5.5. The effect of forage and starch on pH.

Starch	Forage			
	Lucerne	MS	DMS	SEM[1]
No added starch	6.53[a]	6.34[ac]	6.28[bc]	0.0473
Added starch	6.24[a]	6.06[ac]	5.89[bc]	0.0473

[a-c]Means within a row with different superscripts differ ($P < 0.05$).
[1]Standard error of the means.

5.5. CONCLUSIONS

Yeast did not have any significant effect on any of the response variables but pH. Yeast showed a positive effect on pH with increased estimates with the increase in yeast. There were no significant interactions between yeast and either forage, starch level or time for NDF, kd, DMd, OMd or VFA concentrations.

Starch resulted in higher estimates for DMd and OMd, while increasing the VFA concentrations. The decrease in pH would be expected with the increase in VFA concentrations; however, what was not expected was for the starch to have no significant effect on NDFd. With lower pH values (≤ 6) fibre digestion is reduced as the growth of cellulolytic bacteria is hindered as well as the bacteria's ability to bind to forage particles. Yeast appeared to have a more pronounced effect with the addition of starch by increasing digestibility estimates with added starch but having the opposite effect in the absence of starch. As too was seen with pH, where added starch caused a decrease in pH as expected but estimates were increased with the addition of yeast unlike estimates seen without the starch.

This study confirms the drop in pH values with added starch due to the increase in VFA concentrations. Yeast was also shown to have a more stabilizing effect on the rumen environment showing an increase in pH and digestibility when the system was challenged by the presence of starch. The very controlled and stabilized *in vitro* environment needs to be challenged to allow rumen modifiers such as live yeast to express some of their potential effects.

5.6. REFERENCES

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GENERAL CONCLUSIONS

Previous works have shown inconsistent results between *in vivo* and *in vitro* studies when analysing feed additives' effectiveness for ruminants. Being aware that an *in vitro* system will never be able to perfectly simulate a rumen, the aim of the experiments was to better understand an *in vitro* system in relationship to yeast activity. Yeast was capable of increasing digestibility estimates as well as VFA concentrations in the absence of starch or oxygen stress, while interacting with the specific medium used. However, it was unable to show significant differences when being placed in a stressed *in vitro* environment. This could possibly be due to the yeast changing its mode of action when placed in an environment where it is objected to stressors such as high oxygen levels, which could possibly cause the yeast to enter a respiration mode, however, this cannot be confirmed in the present study.

Oxygen had a significantly negative effect on *in vitro* digestibility estimates, VFA concentrations as well as rate of NDF digestion. It has been previously thought that the facultative anaerobic bacteria are capable of utilizing any oxygen which enters the rumen via eating and drinking, however, to our knowledge there is not much research done on the effects of adding oxygen to the *in vitro* environment and measuring variable parameters. Further research can therefore be done to determine more precise oxygen concentrations at which bacteria can withstand without affecting digestibility parameters. Previous research has shown yeast as being capable of utilizing oxygen *in vitro,* however, the concentration of oxygen should be considered when comparing research as well as the strain of yeast. Further research should also be done to determine at which oxygen concentration yeast is capable of utilizing more effectively *in vitro* and the effects on digestibility parameters.

The pH estimates, even when significant, often had very low numerical differences which may not be biologically important as the estimates still remained within normal pH ranges for fermentation to occur. The medium being used should also be considered as the buffering capacity may have played a larger role in stabilizing the pH compared to the yeast. In experiment 3, even though the initial pH was reduced to 6.2, this value increased to 6.53, 6.34 and 6.28 for lucerne, MS and DMS respectively, in the absence of added starch. The interaction between yeast with no added starch resulted in a pH decrease as dose increased which could not be

attributed to an increase in digestibility as there were no significant interactions between dose and starch. This may confirm the buffering capacity of the medium.

It can be concluded that yeast and yeast dose have an effect on digestibility parameters, although not always significant, when not challenged by stressors in the *in vitro* environment. With the addition of either the oxygen- or starch stress, the *in vitro* system is assumed to become more similar to the dynamic rumen environment. The fermentation behaviour changed when the *in vitro* system is challenged, however, the exact reasons as to why cannot always be explained. Further research needs to be done on the effect of yeast *in vitro* when stressors such as oxygen or high starch levels are introduced. Caution should be taken when analysing the effects of feed additives on *in vitro* fermentation parameters, especially when using different media, forages and combinations of feeds, as the outcomes may vary.