The effects of three rumen buffering agents on rumen fermentation parameters, nutrient digestibility, and milk composition in dairy cows

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TABLE OF CONTENTS

DECLARATION

I, Sarah Christine Harrison, declare that this dissertation, which I hereby submit for the degree MSc (Agric.) Animal Science: Animal Nutrition, at the University of Pretoria, is my own work, conducted under the supervision of Prof L.J. Erasmus, and that it has not previously been submitted by me for a degree at this or any other tertiary institution. Where secondary material has been used, this has been carefully acknowledged and referenced in accordance with university requirements. I am aware of university policy and implications regarding plagiarism.

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I can do all things through Christ who strengthens me.

SUMMARY

The effects of three rumen buffering agents on rumen fermentation parameters, nutrient digestibility and milk composition in dairy cows

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Feeding highly fermentable carbohydrate-rich diets to dairy cows is universally practised to achieve the energy intake needed to support higher levels of milk production. However, this dietary regimen can reduce rumen pH, and thereby adversely impact rumen fermentation, milk production and composition. Lithothamnium (Lith), calcified remains of a marine algae, is an alternative buffering agent to negate these effects and may provide advantages over sodium bicarbonate ($NaHCO_3$) due to its slower rumen dissolution rate. Whilst past research has only considered effects on pH, this study looks at new in-vitro methodologies such as the buffer value index (BVI) and buffering capacity (BC) have been developed to simulate the rumen environment and how different buffers will react over different incubation intervals. Furthermore, an *in vivo* 4 x 4 Latin square design experiment, spanning a total length of 92 days was carried out with 4 lactating dairy cows. Four treatment diets were formulated, each differing by the type of buffer included, namely; Control, $NaHCO_3$, Lith A, Lith B. the following parameters were recorded; milk production parameters, feed intake and digestiblity, animal health, ruminal pH parameters. Sodium bicarbonate and Lith A as buffer sources aided in significantly increasing the dry matter and crude protein digestibility. Increased DMI for the control diet may have contributed towards increased milk yields. It may be speculated that lowered SCC for Lith B may be attributed to the effect of the exogenous buffer to aid in udder health and improve cow immunity. Significant differences between Lith B and $NaHCO_3$ were observed for mean pH, with Lith B exhibiting a lower ruminal pH and increased time spent below pH 5.8 in the rumen. This low pH contradicted previous studies that claim lowered milk fat under such ruminal conditions, whilst the milk fat in this study was numerically higher for Lith B. Increased butyrate content for cows fed Lith A versus the control may aid in explaining milk fat numerical differences between the control diet and Lith A. Although buffers do in turn help to alleviate low pH problems in the rumen, however, alternative strategies which investigate fermentation pathway control may be more successful in combating bouts of SARA in the long-term.

LIST OF ABBREVIATIONS

ADF	Acid detergent fibre
BC	Buffering capacity
BVI	Buffer value index
BW	Bodyweight
CLA	Conjugated linoleic acid
CO_2	Carbon dioxide
CP	Crude protein
CV	Coefficient of variation
d	Day
DE	Digestible energy
DIM	Days in milk
DMD	Dry matter digestibility
ECM	Energy corrected milk
EE	Ether extract
FA	Fatty acid
FCM	Fat corrected milk
FI	Feed intake
g	Gram
GE	Gross energy
HCO_3^-	Bicarbonate
IVOMD	*In vitro* organic matter digestibility
$K_2Cr_2O_3$	Potassium dichromate
kg	Kilogram
L	Litre
LA	Lactic acid
LAB	Lactic acid producing bacteria
LPS	Lipopolyssacharide
LUB	Lactic acid utilising bacteria
ME	Metabolisable energy
MEB	Multi-elemental buffer
MFD	Milk fat depression
mg	Milligram
MgO	Magnesium Oxide
min	Minutes
MPO	Milk Producers Organisation
$NaHCO_3$	Sodium bicarbonate
NDF	Neutral detergent fibre
NE	Net energy
NFC	Non-fibrous carbohydrate
NH_3-N	Ammonia nitrogen
NSC	Non-structural carbohydrates
OM	Organic matter
OMD	Organic matter digestibility
pKa	Acid-dissociation constant
PO_3^{-4}	Phosphate ion
PUFA	Polyunsaturated fatty acid
SABC	Sample Buffering Capacity
SAPH	Sample pH
SARA	Sub-acute ruminal acidosis
SD	Standard deviation
SEM	Standard error of the mean
SNF	Solids not fat
STBC	Standard Buffering Capacity
STPH	Standard pH of 6
TiO_2	Titanium dioxide
TMR	Total mixed ration
TS	Total solids
VFA	Volatile fatty acid

LIST OF TABLES

LIST OF FIGURES

PREFACE

Chapter 1 entails a general introduction to the studies topic and gives an overview on what is to be expected from the dissertation.

A literature review, compiled of studies and meta-analysis, forms the second chapter whereby previous research pertaining to the rumen is critically discussed in terms of its anatomical and physiological functions, associated metabolic disorders as well as the role of buffers in the ruminant and applicability to industry.

Materials and methods involved in the two experiments which comprise this study are discussed in Chapter 3.

Chapter 4 explores the results in a discussion of experiment 1's *in vitro* work. Experiment 1.1, 1.2 and 1.3's results may be found here tabulated and mapped over a 24-hour incubation period, focusing on solution pH, treatment buffering capacity and buffer value indices.

The *in vivo* results obtained in experiment 2 are discussed in Chapter 5. Analyses were carried out on feed and refusals, milk production and composition, and rumen parameters including data from continuous rumen pH loggers.

Chapter 6 concludes the findings from the results discussed in chapter 4 and 5.

A critical evaluation of the research conducted as well as future research possibilities are discussed in Chapter 7.

Chapter 1

General Introduction

The increased quantity of readily available carbohydrates to meet the increasing energy requirements of high producing dairy cow is associated with an indirect decrease in ruminal pH (Grant & Mertens, 1992). Failure to regulate the pH of the rumen can result in lower productivity as well as serious animal health implications.

Reduced fibre digestibility may occur as a change in the rumen environment influences the microbial population present. The optimal range of the rumen pH for physiological maintenance of its' microbiome is between 5.8 and 6.4 (Ishler *et al*., 1996). Increased starch intake causes rapid decreases in ruminal pH, which results in reduced numbers of methanogenic and cellulolytic bacteria. This ultimately alters the acetate to propionate production ratio by microbes in the rumen and adversely affects total tract starch digestibility (Clark & Davis,1980).

Subacute ruminal acidosis (SARA) is the most encountered nutritional disease in the dairy industry (Calitz, 2009). Animals with a high dry matter intake (DMI) that experience wide variation in their meal patterns and diet compositions, are at higher risk of developing SARA (Stone, 2004). A ruminal pH below 5.5 for intervals longer than 174 minutes daily will result in this condition (Cerrato-Sánchez *et al*., 2008; O'Grady *et al*., 2008 & Plaizier *et al*., 2008). The sudden high rate of volatile fatty acid production, from increased dietary starch content, in combination with low fibre content causes the reduction in pH. Low ruminal pH leads to increased lysis of gram-negative bacteria which results in a higher concentration of lipopolysaccharide (LPS) in the rumen. Translocation of LPS into the circulatory system evokes an inflammatory response (Khafipour *et al*., 2011), a reduction in fibre digestion, depressed feed intake, milk fat depression, laminitis, liver abscesses and diarrhoea (Plaizier *et al*., 2008).

Milk fat depression (MFD) is linked to incomplete biohydrogenation (BH) by ruminal microbes of ingested polyunsaturated fatty acids (PUFA's) in a low pH rumen environment of cows fed high concentrate diets. This leads to an increase in the trans-C18:1 fatty acid in milk (Kalschear *et al*., 1997). Milk fat depression alters the composition of milk as the fat content in milk is substantially reduced, directly influencing a dairy farmers' income. Maintaining the pH of the rumen above 6.0 for the most part of the day will help to alleviate the formation of incomplete BH isomers (Staples, 2006).

Buffers aid in maintaining the stability of pH in a system (Morel *et al*., 1976). There are primarily three ways by which ruminants' buffers ingested acid or volatile fatty acid (VFA)

production by ruminal microbes, namely, the buffering ability of feed ingredients, saliva and dietary buffers (Erdman, 1988). Different feedstuffs have specific acid-consuming capabilities. Studies into fermentation properties of ensiled alfalfa and maize plants have shown improved buffering capacities of two- to three-fold. Higher buffering capacities in fresh forages have been recorded among legume plant species whilst cereal grains have lower buffering capabilities in comparison (Jasaitis *et al*., 1987). Feed factors have a direct impact on saliva production and flow through the dry matter content, feed intake and particle size of the forage. Bovine saliva concentration of disodium phosphate and sodium bicarbonate makes saliva the primary source of rumen buffering. However, in cases of high producing dairy cattle on high starch diets, supplemented dietary buffers are needed as the buffer flow from saliva is inadequate (Erdman, 1988).

Examples of effective rumen buffers are sodium bicarbonate ($NaHCO_3$), magnesium carbonate, bentonite, calcium carbonate and potassium bicarbonate. Sodium carbonate, magnesium oxide (MgO), calcium hydroxide, potassium carbonate and sodium hydroxide make up the list of alkalizing agents. Sodium carbonate cakes quickly due to hygroscopic properties, thus preventing it's use in majority of diets. Both sodium and potassium hydroxide are hazardous to human health complicating manufacturing procedures, thus highlighting the importance of removing these compounds from dairy diets. Although $NaHCO_3$ is one of the most commonly used buffering agents seen in dairy diets today, its use as well as the use of MgO has been reported to reduce concentrate intake in dairy cattle (Erdman, 1988). Sodium bicarbonate also has a very fast reaction rate in the rumen resulting in ineffective long-term buffering in the presence of continual VFA production (Cruywagen *et al*., 2015), whilst MgO is less soluble at a pH lower than 6.

Lithothamnium can be identified as calcified remains of a sea algae and is marketed as buffering agents, with the ability to release minerals slowly into the rumen environment due to its "honeycomb structure". Different Lithothamnium sources are widely used as buffers in South Africa, but limited research is available that compare buffering agents to Lithothamnium sources *in-vitro*. The research that is available does not explore the concepts of buffering capacities (BC), buffer value index (BVI) or methods of titrating an acid and base into a buffer and ruminal fluid solution at ruminal temperature to simulate the rumen environment over different incubation time intervals.

This study aims to compare the effectiveness of alternative Lithothamnium sources to $NaHCO_3$ through the *in vitro* determination of BC and BVI, as well as the effect on milk production and composition, rumen fermentation and total tract digestibility of lactating dairy cows.

The hypotheses for this study were:

H0: Different buffers will have no influence on the pH, BC and BVI of ruminal fluid *in-vitro*.

H1: Different buffers will influence the pH, BC and BVI of ruminal fluid *in-vitro*.

H0: Different buffering agents will not affect milk production, milk composition and nutrient digestibility in lactating dairy cows.

H1: Different buffering agents will influence milk production, milk composition and nutrient digestibility in lactating dairy cows.

H0: Different buffering agents will not influence rumen pH and rumen fermentation parameters in lactating dairy cows.

H1: Different buffering agents will influence rumen pH and rumen fermentation parameters in lactating dairy cows.

Chapter 2:

Literature review

2.1 Introduction

High producing dairy cattle play an imperative role in food security through the production of protein-rich milk. Dairy product demands will continue to grow, not only as a result of population growth, but more so due to an increase in per capita dairy product consumption. Although South Africa produces 3.4 million tons of solids corrected milk annually, it is only 0.4% of global milk production (Lacto Data, 2021) With shifting climates, looming antibiotic bans, a reduction in the number of South African dairy producers and an ever-growing population, production efficiency must be optimised. Improving production through effective precision nutrition appears to be the obvious choice, however, the increased content of readily available carbohydrates to meet increasing energy requirements of high producing dairy cows' is associated with an indirect decrease in ruminal pH (Grant & Mertens, 1992). Failure to regulate the pH of the rumen will result in undesirable changes in productivity, as well as serious animal health implications. Sub-acute ruminal acidosis (SARA), acidosis, milk fat depression (MFD) and other associated metabolic disorders have negative consequences in terms of animal welfare and production. The 1960's brought about the use of dietary buffers added to high concentrate diets to compensate for the shortfalls in the ruminant's ability to maintain ruminal pH within the physiologically safe threshold (Russell & Chow, 1993). This literature review aims to highlight the functioning of the rumen and associations with metabolic disorders. It will further probe into dietary buffers and their role in maintaining pH stability in the rumen environment.

2.2 Rumen

2.2.1 Anatomical overview of the rumen

The four-component stomach of dairy cattle allows the conversion of low-quality feed, such as grass and human-food by-products, into energy and dairy product components (Van Soest, 1994). The rumen, a 114 L fermentation vat (Ishler *et al.,* 1996), resides on the left-hand side of the body and makes up 65% of the total volume of the four-part stomach. Like all mammals, ruminants are unable to digest structural plant polysaccharides such as cellulose, whilst the microbes in the rumen can.

The rumen provides a suitable environment for microbes to inhabit whilst the microbes partially ferment and degrade the majority of the ruminant's feed so that the host can utilise

fermentation end-products for its own nutritional requirements. The ability of the rumen to integrate different metabolic pathways makes this ecosystem so effective (Harfoot, 1981). The rumen is constantly contracting throughout the day, at least twice a minute. These contractions are responsible for mixing of ruminal contents, allowing fermentation gasses to escape the rumen via the oesophagus, as well as aid the flow of digesta to the other parts of the stomach and lower digestive tract. Small, finger-like projections, called papillae, increase the surface area of the rumen by lining its interior wall and is responsible for the absorption of the fermentation end-products such as water, ammonia and volatile fatty acids (VFA). These papillae give an indication of the health of the rumen environment as their length adapts according to the ruminant's diet. A low-energy diet shortens the papillae length, whilst the high VFA production consistent with a high starch diet stimulates papillae growth for maximum VFA absorption (Van Soest, 1994). Should the absorptive capacity of the ruminal papillae epithelium become impaired due to rumenitis, it becomes increasingly difficult to maintain the pH stability in the rumen as these unabsorbed VFA's will build up in the rumen (Krause & Oetzel 2006).

2.2.2 Overview of microbiome

Colonisation of the rumen by microbes begins in the first 24 hours after calf birth and develops further into a highly competitive community early in life. This community is known as the microbiome and involves symbiosis, resource competition, predation, and antagonism (Firkins & Yu, 2015). The rumen microbiome can be described as a superorganism due to the intimate symbiotic relationship formed between the ruminant and the microbes found within its' rumen. These microbes are crucial for the digestion of the ruminant for maintenance and production. They function in conjunction with the gastrointestinal tract influencing digestion, as well as metabolic and immunological responses to the benefit of the host. Host animals have an individual effect on their own gut microbiota through complex interactions with receptors between the rumen wall, antibodies in saliva, physical structure, as well as gut digesta dynamics (Shabat *et al.,* 2016). The microbial community of the rumen microbiome is dominated by more than 90% bacteria, 2- to 8% Eukarya and less than 1% Archaea (Pitta *et al*., 2015).

Bacteria is the most diverse group in the microbiome. Within the bacterial domain, 26 phyla have been identified, with the most abundant being *Bacteroidetes* (61- to 80%) and *Firmicutes* (12- to 23%) (Pitta *et al.,* 2015). Fibrolytic bacteria are well studied hydrogen producers that are part of the *Firminicute* phylum, whilst *Bacteroidetes* are net hydrogen utilisers that yield energy, carbon skeletons and NH_4^+ for biosynthesis of bacterial cells (Tapio *et al.,* 2017; Harfoot, 1981). Half of the ruminal bacteria are freely suspended in ruminal fluid whilst the rest

are found on the exterior of fragments of plant particles. Bacterial metabolism end-products includes acetate, butyrate, formate, succinate, propionate, lactate, methane gas, hydrogen gas (H_2), carbon dioxide (CO_2), C_4 and C_5 branched chain fatty acids.

Ciliate protozoal species can be found as two types in the rumen: holotrichs and entodiniomorphs. Holotrichs metabolise soluble sugars as their energy and carbon sources and produce butyric-, acetic- and lactic acid in conjunction with CO_2 and H_2. Entodiniomorphs are particle feeders which ingest bacterial and plant cells as well as starch grains with fermentation end-products being CO_2, H_2, VFA's and lactic acid (Harfoot 1981). Protozoal species cannot survive in the ruminal environment when the pH drops below 5.5 for extended periods of time. When fewer microbes are present in the rumen, the ruminal microflora becomes more dynamic and struggles to maintain the pH during periods of dietary change or stressful times in an animal's life (Garry, 2002).

At a pH below 5.5, *Lactobaccilus* begin to proliferate, increasing the rate of lactate production. Ruminant adaptive mechanisms will respond to this through the proliferation of lactate-utilising bacteria, *Megasphaera elsdenii* and *Selenomonas ruminantium*, which converts lactate to other VFA. This mechanism is usually not fast enough to maintain pH stability, as the bacteria's turnover time is substantially slower than the rate of lactate production (Oetzel, 2007). This negative pattern, in turn, causes ruminal pH to further decrease.

2.2.3 Physiology

2.2.3.1 *Rumen pH*

The pH of the rumen is defined by the difference between ruminal acid production and removal via rumen epithelial cell absorption, passage to the lower gastrointestinal tract and buffer neutralisation (Gao & Oba, 2014). Ruminants have the innate ability to regulate and maintain ruminal pH within physiological limits, through the regulation of intake, microbial adaptation, endogenous buffer production and VFA absorption. However, the consumption of readily fermentable carbohydrates may inflate acid production in the rumen past the point that the buffering-system can accommodate, leading to ruminal pH compensation failing and the subsequent reduction in pH (Krause & Oetzel 2006). Reduction in fibre digestion may occur, as a change in the pH of the rumen environment influences the microbial population to shift from fibrolytic- to starch-utilising microorganisms. The optimal range of the rumen pH for physiological maintenance of its' microbiome is between 5.8 and 6.4, whilst the high starch digesting microbes function best under a lower pH of between 5.2 and 6.0 (Ishler *et al.,* 1996). Should the ability of

the ruminal papillae also be impaired due to fibrosis from chronic rumenitis, ruminal pH becomes increasingly difficult to maintain post feeding because the papillae does not have the capacity to remove the excess VFA from the rumen and aid normal rumen pH recovery (Oetzel, 2007).

Throughout the day, there is great variation in the pH of the rumen as shown Figure 2-1. The pH is lowest following consumption of feed and then follows a gradual increase as rumination and absorption continues (Keunun *et al*., 2002).

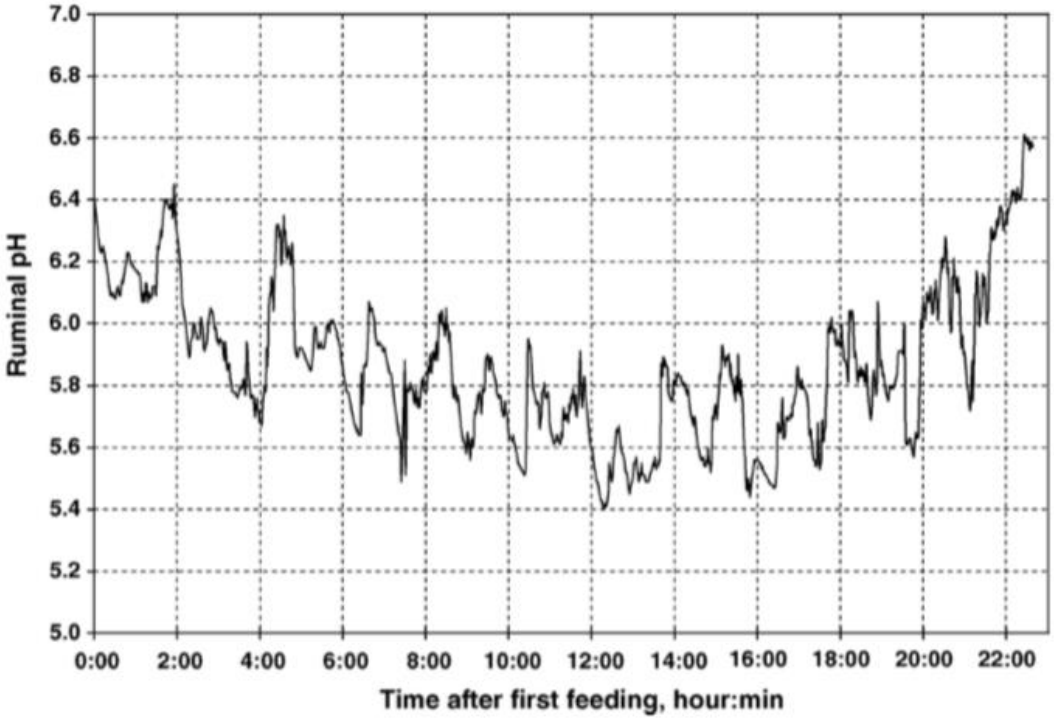

Figure 2-1 Graph showing the typical ruminal pH fluctuations post-feeding over a 24-hour period (Adapted from Krause & Oetzel, 2006)

Ruminal atony may result due to further reductions in ruminal pH as VFA absorption is decreased and lactic acid absorption is increased. The animal senses that their homeostatic acid-base balance has been disrupted and, in an attempt, to preserve its life, will cut off all absorption and interactions with the rumen. This is life-threatening as the cow is essentially trusting that the rumen will use all the fermentable carbohydrates supply before dying (Oetzel, 2007).

Ruminal fluid pH measurement methods influence the pH value recorded, differences between pH-meters as well as indwelling probes are commonly seen. When comparing *in-vitro* and *in-vivo* methodology studies, *in-vivo* methods record significantly lower pH values. This is due to the loss of CO_2 in *in-vitro* sampling methods before pH recording. Rumen fluid samples from

cannulated animals have lower pH values when compared to rumenocentisis collected rumen fluid. Although the correlation in pH between the two methods is significant, it is not as strong as ideal (R^2=0.52) (Stone, 2004). Rumen pH will further fluctuate according to the feeding regime. Animals with feed available *ad libitum* will exhibit a different rumen pH profile over 24-hours compared to animals that are slug-fed or fed twice daily.

2.2.3.2 *Volatile fatty acids*

Volatile fatty acids (VFA) synthesised in the rumen account for 60 to 70% of metabolizable energy (ME) in dairy cows (Seymour *et al.,* 2005). Acetic-, propionic- and butyric acid are three principal VFA that aid in milk production. Propionic acid is gluconeogenic and contributes 65-80% of a dairy cows' net glucose supply (Reynolds, 2006). Butyric and acetic acid are precursors required for milk lipid synthesis and substrates of oxidation (Seymour *et al.,* 2005). Butyrate can be derived from the condensation of two mole acetate. Preferential oxidation of VFA by ruminal epithelial cells determined *in-vitro* occurs in in the following order: butyrate, propionate, acetate (Baldwin and Jesse, 1996). This suggests that a decrease in ruminal epithelium oxidation of acetate and propionate may occur thus enhancing supply for milk fat production, should there be an increase in butyrate concentration in the rumen.

Relating the concentration of VFA's in the rumen to milk production and composition aids in determining which metabolic pathways are stimulated by different feeding regimes. It is, however, currently unclear as to what degree the relationship between milk production and rumen VFA is implicated. Having said this, it is possible to identify the limiting metabolic milk production pathways based off the individual VFA concentrations in the rumen. This can be seen in a comparison study conducted by Seymour *et al. (*2005); it was demonstrated that milk yield was directly correlated to the butyric concentration in the rumen, with a moderate relation to rumen propionate concentration. It was further discovered that dry matter intake (DMI) is directly related to butyric acid which links back to increased milk yields with increased DMI. There is a strong negative correlation between ruminal pH and VFA concentration, as well as milk protein (g/100 g). The ruminal acetate to propionate ratio is strongly positively related to milk fat (g/100 g). These findings further indicate that above a ruminal pH threshold of 6, milk protein and fat content is reduced. This has previously been discussed as a loss in function of starch fermenting microbes reducing the fermentation rate in the rumen (Ishler *et al.,* 1996). This reiterates the narrow range that the microbiome functions at.

This more acidic environment can result in the reduction in numbers of methanogenic and cellulolytic bacteria ultimately changing the acetate to propionate production ratio by microbes in the rumen. In addition, enzyme activity such as that of pancreatic alpha-amylase, may be reduced in this environment, owing to the lower pH of the small intestine, a consequence of reduced ruminal pH. This can adversely affect total tract starch digestibility (Clark & Davis, 1980). Once again, the stability of the rumen's pH depends on the capacity of the rumen to quickly passively absorb VFA which is an important bottleneck.

At ruminal pH's below 5.5, increases in VFA absorption is reduced due to increases in lactate production as *Strep. bovis* ferments glucose to lactate, forgoing VFA production. The danger of this lies in the fact that lactate's pKa is substantially lower than VFA , leading to the further depression in ruminal pH as lactate will accumulate in the rumen (Oetzel, 2007).

2.2.4 External factors affecting rumen function

Calving and gestation have significant effects on ruminal pH and the inherent buffering ability of the cow. The reduced abdominal capacity, due to pregnancy, restricts rumen capacity and necessitates a highly digestible, good quality diet. To stimulate maximum milk production, the diet is changed from predominately fibre-based to high concentrate content referred to by (Counotte *et al.*, 1979). The high concentrate content changes the VFA profile of the rumen and lactic acid production rates through a shift in the microbial population. This change in the microbial population and VFA profile, makes the adaptation period of the transitioning cow imperative. However, it can be argued that reduced feed intakes due to a drop in animal appetite could help by potentially protecting the cow from the sudden diet change (Counotte *et al.,* 1979). This leads to questions as to why the VFA concentration is irregular during this time in the rumen. One theory incorporates the assumption of ruminal net water loss; on average an absorption of 14.2 L of water per hour would account for such VFA irregularities. A further observation of this transitioning period is decreased salivation which will further impair the rumen's buffering ability and account for VFA profile changes (Counotte *et al.,* 1979).

In a study conducted by Kelley *et al.* (1967), cannulated cows were subjected to various environmental temperatures in a climatic laboratory. The study reported that at increasing temperature intervals, the total VFA content in the rumen reduced significantly. Acetic acid exhibited a 50% reduction, whilst propionic acid was reduced by 72%. This ultimately affected the acetate to propionate ratio (A:P), concluding that higher temperatures increase the A:P ratio. Variation in digestibility can be attributed to the microbial population shift that occurs in cattle experiencing heat stress which will subsequently alter fermentation patterns in the rumen (Yadav

et al., 2013). As anticipated, water consumption increased with rising levels of heat stress although this had a non-significant influence on VFA concentrations (Weldy *et al.,* 1964). High environmental temperatures in the region of 38 °C directly reduced rumen activity (Attebery & Johnson, 1969). Heat stress influences the hypothalamus to reduce an animal's appetite to reduce the bodies' heat increment. Weldy *et al.* (1964) and Yadav *et al.* (2013) concluded that temperatures between 25 to 26 °C have been reported to further depress feed intake in ruminants, although upwards temperatures reduce intake at more rapid rates. Saliva is imperative in ruminal pH modulation. Reduced intake results in less rumination time leading to decreased saliva production (Weldy *et al.,* 1964, Yadav *et al.,* 2013).

2.3 Metabolic disorders

2.3.1 Milk composition/production

Proper rumen functioning, a normal milk fat production and maintenance of a high feed intake are factors all heavily dependent on the rations' fibre content. Forage should make up at least 75% of dietary neutral detergent fibre (NDF), according to the NRC (2001). Non-structural carbohydrates (NSC) are added to the diet in rising levels to increase the energy content of the diet to optimise milk production. However, fermentation of NSC results in the production of acids which overcome the buffering ability of the rumen (Sarwar *et al.,* 1992). The optimal inclusion rate of NSC in the diet for milk production is hypothesised to be 40%. The forage to concentrate ratio (forage : concentrate) may be used to manipulate the dietary NSC percentage (Nocek & Russell, 1988). However, cows fed diets exceptionally low in NSC's milk is often seen to be high in fat content but low in protein. A reduction of NSC content in the diet to 25% reduces the microbial protein flow to the duodenum, thus reducing the protein content available for further digestion and absorption. This highlights the importance of ensuring that the dietary NSC content falls in the correct range. Dairy cows seen to be in a negative energy balance undergo lipolysis in their adipose tissues (Sarwar *et al.,* 1992). This metabolic process could be the reason for increased milk fat percentage in cows fed diets with reduced NSC concentrations.

Three major factors affecting milk yield and composition are days in milk (DIM), dairy cow breed and season. According to Dunlap *et al.* (2000), DIM can cause variation of up to 0.75% in milk fat. Milk yields rapidly increase until 50 DIM and then begin to slowly decrease. While this occurs, milk fat percentage follows the opposite trend, with fat content increasing later in lactation (Dunlap *et al.,* 2000). Breed has a direct effect on milk fat percentage as well. The normal threshold for milk fat percentage in Holstein herds is between 3.4 to 4%. Should the milk fat percentage drop below 3.2%, milk fat depression (MFD) can be defined. The same follows for

Jersey herds dropping below 4.2% from the acceptable 4.2 to 5% (Oetzel, 2007). Oetzel (2007) explains further that summer puts cows at a higher risk for SARA development as heat stress increases their respiratory rate, leading to respiratory alkalosis. Cows in respiratory alkalosis have lowered blood bicarbonate concentrations which reduces the natural innate ruminal buffering ability of the ruminant (Oetzel, 2007). An indication of this is a 0.25% milk fat reduction.

The fact that normal milk fat percentage directly depends on DIM, season and dairy cow breed makes it increasingly difficult to determine whether a low milk fat test is due to MFD or one or more of the afore mentioned external environment influences. The problem comes into play when these influences have already been accounted for and there is still a low milk fat test (Oetzel, 2007). From the beginning to the end of a single milking-session, the content of milk fat varies considerably. Therefore, making it important to ensure sampling is done correctly when testing for MFD. Milk fat testing must never under any circumstances be conducted using strip milk samples. A milk fat proportioning device or method is important when taking samples to prevent biased results (Oetzel, 2007).

2.3.2 Milk fat depression

2.3.2.1 *Introduction*

Milk fat depression alters the composition of cow's milk as the fat content in milk is substantially reduced, which has significant financial implications. Milk fat depression can be broadly defined using the milk fat to milk protein ratio of milk samples. The concept that a higher milk protein percentage than milk fat is an indication of MFD is not supported by science (Oetzel, 2007), as the synthesis of the different components are separated physiologically. Ruminal acidosis, overfeeding unsaturated fats, or feeding ionophores such as Monensin (an antibiotic proved to improve feed efficiency, increase rate of weight gain and for the prevention and control of coccidiosis) are the three main causes of MFD. The relationship between MFD and SARA are complex and inconsistent. Based off comparison studies, the correlation between ruminal pH and milk fat concentration is between 0.31 to 0.39 (Gao & Oba, 2014). Sub-acute ruminal acidosis in dairy cattle has the capacity to decrease milk fat production by 0.3%. This can be seen in experimentally induced SARA trials by increasing the grain concentration in the diet or substituting lucerne pellets for lucerne hay (Stone, 1999) or using an oral glucose drench (Gao & Oba, 2014). Milk protein percentage is significantly reduced in dairy cows when feeding diets increased with fat content and reduced NSC (Sarwar *et al.,* 1992) due to changes in post-absorptive metabolism. Further inclusion of ruminal bypass or animal fat sources lowers the risk of MFD (Oetzel, 2007).

In addition, alterations to the ruminal microbial population at a lower pH reduces the acetate to propionate molar ratio, further reducing the milk fat percentage (Russell, 1998).

Table 2-1 compares the VFA content of rumen fluid and milk production parameters between cows fed a normal total mixed ration (TMR) and a high-grain, low-fibre TMR. Milk fat content can be observed to have halved in cows fed a higher NSC content ration. The authors linked this to the reduction in the A:P ratio from 3.2 to 1.0 (Bauman & Griinari, 2001). These were reported to be significant differences.

Table 2-1 Production and volatile fatty acid parameter differences between normal and high grain diet (Adapted from Bauman & Griinari, 2001)

Parameter	Normal diet	High grain, low forage diet
Milk yield (kg/d)	No significant change	
Milk fat (kg/d)	6.83	3.63
Ruminal VFA (molar %)		
Acetate	67	46
Propionate	21	46
Butyrate	11	9
A:P[1]	3.2	1.0
Ruminal VFA production (mol/d)		
Acetate	29.4	28.1
Propionate	13.3	31.0

1 A:P = Acetate to propionate ratio.

2.3.2.2 *Substrate supply theory*

It was previously hypothesised that MFD was caused by insufficient acetate, beta-hydroxybutyrate (BHBA) and the glucogenic-insulin theory of MFD. Beta-hydroxybutyrate is the end-product of biochemical reactions involving butyrate in the rumen. *De novo* fat synthesis from acetate and BHBA is responsible for approximately 50% of fatty acids found in cow's milk (Overton *et al.*, 2006). This stemmed the idea that a reduction in the acetate and butyrate substrates in cases of reduced fibre digestion results in MFD. Although this theory is still mentioned in the dairy industry, it is highly doubtful whether that is the cause of MFD. This is supported by numerous studies (Davis, 1967; Palmquist *et al.,* 1969; Bauman *et al.,* 1999) that reported MFD in cases where high-concentrate, low-fibre diets were fed to lactating dairy cows. Significant increases in

production of rumen propionate was the only factor which changed the molar percentages of VFA's as there were no significant differences in acetate or butyrate content in rumen fluid on a different treatment diet. The glucogenic-insulin theory proposes that MFD is a consequence of insufficient fat-precursors to be metabolised for milk fat production (Corl *et al.,* 2006).

2.3.2.3 *Conjugated linoleic acid theory*

Conjugated linoleic acid (CLA) is the term given to a group of geometric and positional fatty acid isomers comprised of octadecadienoic acid with conjugated double-bonds derived from ruminant products (Booth *et al.,* 1935). Cow's milk predominantly (80 to 90% of total CLA content) includes the CLA isomer *cis-9, trans-11.* This isomer is also found in meat fat. These CLA's can be biosynthesized by means of two pathways, namely: biohydrogenation of linoleic acid (*cis-9, 12*) in the rumen and conversion of the biohydrogenation intermediate *trans*-11 C18:1 in the animals' tissues (Bauman *et al.,* 1999, Hussein *et al.,* 2013). The rumen biohydrogenation pathway is of interest with regards to milk fat depression. A wide range of bacteria in the rumen are responsible for the biohydrogenation of unsaturated fatty acids involving multiple biochemical steps demonstrated in Figure 2.2. Linoleic acid ester linkages are hydrolysed by microbial lipases in the preliminary step of biohydrogenation. The reduction of *trans*-11 C18:1 is the rate-limiting step as it occurs at a slower rate leading to the accumulation of the biohydrogenation intermediate in the rumen that is more available for absorption than the end-product, stearic acid (Bauman *et al.,* 1999).

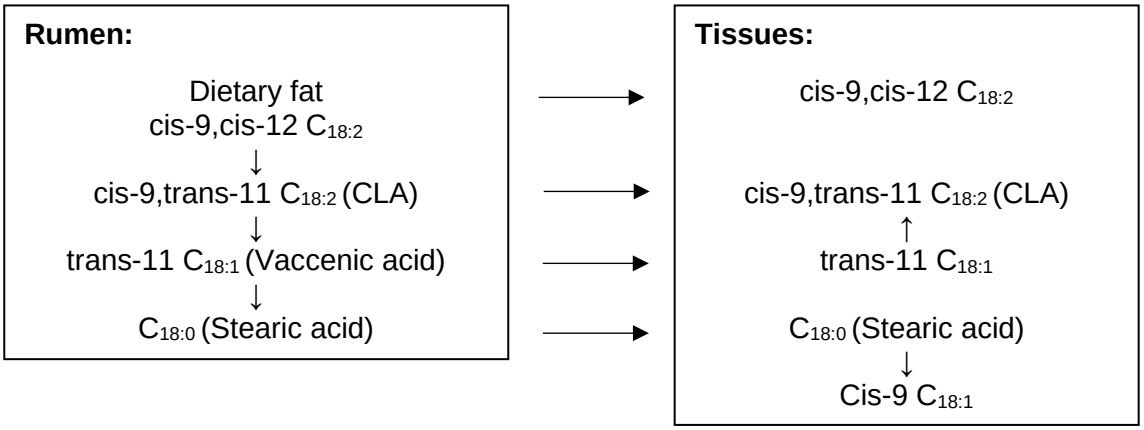

Figure 2.2 Depicts ruminal biohydrogenation processes involved in the production of conjugated linoleic acid (Adapted from Bauman *et al.*, 1999)

Recent studies have reported increased occurrences of MFD cases in herds fed low-fibre, high-concentrate diets, or diets with increased plant fat content. Incomplete biohydrogenation occurs as the microbial population shifts to become primarily amylolytic and alter the rumen

environment by pH reduction. This is the reason why MFD is often seen in cattle battling SARA. Erdman (1996) and Griinari *et al.* (1998) concluded that increased *trans* C18:1 fatty acids, specifically *trans*-10 C18:1, in cow's milk is directly correlated with MFD incidences. Griinari *et al.* (1999) further linked MFD to the *trans*-10, *cis*-12 CLA milk content. In a study involving lactating ewes, a reduction of between 25 to 40% in mRNA abundance of fatty acid synthase, stearoyl-CoA desaturase 1, acetyl-coenzyme A carboxylase ∝ and G_3P acyltransferase-6, as well as a reduction of 55% in the genes involved in lipogenic enzymes, were seen in the ewes fed a lipid protected *trans*-10, *cis*-12 CLA supplement. These intermediate isomers are absorbed through the small intestine and taken up by the mammary gland. This led to the conclusion that induced MFD is caused by downregulation for the enzymes that are involved in milk fat synthesis in the mammary gland (Hussein *et al.,* 2013).

Keeping the pH of the rumen above 6.0 for most of the day will help to alleviate the formation of incomplete biohydrogenation isomers (Staples, 2006). Oetzel (2007) describes experiments where induced SARA for a single day have no effect on MFD, indicating that incomplete biohydrogenation is not induced by a single acidotic event but rather over time and multiple events to cause MFD.

2.3.3 Ruminal Acidosis

Ruminal acidosis can be seperated into two very different clinical diseases, namely acute ruminal acidosis, and subacute ruminal acidosis (SARA). Acute ruminal acidosis is characterised by excessive intakes of highly fermentable carbohydrates which leads to rapid, uncompensated drops in the pH of the rumen to below 5.0 (Krause & Oetzel, 2006). As a result, the concentration of lactic acid in the rumen starts to rise. This is a result of unadjusted microbial populations in the rumen that have not yet developed a large enough population of lactic acid-utilising bacteria to deal with the sudden increase in lactic acid as previously mentioned (Owens *et al.,* 1998). *Megasphaera elsdenii* and *Selenomonas ruminantium* are examples of lactate-utilising bacteria that metabolise and proliferate in its presence (Goad *et al.,* 1998). These bacteria convert lactate to easily protonated and absorbable VFA, however, as the pH of the rumen decreases closer towards 5, their population growth halts resulting in lactate production exceeding utilisation (Russel & Allen, 1984). In contrast, lactate-producing bacteria are not sensitive to low pH. Underdeveloped rumen papillae are too short to absorb high volumes of VFA now being produced in the rumen (Russel & Allen, 1984).

With a reduction in pH comes increased osmotic pressure in the rumen in the presence of free glucose. High osmolarity further reduces the acid VFA absorption rate, exacerbating the ruminal acidity levels (Owens *et al.,* 1998). According to Krause and Oetzel (2006), clinical signs of acute ruminal acidosis include: abdominal pain, complete anorexia, diarrhoea, staggering, tachycardia, tachypnoea, recumbency and death, and the possibility of recurring symptoms should an animal recover. Even in the absence of blood pH depression, the function of the central nervous system will be disrupted in the incidence of low concentrations of bicarbonate (Owens *et al.,* 1998).

Sub-acute ruminal acidosis is the most encountered nutritional disease in the dairy industry (Calitz, 2009). High DMI animals, transition animals and animals fed poorly formulated diets or experience wide variation in their meal patterns and diets, are at higher risk of developing SARA (Stone, 2004). A ruminal pH below 5.5 for intervals longer than 174 minutes daily will result in this condition (Cerrato-Sánchez *et al*., 2008; O'Grady *et al.,* 2008 & Plaizier *et al.,* 2008). The sudden high rate of volatile fatty acid production from increased starch content in rations in combination with low fibre content causes the reduction in pH, compared to the increased lactic acid production commonly seen in cases of acute acidosis. Low ruminal pH leads to increased lysis of gram-negative bacteria which results in higher concentration of LPS (lipopolysaccharide) in the rumen. Translocation of LPS into the circulatory system evokes an inflammatory response (Khafipour *et al.,* 2011), a reduction in fibre digestion, depressed feed intake, milk fat depression, laminitis, liver abscesses and diarrhoea (Plaizier *et al*., 2008). Sub-acute ruminal acidosis is most prevalent in dairy cattle between the time of calving to roughly 5 months post-calving (Oetzel, 2000). Donovan (1997) has estimated that in 1997, SARA cost the U.S. dairy industry between $500 million to $1 billion. In 1999 the study was taken further by Stone (1999), who estimated that SARA reduced milk production by 3 kg/cow/day, as well as milk fat from 37 to 34 g/kg. This estimation does not consider the effect that associated disorders such as lameness and laminitis would have in reducing milk fat and milk production in the dairy industry further. Unfortunately, after the initial exposure to low ruminal pH, most of the clinical signs seen in SARA and acute acidosis are delayed between weeks to months (Krause & Oetzel 2006).

The total intake of ruminal fermentable carbohydrates is equally dependent on the total dry matter intake (DMI, kg) and non-fibre carbohydrates in the diet (Krause & Oetzel 2006). The highly fermentable carbohydrate fraction in dairy cow diets gets increased to keep up with the vast energy demands associated with their current level of milk production. This means that ruminal acidosis and associated diseases will continue to be a problem as genetic progress

alongside improved feeding management is resulting in feed intakes being higher than ever before.

2.3.4 Animal welfare

It is important to remember that short term improved production and economic benefit from increased grain feeding does not negate the long-term unfavourable effects on cow health (Krause & Oetzel 2006). With the importance of public perception in mind, one must remember that lame cows do not portray the animal production industry favourably. According to Stone (2004) lameness is a major cause of involuntary, premature culling in a dairy herd. Lameness is directly linked to ruminal acidosis, with the common thread being laminitis. Solar haemorrhage, sole ulcer and white line abscess are all examples of laminitis-related hoof problems that cause lameness in dairy cows. These are serious animal welfare issues that need to be handled appropriately. Currently, the specific relationship between laminitis and SARA is unclear, however, the leading theory states that the low pH associated with SARA damages the ruminal epithelium wall leading to a reduction in absorption of specific amino acids. The absence of these compounds in the circulatory system causes hoof inflammation and disrupts homeostasis of the body leading to laminitis (Stone, 2004). The issues associated with hooves are generally only identified anywhere from weeks to months after the initial incident of acidosis that caused them, as with other acidosis-associated diseases (Oetzel, 2007).

Seasonal trends have a direct impact on lameness (Clarkson *et al.,* 1996). In weeks following seasonal environmental difficulties, spikes in the incidence of laminitis have been observed. This can be linked to the fact that slug-feeding is induced by heat stress which reduces rumen contractions, rumination and thus rumen pH. Combine this with a ration already higher in grain and the already low rumen pH drops further, inducing SARA (Stone, 2004), and the vicious cycle continues. A well-known issue that is often ignored, is the link between animal health and human health. A low ruminal pH increases the risk of enterohemorrhagic *E. coli* shedding from the intestinal lining of the dairy cow and being spread into the environment to the detriment of farmworkers and other cows in the herd (Nocek, 1997).

2.4 Buffers

2.4.1 Natural ruminant buffering system

The presence of a buffering agent in an aqueous solution, results in a resistance to change the pH of that solution in the incidence that a strong acid or base is added to that solution. The buffers aid in maintaining the stability of the pH in a system (Morel *et al.,* 1976). Buffering agents

must have the following properties: soluble in water, weak acid or weak base or salt thereof and have an equivalence point (pKa) close to the physiological pH of the system in need of buffering. Buffers can be split into two groups: true buffers and alkalinizing agents. True buffers prevent the extent to which pH decreases without increasing the pH whilst alkalinizing agents can neutralise acid and increase pH (Staples & Lough, 1989). There are primarily three ways by which the ruminant buffers ingested acid or volatile fatty acid (VFA) production by ruminal microbes, namely: saliva, buffering capacity of dietary feed, and dietary buffers (Erdman, 1988).

2.4.1.1 *Saliva*

Alkaligenic glands secrete a high concentration of bicarbonate fluid whilst mucogenic glands predominantly secrete mucoproteins (Harfoot, 1981). Feed intake stimulates the activity of these glands and their respective secretions. The greatest stimulation is seen when animals are fed coarse feedstuffs high in fibre. Coarse feed particles stimulate the rumen wall and results in the initiation of salivary reflexes. This may be additionally due to ruminal pressures, such as rate of flow and capacity to some extent. Feed factors have a direct impact on saliva production, secretion, and flow through dry matter content, feed intake and particle size of the forage. Higher rates of feed intake and increasing forage particle size increases saliva production of up to between 108 to 308 L/d in cattle (Bailey, 1961). The chewing of feedstuffs is imperative for entire maceration of plant matter with saliva (Harfoot, 1981). Bovine saliva concentration of disodium phosphate and sodium bicarbonate makes saliva the primary source of rumen buffering, with an average pH of 8 (Bartley, 1976). However, in cases of high producing dairy cattle fed high starch diets, supplemented dietary buffers are needed as the buffer flow from saliva is inadequate (Erdman, 1988).

Another consequence of feeding high concentrate diets is reduced saliva flow to the rumen (Van Campen, 1976) which has a direct impact on the bicarbonate-phosphate buffer system. According to Bartley (1976), the concentration of bicarbonate and phosphate in bovine saliva is 90 to 120 mM and 20 to 25 mM, respectively. Keeping in mind the pH of saliva, 86% of the phosphate present is in the ionic form (HPO_4^{2-}). Upon entering the rumen environment, which is already at a lower pH than that of saliva, the phosphate ions will react with the hydronium ions (H_3O^+) present, resulting in the formation of dihydrogen phosphate ions ($H_2PO_4^-$) and water (H_2O). This reactions' endpoint is reached when 90% of the phosphate is in the dihydrogen phosphate form, which significantly raise the ruminal pH (Counotte *et al.,* 1979). The bicarbonate ions work in a similar way. However, because the reactions equilibrium pH value of bicarbonate ions is 6.25,

bicarbonate theoretically should not play a role in the buffering system until the rumen pH reached this point (Counotte *et al.,* 1979):

$$HCO_3^- + H_3O^+ \leftrightarrow [H_2CO_3 + H_2O] \leftrightarrow CO_2 + 2H_2O.$$

2.4.1.2 *Dietary composition*

The rate of feed fermentation in the rumen is defined by more than just the chemical composition of the feedstuff included in the diet. The physical form and processing of feed ingredients can increase the fermentable extent of the diet, such as whether grains have been steam-flaked, finely-ground or extruded (Oetzel, 2007). This is important when formulating for high producing dairy cattle who are already at risk for SARA development.

Feedstuffs have specific acid-consuming capabilities (Oetzel, 2007). Differences among experimental procedures and buffer capacity calculations complicate comparisons of studies, however there are general trends in results between studies. Higher buffering capacities (BC's) in fresh forages have been recorded among legume plant species and high protein feeds, whilst cereal grains and feedstuffs high in energy have lower buffering capabilities and low protein feeds have been reported to have an intermediate BC (Jasaitis *et al.,* 1987). As forages mature, their BC tends to decline. Studies into fermentation properties of ensiled forages and by-product feeds lead to a lower pH and higher water content of diets. The composition of the fermented plant substrate has a direct effect on the BC of silage and subsequently a large variation in rumen pH. Microbes found in silage and the rumen are responsible for the production of two forms of lactate: namely, D+ and L forms. Whilst the L form is readily metabolised by tissue in the liver and heart, as it is identical to the form produced in the conversion of glucose to energy, D+ makes up 30 to 38% of the total ruminal lactate, is not produced by the body and will therefore accumulate (Owens *et al.,* 1998). This will have a role in further reducing ruminal pH. Estimating the NDF digestibility of maize silages may be incredibly useful when identifying whether the diet may increase the rate of ruminal fermentation and putting cows at risk for SARA (Oetzel, 2007).

Mineral additives contribute to the BC of a ration. The chemical composition of specific compounds is the biggest influencer of BC, as the acidic mineral additives and phosphates have a lower BC when compared to carbonates and di- or tribasic mineral additives (Jasaitis *et al.,* 1987). The mineral content of a feed is directly impacted by the origin (be it animal or plant-based) and type of processing. The ion concentrations in plants will differ according to stage of plant maturity, soil fertility, fertilisation, season as well as the geographic origin (Jasaitis *et al.,* 1987).

An increase in the crude protein (CP) content in a feed result in the increase in BC and pH of the feed. This is because amino groups have high buffering abilities. Crude protein influences BC more-so in *in-vitro* or *in-vivo* fermentation as deamination of proteins increases the concentration of ammonia present (Jasaitis *et al.,* 1987).

The dietary cation-anion difference (DCAD) can be used to describe the BC of the diet. Dietary cation-anion difference is calculated as follows; (Na + K) – (Cl + S), and an optimal level for early lactation diet is +400 mEq/kg (DM) feed (Oetzel, 2007). The higher the Sodium (Na) and Potassium (K) in relation to Chloride (Cl) and Sulphur (S), the higher the DCAD value of the diet and subsequently supports increased ruminal pH, DMI and milk yield. Another major buffer reservoir of bodily fluid is bone. A decrease in ruminal pH in response to acidic diets subsequently reduces blood pH. To maintain blood-pH homeostasis, cations (mostly calcium ions) are released from the bone into the bloodstream. This may be problematic over long periods as cows will essentially leach calcium out of their bones to maintain a constant blood pH, resulting in brittle bones and hypocalcemia when calcium requirements are high during lactation (Goff & Horst, 2003). Supplemented dietary buffers are required when a DCAD value drops below +275 mEq/kg DM feed to increase the relative Na or K in the diet to prevent such occurrences.

2.4.1.3 *Animal behaviour*

Sorting is a behaviour seen in dairy cattle fed total mixed rations (TMR's). Individual cows will select certain ingredients in the diet for consumption, thereby changing the nutritive value and intake quantity of the original diet fed (Miller-Cushon & DeVries, 2017). This type of sorting behaviour results in the overconsumption of non-structural carbohydrates (NSC) that are rapidly fermentable in the rumen (Gao & Oba, 2014). Avoiding excess material longer than 2.5 to 5 cm aids in minimising sorting in TMR rations (Stone, 2004 & Oetzel, 2007). Wetting rations with water or by making use of wetter feed ingredients creates a more viscous feed that is more difficult to sort. Ensuring the diet is palatable and available *ad libitum* encourages maximum intake and prevents further sorting. Table 2.2 summarises the results found in a study by Prentice *et al.* (2000). Forage with lower DM content (%) has a much lower average daily pH in conjunction with increased time spent below a pH of 5.5 in the rumen. Even in animals fed the same diets, there appears to be considerable variation in sorting behaviour of individuals (Gao & Oba, 2014).

Table 2.2 Effect of target ruminal pH goal on pH parameters and feed intake (Adapted from Prentice *et al*., 2000)

Result	Target pH = 6.0	Target pH = 5.6
Forage (DM %)	26.3	9.40
Daily mean ruminal pH	5.99	5.78
Time spent under pH 5.5 (hour)	0.74	1.68
DMI[1] (kg/d)	13.2	9.43

[1]DMI = Dry matter intake

Access to feed is often overlooked when identifying SARA contributors. Maximising milk yield through DMI leads to cattle being fed *ad libitum;* however, the risk of SARA may be limited by slightly limiting consumption. Slightly underfeeding does not in any way equate to feed deprivation. Feed deprivation not only disrupts the microbiome of the ruminant, but further causes overeating as soon as feed is re-introduced. Unfortunately, this has detrimental effects in lowering ruminal pH even more (Oetzel, 2007). Post-feeding ruminal pH variation cannot necessarily be solved by increasing the frequency of TMR feeding as DMI is reduced resulting in a further reduction in ruminal pH. Intake depression is governed by the decrease in ruminal pH following a meal. Increases in ruminal content osmolarity is associated with low ruminal pH and rumenitis (Oetzel, 2007). Dry matter intake depression is even more evident following a reduction in ruminal pH below 5.5.

2.4.2 Dietary buffers

2.4.2.1 *Introduction*

The early theory that offering volatile fatty acids (VFA's) in correct ratios to allow for improved animal production on diets high in concentrates led to the addition of sodium salts of butyric, acetic, and propionic acids to the ruminant's diet (Van Campen, 1976). Improvements in weight gains were observed with the addition of potassium and sodium bicarbonates to high energy diets in steers (Matrone *et al.,* 1959). Adding of the afore mentioned VFA's in ester- rather than salt-form to the diet did not improve animal performance. This led to the conclusion that forms the basis of dietary buffers used today; the increased production was due to improved buffering capacity of the rumen provided by the free ions in exogenous agents (Matrone *et al.,* 1959 & Van Campen, 1976).

Although the causes of ruminal acidosis cannot be eliminated by dietary buffers, it can aid in managing the problem (Krause & Oetzel 2006). Some examples of effective rumen buffers are sodium bicarbonate ($NaHCO_3$), magnesium carbonate, bentonite, calcium carbonate and

potassium bicarbonate. Sodium carbonate, magnesium oxide (MgO), calcium hydroxide, potassium carbonate and sodium hydroxide, all of which are alkalizing agents (Krause & Oetzel 2006). Sodium carbonate cakes quickly due to its' hygroscopic properties, thus hindering it's use in majority of ruminant rations (de Vos, 2019). Both sodium and potassium hydroxide are hazardous to human health, which complicate the manufacturing process and is therefore not used in dairy rations. Although observations of buffers on ruminal fluid VFA content vary in terms of the extent of animal production performance, research has demonstrated that adding an exogenous dietary buffer will benefit production, provided that the correct buffer at the appropriate concentration specific to a dietary situation is employed (Van Campen, 1976).

2.4.2.2 *Bicarbonates*

Early studies conducted by Bunn and Matrone (1968) indicated that the use of bicarbonate as a dietary buffer maintains a higher ruminal pH, reduces ruminal lactate production, as well as decreases the acetate to butyrate (A:P) ratio in the rumen. Further studies confirmed these results and state that the rapid pH decline of the rumen following a meal may be prevented to some extent with the addition of such buffers to the diet (Lee & Matrone, 1971). Alternatively, there have been cases where no significant changes have been reported (Van Campen, 1976). Improved feed intake, milk yield and milk components, particularly milk fat, have additionally been observed to improve with the use of bicarbonates (de Vos, 2019). Sodium bicarbonates popularity as a buffer stems from its improved water solubility capacity (Enemark, 2008) and acid-dissociation constant, which ultimately supports ruminal fluid homeostasis (Marden *et al.,* 2008). Further improvements have been reported with regards to favourable changes in ruminal fermentation patterns, specifically an increase in the A:P ratio, increased apparent digestibility of acid detergent fibre (ADF), neutral digested fibre (NDF) and dry matter (DM) (Erdman *et al.,* 1982; Erdman, 1988; Marden *et al.,* 2008)

Sodium bicarbonate ($NaHCO_3$) can be produced commercially by mixing carbon dioxide (CO_2) with sodium carbonate, also known as soda ash (Weinberg, 1976). This chemical compound is not inherently stable as different ranges in temperature results in decomposition back to sodium carbonate. In the presence of water and temperature differences, sodium carbonate may become a mono-, deca- and/or hepta-hydrate. Despite this, the neutralising capacity of the compound will remain the same as sodium is present (Weinberg, 1976). $NaHCO_3$ has a very fast reaction rate in the rumen resulting in ineffective long-term buffering in the presence of continual VFA production (Cruywagen *et al.,* 2015). Although $NaHCO_3$ is one of the most used buffering agents seen in dairy diets today, it can have an effect on the concentrate

intake of dairy cattle. Palatability is claimed to not be an issue when these agents are fed in a TMR (Erdman, 1988) with multiple studies reporting an increased DMI and subsequent improved feed efficiency (FE) when sodium bicarbonate was used in conjunction with a high maize silage TMR (Brethour & Duitsman, 1972; Embry *et al.,* 1968; McLeod *et al.*,1970). However, inclusion rates above 3% DM in a TMR diet have been shown to depress feed intake and have negative effects on animal performance (Lassiter & Alligood, 1967).

According to Le Ruyet and Tucker's (1992) *in-vitro* work, the buffering ability of Sodium bicarbonate is pronounced in the first 12-hours post-consumption. It ss recommended that sodium bicarbonate is ideal for treating the short-term rapid reduction in ruminal pH, whilst a buffer with the capability of slower mineral release may have better success in ruminal pH maintenance until the next meal. Sodium bicarbonate's pKa value is 6.25 (Enemark, 2008) making it less soluble at a pH lower than 6. This reduce its claim as an aid to combat SARA, rather to be used as a rumen pH modulator. Sodium bicarbonate is an alkalinising agent, thus has an alkali-forming effect with regards to urine quality. Consequently, one must consider the potential for formation of phosphatic urinary calculi. The phosphorus content in the diet might have an influence on the formation of phosphatic urinary calculi, as the inclusion of more than 2% DM of $NaHCO_3$ in a diet with less than 0.28% phosphorus resulted in this condition in lambs (Emerick, 1976).

Buffer mixtures and combinations are regularly seen with sodium bicarbonate (Emerick, 1976). Magnesium oxide, calcium hydroxide and bentonite fall into this category. The magnesium in MgO is successful in alleviating the effects of MFD through increasing the uptake of tri-acyl glycerides and acetate in the mammary gland. This, in combination with alterations to fermentation parameters caused by $NaHCO_3$, may lead to improved animal productivity (Van Campen, 1976). However, the buffering ability of MgO has been shown to reach peak capacity at 24 hours, meaning that rumen passage eliminates the efficacy of this buffer (Le Ruyet & Tucker, 1992). Various limitations in terms of diet preparation and human safety bring additional questions to light with regards to MgO in combination with sodium bicarbonate as a commercially used buffer (Van Campen, 1976).

2.4.2.3 *Lithothamnium calcareum*

The Lithothamnium genus is comprised of 103 species of thalloid red alga. *Lithothamnium calcareum* is a species of sea algae which transforms into a significant natural buffer upon calcification (Almeida *et al.,* 2012). This particular calcified marine alga (CMA), flourishes in the cold temperatures of Atlantic waters. Calcified marine algae has been mined from sites on the Southwest coast of Ireland and Northwest coasts of Iceland (Almeida *et al.,* 2012). The mineral

composition of CMA is mostly determined by the water in which the organisms grow. In general, calcium carbonate in three alternative structures forms the majority of CMA. Calcium (300 g/kg), magnesium (55 g/kg) and potassium (7 g/kg) are three of the main highly bioavailable minerals (Celtic Sea Minerals, Cork, Ireland). The location or source from which the CMA is harvested, directly impacts the properties of the CMA. The buffering ability of CMA is attributed to the "honeycomb structure" formed by the alternative calcium carbonate structures, namely, calcite (65%), aragonite (23%) and vaterite (12%) (Celtic Sea Minerals, Cork, Ireland). This gives CMA the ability to release minerals slowly into the rumen environment. Using Lithothamnium as a base compound for alternative buffering agents may provide advantages over $NaHCO_3$ due to its slower rumen dissolution rate. Cruywagen *et al.* (2015) reported that due to this property of CMA, it has a substantial increase in the amount of ruminal acid neutralized for longer periods when compared to other chemical-based buffers used in industry.

Currently, Lithothamnium-based buffers are amongst the most used exogenous buffers in the dairy industry, with recommended inclusion rates of between 3- to 4% in the diet (Cruywagen *et al.,* 2015). Despite this, very little research has been published regarding the comparative efficacy between buffers, as well as their influence on animal production. Claimed advantages of existing CMA-buffers used in lactating cows' diets consist of improved ruminal environment neutralisation for longer time periods, greater milk yields and milk component parameters, enhanced fibre digestion and reduced methanogenesis (Calitz, 2009). In a comparative study by Cruywagen *et al.* (2015), sodium bicarbonate, due to its rapid solubilisation, maintained ruminal pH of pre-fed cows for an hour longer than both the control and a CMA-based buffer after feeding. However, the $NaHCO_3$ treatment was unable to maintain a higher pH in the rumen for an extended period, as seen with the CMA-based buffer treatment. It was further observed that the time the rumen pH spent below 5.5 was reduced from 13.8 to 4 hours in cows fed a diet supplemented with the Lithothamnium-based buffer compared to sodium bicarbonate, where the pH was below 5.5 for 7.5 hours, demonstrating CMA's ability to aid in prevention of SARA. However, one must consider the components of the diet to identify whether the requirements for fibre, non-structural carbohydrates and starch were met; this will be later discussed. Total VFA production has not yet been reported to be significantly affected by CMA-products, although the same study described an increase in acetate concentrations ($P < 0.01$). This led to the conclusion that since this particular CMA-based buffer created a more favourable acetate to propionate ratio, methane production would theoretically be reduced (Calitz, 2009). Others have seen no alterations in ruminal fermentation parameters nor molar proportions of VFA's produced (Mubiayi Beya, 2007; Montanez-Valdez *et al.,* 2012; Bilik *et al.,* 2014) with the use of CMA buffers. Cruywagen *et al.*

(2015) reported reduced lactic acid concentrations in the rumen due to lowered lactic acid producing bacteria after ingestion of this Lithothamnium-based buffer, therefore aiding in ruminal pH stabilisation.

No significant changes have been observed in DMI, body weight (BW), BW-change or body condition score (BCS) in lactating dairy cattle on CMA-product's additives in their diet (Calitz, 2009; Bernard *et al.,* 2014; Wu *et al.,* 2015; Cruywagen *et al.,* 2015). Milk production parameters however do appear to be significantly impacted by addition of *L. calcareum* to high producing dairy cows' diets (Cruywagen *et al.,* 2015). Increased total milk yields, milk fat percentages in conjunction with milk fat yields, as well as increased fat corrected milk (FCM) and energy corrected milk (ECM) were reported when CMA's were included at industry standards of 3 to 4% DM (Mubiayi Beya, 2007; Cruywagen *et al.,* 2015). Improved ECM with an unchanged DMI lead to the conclusion that CMA-based buffers have the capacity to improve a lactating cows' feed efficiency (FE) to produce milk (Cruywagen *et al.,* 2015). These improvements were above what could be obtained through using sodium bicarbonate as a dietary buffer which is significant as these results were achieved by using half the inclusion rate of sodium bicarbonate. In a study conducted by Wu *et al.* (2015), it was suggested that the use of Lithothamnium-based buffers may aid in a smoother transitioning period if fed in both pre- and post-partum stages of a cows' lactation cycle.

2.5 Application to industry

Disorders caused by high-starch, low fibre diets consistent with high producing animals have devastating effects on ruminant welfare and reduced production leading to financial losses. With livestock farming having such narrow margins, prevention is always better than cure. A comparative calculation included in an Intelact presentation by Anton Venter (2020) evaluated at whether the inclusion of an industry used buffer would be financially viable.

Assuming on a pasture-based system:

- Daily milk yield – 20 L
- Fat content – 3.2%
- Concentrate intake – 6 kg
- Income for butterfat – R 28/kg
- Penalty for butterfat percentages lower than 3.3% – 10 c/L
- Dietary buffer price – R 8500/ton

Pre-buffer use, daily income per cow (based off butterfat yield) is R 17.92. Considering the 0.3% butterfat increase with the addition of an exogenous buffer to the diet as well as taking the daily buffer cost of R 1.00 per cow, the post-buffer use daily income per cow is R 20.60. This is an additional R 2.68 per cow daily due to buffer supplementation. Taking into consideration the fact that buffers have been proven to assist in preventing metabolic diseases, one has to additionally consider the further losses experienced in these cases with regards to treatment, production losses, recurring cases, and possible death of animals (Oetzel, 2007). The value of dietary buffers to the ruminant livestock industry is substantial once all of this has been taken into consideration.

Buffers are not confined to the dairy industry alone. The feedlot industry in South Africa battle acidosis too. Additionally, sub-acute ruminal acidosis is a not only confined to South Africa, nor Africa; it is experienced in animals worldwide (de Vos, 2019). Beef feedlot cattle have similar risks as dairy cattle to develop SARA. The diet of a dairy cow is higher in fibre and forage when compared to feedlot cattle, however the significantly higher intakes of a high producing dairy cow offsets this (Oetzel, 2007). Monensin is commonly included as an ionophore in beef feedlot and dairy cow rations. Including Monensin at the upper level of inclusion rates can depress milk fat by 0.1- to 0.2%. Even though Monensin reduces acidosis risk, due to LAB inhibition and favouring lactic acid utilizers, one would expect increased milk fat percent, however MFD is still commonly seen (Oetzel, 2007). Due to inconsistencies in the effects of buffers on ruminal ammonia-N (NH_3-N), the effect of buffers on bacterial protein synthesis is often overlooked. However, should a buffer consistently demonstrate reduced NH_3-N, it may be deduced that bacterial nitrogen flow has been increased, thus enhanced bacterial protein synthesis efficiency (de Vos, 2019). This will be beneficial to both the dairy and beef industry.

2.6 Conclusion:

Feeding highly digestible, fermentable carbohydrate-rich diets to dairy cows is universally practised, owing to the emphasis on elevated levels of production. This results in the reduction of ruminal pH, which could prompt negative influences on production. Although the inhibition of the milk fat synthesis mechanism is not yet clearly defined, what can be concluded is the CLA isomers with a double bond at position-10 do have an inhibitory effect on milk fat production. Sub-acute ruminal acidosis accounts for substantial milk production losses yearly, and together with additional veterinary costs have significant financial implications.

Buffers play a key role in ruminal animal homeostasis. The addition of exogenous buffers to the diet has the capability to simulate the ruminants natural buffering system and aid in maximising rumen function and subsequent animal production. Several buffering agents are commercially available for use in dairy diets and is most effective when feeding high starch diets with a low effective fibre content. Variances in animal performance are to be expected when comparing studies, as differences in diet composition will impact the efficiency of the dietary buffer due to differences in the buffering capacity of individual feed ingredients as well as the interaction between the buffer, feedstuff, and the degree to which the animals are adapted to the diet in terms of their microbiome.

Buffers using Lithothamnium sources are among the leading dietary buffers sold in South Africa for use in dairy rations. There is limited research available on comparisons between other buffering agents and Lithothamnium sources. Studies are highly variable when reporting efficacy results of Lithothamnium-based products that are currently used in industry. It should be kept in mind that dairy cow diet, stage of lactation, age, environmental stressors, as well as management should be considered when interpreting the results of these studies. Furthermore, there is limited *in vitro* research on Lithothamnium using techniques such as BVI and acid-base titration studies with rumen fluid at body temperature investigating different incubation intervals.

Chapter 3:

Materials and methods

3.1 Experiment 1:

Experiment 1 was composed of three *in-vitro* studies; a buffer dissolution study (Experiment 1.1), a titration into incubated buffer solution study (Experiment 1.2) and a titration into ruminal fluid buffer solution (Experiment 1.3). Both experiment 1.1 and 1.2 were completed as background research and therefore were designed with statistical analysis in mind.

For all experiments, the following treatments were used in various quantities and procedures relative to the experimental design:

Treatment 1: No buffer (control)

Treatment 2: Sodium bicarbonate (NaHCO3)

Treatment 3: Lithothamnium source A (Lith A)

Treatment 4: Lithothamnium source B (Lith B)

3.1.1 Experiment 1.1:

3.1.1.1 Experimental design and treatments

The preliminary experiments of 1.1 and 1.2 were conducted at the Chemuniqué research laboratory (Chemuniqué (Pty) Ltd, 28 Eagle Lane Lanseria Business Park Lanseria, 1739). Experiment 1.1 took place on June 30, 2020 and was comprised of a buffer dissolution study in which 214 mg of the three buffer treatments (Sodium bicarbonate, Lith A and Lith B) were dissolved into 30 ml of distilled, deionised water (Le Ruyet & Tucker, 1992). The pH of each buffer-solution was recorded every 20 seconds for an hour using the Hanna portable pH meter (HI98190, Professional Waterproof Portable pH/ORP Meter, Hanna Instruments). A control was used in which the pH of distilled, deionised water was observed for the same time intervals to use as a basis to compare buffer results to. No statistical analyses conducted on this research as it was to be treated as background work.

3.1.2 Experiment 1.2:

3.1.2.1 Experimental design and treatments

Experiment 1.2 involved the incubation of three buffer treatments in a shaking water bath heated to 39.2°C for various time periods and subsequent acid-base titrations. This experiment took place between June 29 and July 2, 2020. As in Experiment 1.1, the same control of distilled, deionised water was used. Each buffer treatment (0.5 g) was respectively mixed with 70 ml of

distilled, deionised water in a 150 ml Erlenmeyer flask and placed in the shaking water-bath for various incubation periods; 2-, 4-, 6-, 8-, 10-, 12- and 24-hours. Upon removal from the water-bath, as well as with the 0-hour incubation period, the Erlenmeyer flasks were swirled five times and 30 ml of buffer-solution was dispensed into a 100 ml beaker for acid titration and repeated for base titrations. The pH of the solution was measured using a Hana pH portable meter (HI98190, Professional Waterproof Portable pH/ORP Meter, Hanna Instruments) every 20 seconds after titrating 2 ml of HCl (0.1 *M*) (Hydrogen chloride) and NaOH (0.1 *M*) (Sodium hydroxide) respectively at a time. The volume required to obtain end points of 2 and 12 for acid and base titrations respectively was recoded (Le Ruyet & Tucker, 1992). As with Experiment 1.1, no statistics were run for this experiment.

Figure 3-1 Image illustrating the arrangement of equipment for titration into aqueous-buffer solutions to determine a titration curve using a Hana portable pH meter.

3.1.3 Experiment 1.3:

3.1.3.1 Farm and management

Four cannulated Holstein cows were individually housed at the University of Pretoria's Hatfield Experimental farm (University of Pretoria Experimental Farm, Koedoespoort 456-Jr, Pretoria, 0186). The trial commenced on August 3 and ended on August 29, 2020. Animals were kept in semi-covered pens with concrete flooring, each with an area of 53 m^2. Pens were cleaned by removing any excess excrement and waste build-up whilst water troughs were drained and

scrubbed on a regular basis. This occurred during the morning milking while two cows of the four cows were out of their pens to prevent any disturbances to their usual eating behaviour or induce stress. The two remaining cows that were not milked were moved to a neighbouring pen to have then pens cleaned for minimal stress. The experimental pens allowed for sufficient cow movement and shelter and additionally were bedded with dried manure solids which aided in the prevention of claw problems or lameness incidences. Feeding troughs were cleaned every morning before morning feeding. Cows were fed twice daily; 07:00 and 16h00, to ensure fresh feed was available *ad libitum.* The diet adaptation period lasted for three weeks to ensure adequate adaptation before rumen fluid collection commenced.

Two out of the four cannulated cows were in lactation and were milked three times daily at the following times: 06h00, 12h00 and 19h00. The other two cows were dry and remained in their assigned pens for the duration of the study.

Table 3-1 Animals were assigned to 1 of 4 pens, whereby cow identification number (ID), body condition score (BCS), body weight (kg) and lactation number were noted

Pen Number	Cow ID	BCS	Body weight (kg)
1	1402	3.5	805
2	1303	2.25	611
3	1302	3.25	789
4	1401	3.25	753

The University of Pretoria's Animal Ethics Committee (AEC) gave experimental approval to the protocol number NAS038/2020 on July 3, 2020 prior to the commencement of the trial. Cattle were handled according to the standard operating procedure followed by all staff for dairy cattle at the University of Pretoria Experimental Farm.

3.1.3.2 Experimental design

The total duration of the experimental period lasted 27 days. This was comprised of an initial adaptation period of three weeks, followed by three rumen fluid collections on days 22, 24 and 26 of the total experimental period. Cows remained housed in their assigned pens for the duration of the experiment unless taken for milking in the parlour or rumen fluid collection in the crush.

3.1.3.3 Feeding and total mixed ration mixing

All four cows received the same basal TMR *ad libitum* basis and was mixed on farm every morning before feeding at 07:00 (Table 3.2). The three feed component; two roughage sources (silage and wheat straw) and a premixed dairy concentrate, were weighed on a battery-operated scale and mixed using spades on a plastic tarpaulin. This task was completed in the same manner daily to ensure fresh feed and preventing silage spoilage. Once mixed, the TMR was weighed into 20 L sealable plastic buckets for both morning and afternoon feeding. Afternoon feeding occurred at 16h00.

The concentrate was a custom-made premix, manufactured on-site of Chalmar Beef (Chalmar Beef, Section 78, Tweefontein 413 JR, Bapsfontein, Gauteng, 1510).

Table 3-2 Total mixed ration ingredient composition of the experimental basal diet (%DM)

Ingredient (% DM)	
Maize silage	26.4
Yellow maize (finely ground)	18.7
Soybean oilcake	13.9
Wheat straw	13.6
Hominy chop	9.5
Wheat bran	8.4
Canola oilcake	5.2
Megalac	1.9
Limestone	1.3
Molasses (Liquid)	0.4
Salt (NaCl)	0.4
Urea	0.3
ENS Dairy Premix[1]	0.01

[1] Essential Nutrient Solution (ENS) Dairy Premix (PO Box 68544, Highveld, 0169, Gauteng) is comprised of the following components: Nutroteq Dairy microminerals, Monensin and flavour enhancers

3.1.3.4 Monitoring of body weight and body condition

Cows were weighed at the beginning of the experimental period and additionally on a weekly basis thereafter. The body weights of the two cows in milk were also automatically recorded three times a day as the cow leaves the milking parlour. Should one of the experimental

animals have lost more than 15% of her starting body weight, she would have been removed from the trial. Animal body condition scoring (BCS) occurred on the first day, halfway and the last day of the trial according to the 5-point scoring system (Wildman *et al.*, 1982). On the experimental farm, BCS's are routinely carried out by staff in the milking parlour after morning milking.

Cannulae were inspected weekly throughout the duration of the trial. After each inspection, cannulae were cleaned with luke-warm water mixed with a medical soap. Coopers spray was afterwards applied to the perimeter of the cannula for fly control.

3.1.3.5 Sample collection, preparation, and laboratory work

Ruminal fluid was collected by hand at 9h00 on collection days and strained through three layers of cheesecloth into a preheated thermo-flask. A total of 2.4 L of ruminal fluid was then decanted into a flask being held in a water bath in the University of Pretoria's NutriLab (University of Pretoria, Lynnwood Rd, Hatfield, Pretoria, 0002). A measurement of 0.5 g of each buffer treatment (bar T1, as it was the control) was weighed into 32, 100 ml labelled glass bottles. Carbon dioxide was bubbled through the flask to ensure the microbes in the rumen fluid were kept in an anaerobic environment. Thereafter, 70 ml of rumen fluid was drawn into a 100 ml syringe and dispensed into the 32 labelled glass bottles. Rubber stoppers with a cockstop-needle attachment were used to seal off the rumen fluid solution in the glass bottles. These cockstop-needle devices allow for gas build-up release *in-vitro* as a result of microbial activity in the ruminal fluid during incubation period whilst ensuring the solution is not contaminated with environmental air. After the addition of the rubber stopper to a bottle, the bottle was swirled 5 times before being placed into the incubating shaking water bath, with the temperature maintained at 39 °C (Figure 3-2).

Flasks were removed from the shaking water bath at the following time intervals; 0-, 2-, 4-, 6-, 8-, 10-, 12- and 24-hours for titrations and further analysis. Upon removal, each solution was swirled five times and 30 ml were decanted into two separate flasks that were used for acid and base titrations. These flasks were placed onto heated stir-plates and whereby 0.1 *M* of HCl (Hydrochloric acid) was titrated into one of the flasks solutions to an endpoint of pH 5 and 0.1 *M* of NaOH (Sodium hydroxide) was titrated into the second flask to an endpoint of pH 7. The pH was measured and noted using two Hanna portable pH meters (HI98190, Professional Waterproof Portable pH/ORP Meter, Hanna Instruments) 1 minute after each titration was performed (Figure 3-3). In cases where the starting pH of the solution was above 7, only the volume of acid required to reduce the pH to 5 was recorded (Le Ruyet & Tucker, 1992).

Figure 3-2 Image illustrates the 32, 100 ml bottles with rubber stoppers and a cockstopper-needle attachment in the shaking water bath maintained at 39 °C

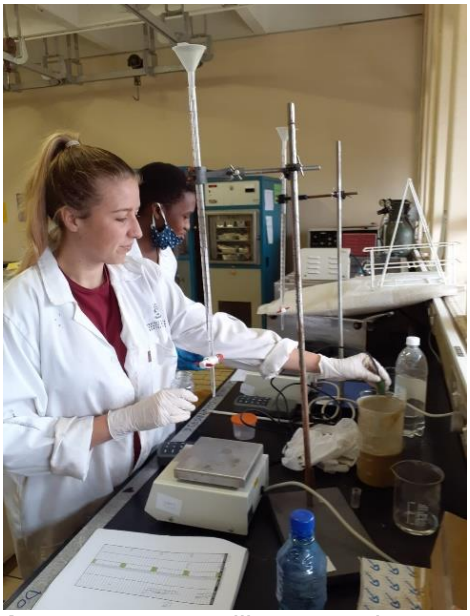

Figure 3-3 Image illustrates the setup of the titration equipment so that researchers may titrate acid and base into separate solutions simultaneously

Solution pH as well as the volumes of acid and base required to reach the appropriate endpoints were used to calculate solution hydrogen ion content (H+) (nano-equivalents/L) (Fiorica, 1968), buffer's buffering capacity (BC) (milli-equivalents/L) and individual buffer's buffer value index (BVI) (Tucker *et al.,*1992).

Buffering Capacity (BC) of a buffer (milliequivalents/L) = [1*N* HCl (ml) x 1*N* NaOH (ml)] x 10^3/30

Buffer value index (BVI) of a buffer = ({[(antilog$_{10}$(-STPH)) – (antilog$_{10}$(-SAPH))]/(antilog$_{10}$(-STPH)) + [(SABC – STBC)/STBC]} x 10) + 100

Whereby: STPH = the standard pH of 6, SAPH = initial pH of the rumen fluid solution before titrations had begun, SABC = the initial BC of the rumen fluid solution before titrations had begun (mEq/L) and STBC = the standard BC of mEq/L.

3.1.3.6 Statistical analysis

Experiments were repeated in the same manner over three different days, each day serving as a replicate. The data was analysed statistically with the Proc Mixed model (Statistical Analysis System, 2021) for the average effects within each period. The means and standard error

were calculated afterwhich a significance of difference (P<0.05) between means was determined by Fischers test (Samuels, 1989).

The linear mix model used is describe by the following equation:

One way

$$Yi = \mu + Ti + ei$$

Where Yi = variable studied during the period

μ = overall mean of the population

Ti = effect of the i^{th} treatment

ei = error associated with each Y

3.2 Experiment 2:

3.2.1 *Farm and management*

Experiment 2 commenced on September 14, 2020 and ended on December 14, 2020. Animals were individually housed at the Outeniqua Research Farm (Outeniqua Research Farm, Western Cape Department of Agriculture, George, 6530). Cows were fed respective treatment diets twice daily at 08:00 and 16h00 to ensure fresh feed was available *ad libitum.* Furthermore, cows had access to fresh, cool water *ad libitum.*

Separate adjoined pens of 36 m^2 housed experimental animals under trees which provided shade. Cows remained in their respective pens for the duration of the study and were removed twice daily for milking at 05:00 and 15h00. Pens were bedded with woodchips and cleaned three times daily prevent any manure build-up and promote udder health (Figure3-4). Feed troughs were placed in the corner of each pen at the highest point to prevent water flooding the area around the trough during rainy conditions. Wooden backboards were installed behind the feed troughs, depicted in images below, to ensure minimal feed wastage. Roofs were built over the feed troughs according to the height of each animal to prevent decreases in feed intakes due to wet feed during rainy conditions (Figure 3-5). Rubber mats were placed under feed troughs to keep the area stable to catch any wasted or spilt feed so that it could be put back into the trough for accurate feed intake estimations.

Figure 3-4 Image depicts 36 m^2 pens, floored with woodchips setup under trees for shade; furthermore, buckets used for morning and afternoon feeding can be seen here.

Figure 3-5 Illustrates the wooded back-boards placed behind the feed-troughs to prevent excess spilling as well as the wooded roofs placed over the feed-troughs, ensuring dry feed during rain conditions.

Cannulated animals were selected according to their lactation number and age (Table 3-3). All cows were the same age and in the same lactation. The younger the cow, the higher her resilience during stressful changes, for example being adapted from a herd pasture grazing system to being separated into individual pens on a TMR.

Table 3-3 Animals assigned pen numbers, identification number (ID), weight, body condition score (BCS), lactation number, daily milk yield (kg/day) and days in milk (DIM) the day before the trial began

Pen	Cow ID	BCS	Body weight (kg)	Lactation	DIM	Milk Production (kg/day)
1	16197	2.25	364	3	19	18
2	16205	2	346	3	99	20.4
3	16212	2	387.5	3	81	18.9
4	16226	2	337	3	52	24.8

The University of Pretoria's Animal Ethics Committee (AEC) gave experimental approval to the protocol number NAS038/2020 on July 3, 2020. The Western Cape Governmental Research committee also approved the trial protocol. Cattle were handled according to the standard operating procedure followed by all staff for dairy cattle at the Outeniqua Research Farm.

3.2.2 *Experimental design and treatments*

The study composed of a 4 x 4 Latin square design experiment with a total experimental length of 92 days (Table 3-4). There were 4 experimental periods each spanning 23 days. Within a period, animals were allocated 14 days for adaptation (de Vos, 2019) followed by a 9-day sampling period (Cruywagen *et al*, 2015).

Table 3-4 Experimental animal diet rotation schedule between experimental periods for the duration of the trial

Pen Number	ID	Period 1	Period 2	Period 3	Period 4
1	16197	T1[1]	T4	T3	T2
2	16205	T2[2]	T1	T4	T3
3	16212	T3[3]	T2	T1	T4
4	16226	T4[4]	T3	T2	T1

[1] Treatment 1, Control.
[2] Treatment 2, Sodium bicarbonate ($NaHCO_3$).
[3] Treatment 3, Lithothamnium Source A (Lith A).
[4] Treatment 4, Lithothamnium Source B (Lith B).

3.2.3 *Feeding and total mixed ration mixing*

The ingredient composition of the four experimental treatments is shown in Table 3-5.

Table 3-5 Total mixed ration (TMR) composition of individual treatment diets mixed on experimental farm for cattle (on a %DM basis)

Ingredient (% DM)	T1 (Control)	T2 ($NaHCO_3$)[2]	T3 (Lith A)[3]	T4 (Lith B)[4]
Maize silage	26.4	26.4	26.4	26.4
White maize (finely ground)	18.7	18.7	18.7	18.7
Soybean oilcake	14.1	14.1	14.1	14.1
Wheat straw	13.6	13.6	13.6	13.6
Hominy chop	9.5	9.5	9.5	9.5
Wheat bran	8.5	7.7	8.1	8.1
Canola oilcake	5.2	5.2	5.2	5.2
Megalac	1.7	1.7	1.7	1.7
Limestone	1.2	1.2	1.2	1.2
Molasses (Liquid)	0.5	0.5	0.5	0.5
Salt (NaCl)	0.4	0.3	0.3	0.3

Urea	0.2	0.2	0.2	0.2
CFG Dairy Premix[1]	0.06	0.06	0.06	0.06
Buffer treatments	-	0.7	0.4	0.4

[1] Cape feed and Grain (CFG) Dairy Premix (Cape Feed and Grain, George, Western Cape) contains per kg of premix: 6 000 000 IU of Vitamin A; 1 000 000 IU of Vitamin D3; 8000 IU of Vitamin E; 50 g of Manganese (Manganese Sulphate 31%); 100 g Zinc (Zinc SO4-Mono 35%); 20 g Copper (Copper SO4 25.2% Penta); 1 g Cobalt (Cobalt SO4 21%); 1.7 g Iodine (Potassium Iodine 76.45%); 0.3 g Selenium (Sodium Selenite 4.5%) with a Dolomitic Carrier of 440.0045 g.
[2] Sodium bicarbonate ($NaHCO_3$) (Simple Grow, Centurion, Gauteng).
[3] Lithothamnium Source A.
[4] Lithothamnium Source B.

The concentrate portion of the experimental treatments were mixed at Nova Feeds, George (Saagmeul St, George Industria, George, 6536, South Africa, Western Cape). The concentrate was then blended on farm with the roughage components of the diet (silage and wheat straw) through a series of mixing techniques on a plastic tarpaulin. To simply daily TMR mixing, feeding and sample collection, each treatment was assigned a different colour that was printed onto the concentrate feed bags (in which the respective buffers were already blended), on the pen tag of each cow on the relative treatment, as well as on respective feed troughs:

Treatment 1 – Red

Treatment 2 – Green

Treatment 3 – Pink

Treatment 4 – Orange

The total mixed ration (TMR) components were weighed out separately for each treatment every morning and the three components (concentrate, wheat straw and maize silage) were placed onto a 3 x 6 m plastic tarpaulin (0.5 mm thick), evenly spread over one of the long ends of the tarpaulin. Feed was mixed by taking hold of the corners of the plastic sheeting where the feed components were placed and pulling the corners to the other side of the sheeting, causing the feed components to mix in a similar manner than that of a cement mixer (Figure 3-6). This technique was repeated three times for each treatment, after which the TMR was placed into feeding bins and buckets for morning and afternoon feeding respectively.

Figure 3-6 The feed is placed on the one end of the plastic tarpaulin before being mixed in a rotational mixing manner to ensure an evenly-homogenized experimental ration.

Animals remained in the same assigned pen for the duration of the study whilst the treatment rotates between them for each period. Upon completion of the trial, each cow would have received each of the experimental diets during a different experimental period. This ensured that the cattle were not disrupted between periods and minimised stress when returning to pens after milking. This method also safeguarded differences in treatments that could be due to cow differences and not cow x pen interactions, thus further reducing variation.

3.2.4 *Monitoring of body weight and body condition*

Feed intakes were monitored and recorded throughout the experimental trial to aid in herd-health evaluation through sudden changes in diet intakes. Cows were weighed and given a body condition score (using the 5-point scoring system) at the beginning of each period, as well as at the end of the trial at the same time of the day. Furthermore, body weights were recorded twice daily as each cow entered the milking parlour. Should an experimental animal have lost more than 15% of her body weight during an experimental period, she would have been removed from the trial. Rumen cannulae had previously been fitted with easy-in-easy-out stoppers, which ensured that animals could be handled for rumen fluid collection with very little stress and no restraints. Agita Granular fly Bait (Novartis) was painted onto the wooden backboards behind each feed trough, to keep the fly irritants to a minimum. Chains used for animal identification purposes with different coloured tags corresponding to pen numbers were placed around cows' necks (Figure 3-7).

Figure 3-7 One of the on-farmhands, Andrew Petoors, walking Lin 58 to her afternoon milking; chains used for animal identification purposes with different coloured tags corresponding to their pen numbers can be seen around her neck.

3.2.5 Sample collection, preparation, and analytical methodology

3.2.5.1 Total mixed ration and refusals

Feed was supplied *ad lib* daily, aiming at 10% of the daily allowance left as refusals. Daily allowances were weighed, recorded and sampled on the relevant sampling days (day 15-20). Feed samples were collected from each batch of TMR using five grab samples at random, post-mixing (Robinson & Meyer, 2010). Samples were then weighed and dried for 24-hours in an oven at 105 °C in order to determine the dry matter content of the feed (AOAC method 934.01). Feed samples were further pooled per period as well as per treatment and processed further by being milled through a 1 mm-sieve. These samples were then frozen until being analysed by ChemNutri Laboratories (Cedar Lake Industrial Park, c/o M57 &, Porcelain Rd, Olifantsfontein, 1665) for ash (AOAC Method 942.05), starch (Megazyme starch assay kit, Megazyme International, County Wicklow, Ireland; further adapted to AACC International (2010), method 996.11 and 76-13.01, respectively), crude protein (AOAC Method 988.05), neutral detergent fibre and acid detergent fibre (Van Soest *et al.*, 1991), ether extract (Soxhlet extraction, Method 920.39), Calcium and Phosphorus (Methods 965.09 and 965.17, respectively after acid digestion using Method 935.13), and Sulphur, Potassium, Magnesium, Manganese, Sodium and Chloride using AOAC (2000) official methodologies for analysis. The pH of each TMR treatment was measured using a Hanna pH meter by mixing 1 g of dried and milled TMR samples with 5 ml of distilled, deionised water after 1 minute (Harrison *et al.,* 1989).

In order to ensure that the TMR had consistent fibre fractions between treatments additional to within and across periods, further grab samples were taken post mixing to pass through the Penn-State particle separator (PSPS). Samples were placed on the top sieve of PSPS and shaken in accordance with the PSPS instructions (Figure 3-8). Separate fractions are then

weighed and inserted into the PSPS formulae to determine whether the fibre particle size distribution was optimal and consistent over days and periods. The following fibre fraction percentage separation was aimed for; Top sieve of 2-8%, Middle sieve of 30-50%, Lower sieve of 10-20% and bottom pan of 30-40% (Heinrichs, 2013). This was carried out for all four treatment TMR's on day 15 and day 16 of each experimental period shorty after morning mixing. Refused treatment feed (orts) was collected in the afternoon, before 16h00 feeding, weighed, recorded and sub-sampled using the afore mentioned method on the equivalent sampling days (day 15-19). Refusal samples were subsequently weighed and dried in the oven at 105 °C for 24-hours. After drying, samples were pooled, frozen and analysed in the same manner as the feed samples.

Figure 3-8 The use of a Penn-State Particle Seperator (PSPS) was used to ensure that the TMR had the correct portions of specific fibre lengths to ensure rumen health.

3.2.5.2 Faeces

Titanium dioxide (TiO_2) was used in this study as an external marker to estimate total faecal excretion. A marker adaptation period of seven days ran concurrently with the second week of the adaptation period of each experimental period, starting on day 8 until day 14. This was done to ensure accurate digestibility results. TiO_2 was weighed out as 5 g quantities into biodegradable coffee filters. During both the adaptation and sample collection period, the TiO_2 wrapped parcels were placed directly into the rumen twice daily, at each feeding (de Souza *et al*., 2015). Faecal sampling ensued from day 15-20 of the experimental period. Grab samples were taken from excreta piles in the experimental pens whilst animals were at morning and afternoon milking. Whilst sampling, care was taken to ensure that the freshest excreta was sampled from and there was no contamination from the surrounding environment. These samples were instantly

placed in the oven to be dried for 24-hours at 105 °C. After drying, faecal samples were milled through a 1 mm-sieve and pooled in accordance with cow per period per treatment. These samples were then frozen until being analysed by ChemNutri Laboratories (Cedar Lake Industrial Park, c/o M57 &, Porcelain Rd, Olifantsfontein, 1665) for titanium dioxide (Myers *et al*., 2004), starch content (Megazyme starch assay kit, Megazyme International, County Wicklow, Ireland; further adapted to AACC International (2010), method 996.11 and 76-13.01, respectively), crude protein (Dumas Method 988.05) and neutral detergent fibre, using AOAC (2000) official methodologies for analysis.

Figure 3-9 Daily faecal samples were milled through a 1 mm-sieve and pooled according to cow per treatment per period into 1 kg buckets.

3.2.5.3 Milk production parameters

Trial animals were milked twice daily where daily milk yield was monitored and recorded throughout the experimental period. During the sampling period of each experimental period (day 15-20), milk samples were deposited directly into a 250 ml bottle, dividing 2 ml of milk into the bottle for every 100 ml of milk pumped into the tank (Figure 3-10). This ensured that a representative milk sample over the full milking time was taken. The 250 ml bottle was then slowly canted 4 times to ensure evenly distributed milk solids throughout the sample. A subsample was then poured with a constant stream into 30 ml bottles with pre-added potassium dichromate preservative in them supplied by Mérieux NutriSciences (4 Pearl St, Tamsui Industria, George, 6529) (Figure 3-11).

In order to ensure a good representation sample from a full day of milk production, two-thirds of the 30 ml sample bottle was filled during morning milking, and one-third during afternoon milking. This is because the experimental cows were milking two-thirds of their daily milk yield in the morning. Samples were then stored at 4 °C for no longer than 48 hours before being analysed for protein (%), fat (%), lactose (%), MUN (mg/dl) (Milkoscan TM FT, FOSS ®) and SCC (1000 cells/ml) (Fossomatic instrument) (Cruywagen *et al*., 2015).

Figure 3-10 Image demonstrates the bottle-contraption used to collect milk samples during morning and afternoon milking; for every 100 ml milked, 2 ml is deposited into the sample bottle.

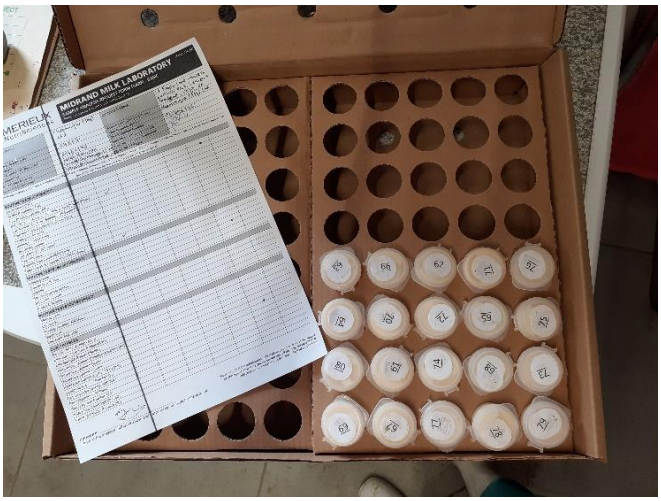

Figure 3-11 Sub-sampled milk capsuled in 30 ml bottles with Potassium dichromate preservative can be seen here to be analysed by Mérieux Milk Laboratory (4 Pearl St, Tamsui Industria, George, 6529).

3.2.5.4 Ruminal pH profile

Ruminal pH was continually measured using a SmaXtec pH Plus bolus (US-1042A). Boluses were activated, as per manufacturer's instructions, after which were inserted into each experimental animals' reticulum at the beginning of the trial via the rumen cannula (Figure 3-12). These boluses relay the ruminal pH at 10-minute intervals to a base station installed in a farm shed nearby. The data is then uploaded to a cloud and can be accessed via an application or by logging into your account with SmaXtec.com online (Figure 3-13). This allowed for pH and continual pH changes to be recorded in real time in this way for the entirety of the trial, however pH information was only analysed statistically over days 21-24. This correlated with the days on which rumen fluid collection was performed. Rumen fluid was collected during the following times on the following days; Day 21 at 09h00 and 21h00, day 22 at 12h00 and 24h00, day 23 at 03h00 and 15h00 and day 24 at 06h00 and 18h00 (Van Niekerk & Hassen, 2009). At each of the

collection times, rumen fluid pH was measured instantly in the pen with each animal using a using a portable pH meter post rumen fluid collection (de Vos, 2019) (HI98190, Professional Waterproof Portable pH/ORP Meter, Hanna Instruments). SmaXtec pH boluses were removed by hand at the end of the trial, cleaned in distilled water and placed into the same bucket with water to determine if there was any large variance in the pH readings of boluses in the same pH medium.

Figure 3-12 Image depicts a SmaXtec pH bolus before insertion into the reticulum via the rumen cannula.

Figure 3-13 Demonstrated here are the pH-, temperature-, activity- and heat-detection-curves for a partiular cow, generated every 10 minutes by the SmaXtec bolus inserted in the reticulum.

Modified Continual pH Omni-Loggers (Omni Instruments) were inserted into the rumen of experimental animals through the cannula in order to track the reliability of the SmaXtec loggers. This was performed during experimental period three's adaptation period, for a total of seven days. The Omni-Logger is a ruminal pH measurement tool similar to that of the SmaXtec bolus, however animals must be cannulated as the logger fits into the rumen fistula, as illustrated in Figure 3.14. Upon removal, ruminal pH data is directly downloaded off the device and onto a computer program which correlates pH's to specific timestamps, which similarly fall in 10-minute intervals. Due to the inherent differences in pH between the rumen, reticulum and inter-cow differences, it was imperative to determine specific differences between cows so that the continually logged reticulum pH's could be extrapolated for ruminal pH explanations (Neubauer *et al.,* 2018). This information was purely used for monitoring the efficacy of SmaXtec pH bolus data as well as the measured ruminal pH during rumen fluid collection days.

Figure 3-14 Image displays a continual pH Omni-Logger, inserted via the cannula into the rumen of an experimental animal.

3.2.5.5 Ruminal fermentation

Ruminal fluid was collected, by hand, via the cannula over four days in order to monitor changes in ruminal fermentation end products (Figure 3-15). The following collection schedule was followed to gain insight into a 24-hour period in the rumen; day 21 at 09h00 and 21h00, day 22 at 12h00 and 24h00, day 23 at 03h00 and 15h00 and day 24 at 06h00 and 18h00 (Van Niekerk & Hassen, 2009). Ruminal contents were collected from the ventral sac in the same fashion between animals, treatments, and periods by starting at the proximal end of the rumen and moving caudally in a systemic manner. Collected digesta was then strained into a sealed ruminal fluid collection jar. These samples were swiftly taken to the laboratory on-farm and strained through three layers of cheesecloth to remove any coarse material. Subsequently, two 20 ml subsamples of rumen fluid were drawn up per treatment and deposited into two 50 ml double-seal rumen fluid bottles respectively (Figure 3-16).

Upon request by the University of Free State Biochemistry Laboratory, no ruminal fluid preservatives were used. Instead, rumen fluid was flash-frozen in liquid nitrogen, and then stored at – 20 °C for analysis (De Vos, 2019). Rumen fluid was analysed for volatile fatty acids (VFA) and lactic acid (LA) at the University of Free State Biochemistry Laboratory (Biotechnology Annex, Swot Street, University of the Free State, Bloemfontein, 9300). For each of these parameters, 128 samples were available for analysis across the entire experimental period.

Figure 3-15 Image showing titanium dioxide, held in a coffee-filter, insertion into the rumen via the cannula.

Figure 3-16 50 ml Bottles used to store rumen fluid samples for further analysis after filtration through three layers of cheese-cloth depicted here.

3.2.5.6 Calculations

Daily dry matter intake (DMI) = (Total daily feed on offer (kg) x Dry matter % of feed) – (Total daily refusals (kg) x Dry matter % of refusals).

Individual nutrient intakes = (Individual nutrient of analysed daily feed on offer (g/kg) x DMI (kg)) – (Individual nutrient of analysed daily refusals (g/kg) x total daily refusals (kg)).

DMI as percent of bodyweight (BW) (%) = [DMI (kg) / individual cow BW (kg)] x 100.

DMI as percent of metabolic BW (%) = [DMI (kg) / (individual cow BW (kg))$^{0.75}$] x 100.

Faecal output estimation using titanium dioxide as a marker (g) = TiO2 consumed (g/d) / TiO2 concentration in faeces (g/g DM).

DM Digestibility (%) = [(Dry matter intake (g) – Faecal output (g)) / Dry matter intake (g)] × 100.

4% FCM (Fat corrected milk) (kg) = (0.4 x milk yield (kg)) + (15 x milk fat yield (kg)) (NRC, 2001).

ECM (Energy corrected milk) (kg) = Milk production (kg) x (383 x fat (%) +242 x protein (%) + 783.2) / 3140 (Sjaunja *et al.,* 1990).

Milk efficiency = Milk yield (kg/d) / DMI (kg).

Milk efficiency 4% FCM = 4% FCM yield (kg/d) / DMI (kg).

Milk efficiency ECM = ECM yield (kg) / DMI (kg).

3.2.5.7 Statistical analysis

Data was statistically analysed as a 4 x 4 Latin Square design, using the general liner model (GLM) analysis of variance (Statistical Analysis System, 2021) for the average effects over time. The linear model used is described by the following equation: $Yijk = \mu + Ti + Cj + Pk + eijk$; where Yijk is the response variable studied; μ is the overall mean; Ti is the fixed effect of the i^{th} treatment (i =T1, T2, T3, T4); Cj is the random effect of the j^{th} cow (j = 1, 2, 3, 4); Pk is the fixed effect of the kth period (k = 1, 2, 3, 4); and eijk is the random residual error associated with the related observation. For the statistical analysis of repeated period measures; ruminal fermentation parameters, sampling time, and sampling time-treatment interaction was added to the model and analysed as Repeated Measures Analysis of Variance using the GLM model. Results were reported as least square means ± standard error of the means (SEM). For the different statistical tests, significance of difference between means were declared at $P < 0.05$ and a tendency of difference at $0.05 < P \leq 0.10$, as determined by Fisher's test (Samuels *et al.*, 1989).

Certain data sets were analysed statistically with the Proc Mixed Contrasts model (Statistical Analysis System, 2021) for the average effects within each period. This was done to directly compare treatments 1 to treatment 3 and 4 as well as treatment, treatment 1 to 2 and treatment 3 to 4. Means and standard error were calculated and significance of difference ($P<0.05$) between means was determined by Fischers test (Samuels, 1989).

The linear mix model used is describe by the following equation:

One way

$Yi = \mu + Ti + ei$

Where Yi = variable studied during the period

μ = overall mean of the population

Ti = effect of the i^{th} treatment

ei = error associated with each Y

Chapter 4:

Results and Discussion I: *In-vitro*

4.1 Solution pH

The capacity of an endogenous dietary buffer to neutralise acid after being consumed differs according to the buffers' physical and chemical characteristics. The nature of a buffer varies from quick dissolving upon rumen entry to rumen-insoluble buffers which largely bypass the rumen. Prevention of fermentation-acid build-up through continual buffer release in the rumen or release during the time of most inflated acid production makes for an ideal, efficient buffer (Le Ruyet & Tucker, 1992). The initial pH represents the pH of solution after incubation but before titration had begun to determine the buffering capacity. Whilst the pH of each treatments' solution remained relatively constant over the 24-hour incubation period, significant differences were observed between treatments (P<0.05). Treatment 2's average solution pH maintained the highest pH of between 6.76 and 6.88. This was higher than treatment 3 and 4 (P<0.05), which exhibited no differences between each other (pH T3=6.09-6.29, pH T4=6.12-6.32) (P>0.05).

Treatment 1's solution pH was significantly lower than treatments 3 and 4 over the 24-hour incubation period (5.57-6) (Table 4-1 and Figure 4-1). Similarly, Tucker *et al.* (1992) and Le Ruyet & Tucker (1992) each described pH increases with the addition of dietary buffers ($NaHCO_3$, MgO and sodium sesquicarbonate) to solution *in-vitro*. Furthermore, the most marked increase was seen with the addition of sodium bicarbonate after 24-hours of incubation. This was supported by the results seen in the background buffer dissolution study (See results in Appendix figures A.1, A2, A3, A4, A4). However, in terms of pH change over time, no significant differences were observed between Lithothamnium sources *in vitro.*

Table 4-1 The pH change over time for eight different incubation periods in a water bath of four buffer treatments dissolved in rumen fluid solution

	Treatment[1]				SEM[2]
	T1	T2	T3	T4	
Incubation Period (hours):					
0	6.00[c]	6.82[a]	6.29[b]	6.22[b]	0.0334
2	5.87[c]	6.87[a]	6.25[b]	6.32[b]	0.0661
4	5.71[c]	6.88[a]	6.15[b]	6.26[b]	0.0710
6	5.64[c]	6.81[a]	6.15[b]	6.18[b]	0.0740
8	5.64[c]	6.83[a]	6.15[b]	6.23[b]	0.0854
10	5.57[c]	6.79[a]	6.09[b]	6.12[b]	0.0749
12	5.59[c]	6.77[a]	6.09[b]	6.12[b]	0.0797
24	5.59[c]	6.76[a]	6.11[b]	6.18[b]	0.0713

[1] T1 = Control (no buffer added to solution); T2 = 0.5 g Sodium bicarbonate; T3 = 0.5 g Lithothamnium buffer source A; T4: 0.5 g Lithothamnium buffer source B.
[2] SEM = Standard error of the mean.
[a, b, c] Means within a row with different superscripts differ significantly (P<0.05).

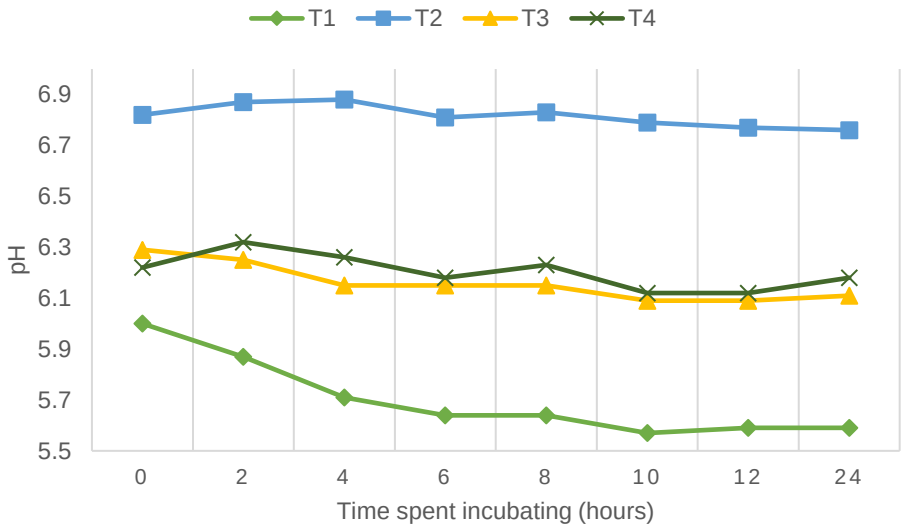

Figure 4-1 Graph depicting the change in pH over time whilst 0.5 g of various treatment buffers (T1 = Control, no buffer; T2 = Sodium bicarbonate; T3 = Lithothamnium source A; T4 = Lithothamnium source B) in rumen fluid solution incubate in a water bath (n=12).

4.2 Buffering capacity

The buffering capacity (BC) is defined by the number of moles of hydrogen ions to be added to 1 L of solution in order to decrease the pH by 1 unit (Segel, 1976) and is calculated by using the total volume of acid and base added to the rumen fluid solution in order to reach specific pH endpoints. It is important to note that this BC value depends on both the buffer system as well as the systems pH. Results showed that solutions that were incubated for up until 10 hours maintained the same trend where differences in treatment BC were seen ($P<0.05$) between treatment 2, 3 and 4, and 1 (listed in descending BC order) (Table 4-2). However, the trend was broken for hours 12 and 24. The BC of treatment 4 did not differ from treatments 3 and 4 ($P>0.05$), but differences were observed between treatments 1, 2, and 3, respectively ($P<0.05$). All four treatments exhibited increases in BC values in the 24-hour incubation period (Figure 4-2).

In a similar study conducted by Le Ruyet & Tucker (1992), the BC of sodium bicarbonate appeared to increase until 12-hours of incubation and remained constant for the remainder of the incubation period. The multi-elemental buffer (MEB), which Le Ruyet & Tucker (1992) has previously likened to treatment 4, exhibited an increase in BC over time. This was attributed to the more gradual buffer dissolution. Sodium bicarbonate used as a dietary buffer resulted in carbon dioxide release from rumen fluid solution and subsequent removal via eructation. The degree to which this occurs depends on the partial pressure of CO_2 in the gas phase, pH as well as the 7.74 constant. *In-vitro,* the addition of sodium bicarbonate to rumen fluid solutions in anaerobic conditions is expected to increase pH. Kohn & Dunlap (1998) demonstrated that rumen fluid incubated *in-vitro* under these *in-vitro* conditions had a greater buffering capacity at a low pH.

Titratable acidity and buffering capacity differences between treatments can be explained by the higher proportion of HCO_3^- in different buffer treatments. This, in turn, may help to explain the different rumen fluid pH starting points for the titrations that were performed here (Xu *et al*., 1994). It is important to note that any reduction of dietary acidity due to buffer inclusion may aid in increasing dry matter intake and subsequently improve rumen acid-base status (Cassida *et al*., 1988; Erdman 1982). Whilst the BC of both Lithothamnium sources change over time, they did not exhibit differences between each other ($P>0.05$).

Table 4-2 Buffering capacity (BC) change over time for eight different incubation periods in a water bath of four buffer treatments dissolved in rumen fluid solution

	Treatment[1]				SEM[2]
	T1	T2	T3	T4	
Incubation Period (hours):					
0	71.1^{c}	113^{a}	86.7^{b}	91.1^{b}	4.1574
2	66.7^{c}	104^{a}	88.9^{b}	86.7^{b}	2.4845
4	66.7^{c}	108^{a}	84.4^{b}	88.9^{b}	1.9245
6	64.4^{c}	109^{a}	88.9^{b}	88.9^{b}	2.2222
8	66.7^{c}	106^{a}	88.9^{b}	91.1^{b}	4.1574
10	68.9^{c}	107^{a}	88.9^{b}	93.3^{b}	2.4845
12	66.7^{c}	104^{a}	93.3^{b}	96.0^{ab}	3.6851
24	82.2^{c}	109^{a}	100^{b}	104^{ab}	2.7217

[1] T1 = Control (no buffer added to solution); T2 = 0.5 g Sodium bicarbonate; T3 = 0.5 g Lithothamnium buffer source A; T4: 0.5 g Lithothamnium buffer source B.
[2] SEM = Standard error of the mean.
[a, b, c] Means within a row with different superscripts differ significantly (P<0.05).

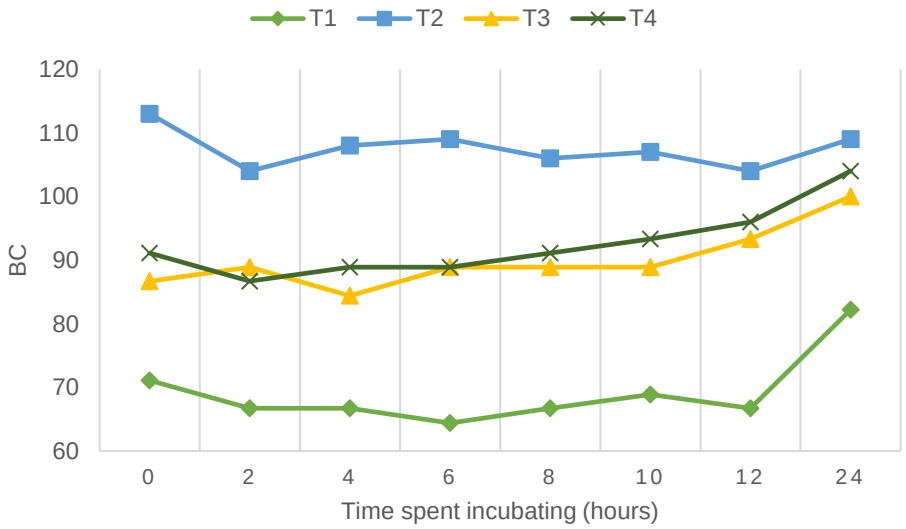

Figure 4-2 Graph depicting the change in buffering capacity (BC) of buffers over time whilst 0.5 g of various treatment buffers (T1 = Control, no buffer; T2 = Sodium bicarbonate; T3 = Lithothamnium source A; T4 = Lithothamnium source B) in rumen fluid solution incubate in a water bath (n=12).

4.3 Buffer Value Index

The BVI takes both the buffer-induced alterations of H+ concentration and BC into account, thus providing a more accurate evaluation of the strength of a buffer (Le Ruyet & Tucker, 1992). A study conducted by Tucker *et al*. (1992) showed that endogenous dietary buffers proved to increase both ruminal fluid pH and buffering capacity *in-vitro*, which carries over buffer advantages to the cow itself. Because the buffer value index accounts for both of these parameters, Tucker *et al.* (1992) concluded that it provides the most complete evaluation of buffer induced changes in the rumen.

Treatments 2, 3 and 4 all managed to maintain their BVI and thus buffer efficacy over 24-hours whilst treatment 1 lost efficacy as time progressed from 104- to 90.5-BVI units (Figure 4-3). Furthermore, the BVI of treatment 1 was lower than treatment 2, 3 and 4 (P<0.05). Treatment 2 maintained an average BVI of 119.8, treatment 3 a mean BVI of 110.6 and treatment 4 a mean BVI of 112 over the 24-hour incubation period. The BVI of treatment 2 was observed to be larger than treatment 3 and 4 (P<0.05). However, at 8 hours of incubation the BVI of treatment 4 did not differ from treatment 3 and 2 (Table 4-3) (P>0.05). Although numerical differences were observed between the BVI of Lithothamnium sources, no significant differences were recorded *in vitro.*

Table 4-3 Buffer value index (BVI) change over time for eight different incubation periods in a water bath of four buffer treatments dissolved in rumen fluid solution

	Treatment[1]				SEM[2]
	T1	T2	T3	T4	
Incubation Period (hours):					
0	104^{c}	121^{a}	112^{b}	112^{b}	0.9864
2	99.5^{c}	120^{a}	112^{b}	112^{b}	1.6286
4	93.0^{c}	120^{a}	110^{b}	112^{b}	1.7919
6	89.0^{c}	120^{a}	110^{b}	111^{b}	1.9996
8	89.3^{c}	119^{a}	110^{b}	112^{ab}	2.5489
10	86.5^{c}	119^{a}	109^{b}	111^{b}	2.2580
12	87.1^{c}	119^{a}	110^{b}	112^{b}	1.9707
24	90.5^{c}	120^{a}	112^{b}	114^{b}	1.6353

[1] T1 = Control (no buffer added to solution); T2 = 0.5 g Sodium bicarbonate; T3 = 0.5 g Lithothamnium buffer source A; T4: 0.5 g Lithothamnium buffer source B.

[2] SEM = Standard error of the mean.

[a, b, c] Means within a row with different superscripts differ significantly (P<0.05).

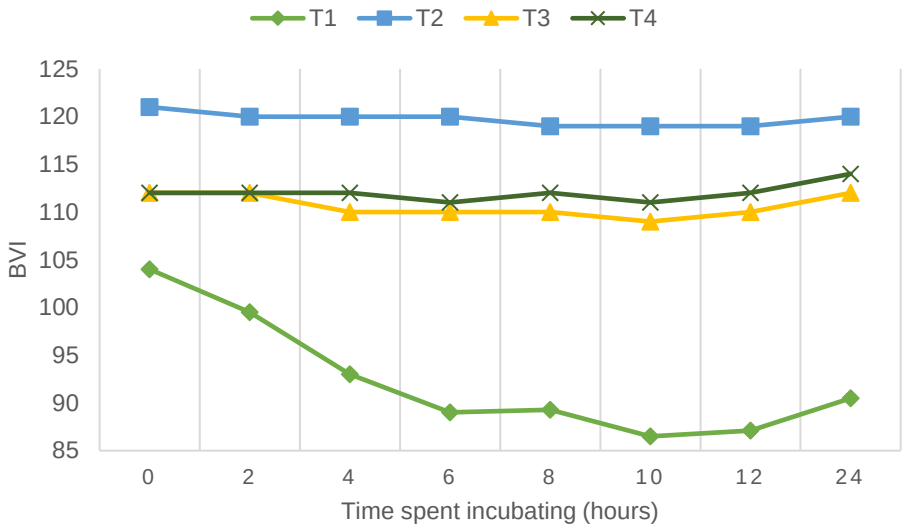

Figure 4-3 Graph depicting the change in the buffer value index (BVI) of buffers over time whilst 0.5 g of various treatment buffers (T1 = Control, no buffer; T2 = Sodium bicarbonate; T3 = Lithothamnium source A; T4 = Lithothamnium source B) in rumen fluid solution incubate in a water bath (n=12).

4.4 Hydrogen ion concentration

Hydrogen ion concentration (mol/L) of solutions followed a similar trend to what was observed in solution pH, as it is a function of pH measured in solution. The hydrogen ion concentration (mol/L) in the rumen fluid solution increased logarithmically from 1001 mol/L to 2498 mol/L over the 24-hour period whilst treatments 2, 3 and 4 maintained their hydrogen ion concentrations of 159, 729 and 642 mol/L, respectively (Table 4-4). Treatment 1 had the highest concentration in solution compared to the other treatments ($P<0.05$) (Figure 4-4). At 0-hours of incubation, treatment 2 was observed to have a lower hydrogen ion concentration than 3 and 4. Over 2- and 4-hour incubation periods, treatment 2 exhibited lower concentrations than treatment 3 ($P<0.05$). From the 6-hour incubation period to the 24-hour incubation period, there were no differences between treatments 2, 3, and 4 ($P>0.05$).

Similarly, in a study conducted by Le Ruyet & Tucker, 1992; buffer addition to rumen fluid solution resulted in ruminal fluid hydrogen ion concentration reduction. In comparison, initially the H^+ concentration increased and subsequently remained inflated for the control solution. This was concluded to be due to the microbial fermentation-acid production which was not neutralised because of the lack of a buffer in solution. Furthermore, $NaHCO_3$ most markedly reduced the H^+ concentration (Tucker *et al*., 1992). These authors theorise that the multi-elemental buffer (MEB) that was compared to sodium bicarbonate, among other buffers in the study, requires more than

5-hours in solution before fully releasing its buffering potential. This may align with treatment 4 in this study, which tended to pull ahead of treatment 3 after 2-hours of incubation. The sharp decline of H^+ concentration at 10-hours of incubation had a knock-on effect in the BVI of the control treatment, which increased along with the BC.

Table 4-4 Hydrogen ion (mol/L) concentration change over time for eight different incubation periods in a water bath of four buffer treatments dissolved in rumen fluid solution

	Treatment[1]				SEM[2]
	T1	T2	T3	T4	
Incubation Period (hours):					
0	1001[a]	151.8[c]	521.2[b]	599.8[b]	41.2354
2	1370[a]	136.2[c]	585.7[b]	490.6[bc]	117.59
4	2073[a]	134.4[c]	732.0[b]	563.8[bc]	147.11
6	2309[a]	167.1[b]	733.6[b]	667.9[b]	238.73
8	2405[a]	156.5[b]	733.9[b]	611.7[b]	238.81
10	2783[a]	166.3[b]	837.3[b]	765.1[b]	255.43
12	2622[a]	174.5[b]	850.6[b]	768.9[b]	248.96
24	2498[a]	185.2[b]	835.7[b]	670.2[b]	236.08

[1] T1 Control (no buffer added to solution); T2 = 0.5 g Sodium bicarbonate; T3 = 0.5 g Lithothamnium buffer source A; T4: 0.5 g Lithothamnium buffer source B.

[2] SEM = Standard error of the mean.

[a, b, c] Means within a row with different superscripts differ significantly (P<0.05).

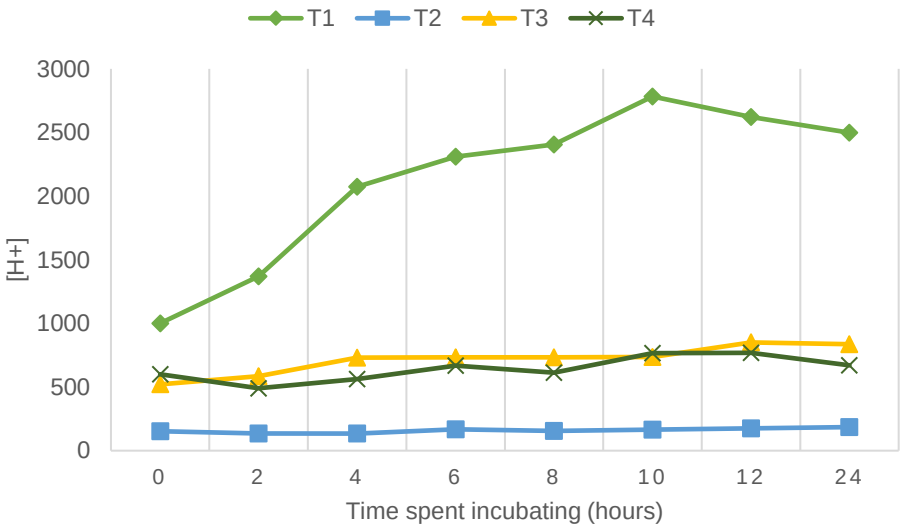

Figure 4-4 Graph depicting the change in hydrogen ion concentration of solution over time whilst 0.5 g of various treatment buffers (T1 = Control, no buffer; T2 = Sodium bicarbonate; T3 = Lithothamnium source A; T4 = Lithothamnium source B) in rumen fluid solution incubate in a water bath (n=12).

Improving prediction models on the effects of ruminal pH on metabolism and VFA production will aid in optimization of ruminant nutrition. Results from the *in vitro* studies revealed that $NaHCO_3$ had the highest *in-vitro* buffering efficacy, followed by Lith sources and then the control, which had the lowest buffering ability. The buffer value index provided the most complete evaluation of buffer efficacy in the rumen according to the *in vitro* results. The *in vitro* buffer value index was the highest for the $NaHCO_3$ treatment compared to the other treatments, while the Lith sources did not differ from each other. *In vitro* results suggest that $NaHCO_3$ has a higher buffering capacity when compared to the control and two different Lith sources.

Chapter 5:

Results and Discussion II: *In-vivo*

5.1 Feed, refusals, and nutrient intakes

5.1.1 Experimental rations comparison

Experimental diets were formulated according to NRC (2001) recommendations for a 454 kg, small breed cow, in mid-lactation (estimated at 90 DIM), producing 20 kg of milk a day. The concentrate was manufactured by Nova Feeds feed (1-9 Saagmeul St, George Industria, George, 6536). The concentrate treatments were mixed together with maize silage and wheat straw by hand, daily, to ensure fresh TMR was available to experimental animals. All four diets were formulated to be identical in chemical composition, differing only in the buffer supplementation. The nutrient composition of the experimental diets and feed refusals are shown in Table 5-1. The small numerical differences can be ascribed to sampling and relatively small samples needed when performing the feed analyses.

Higher calcium content in treatment 3 and treatment 4 is attributed to the chemical composition of the buffer itself. Lithothamnium is derived from calcified species of algae which contains calcium in the form of calcium carbonate (de Vos, 2019), thus resulting in inflated calcium content in those diets. Overall, the calcium content of all treatments was considerably higher than the recommended level of 0.57%. Excess calcium in the dairy cow diets has not been related to a particular toxicity. To the contrary, diets high in maize silage content, such as this diet, shows that inflated calcium levels aided in increasing cow performance. The NRC (2001) explains that this is caused by the alkalinizing effect that calcium carbonate has on the rumen. However, the phosphorus (P) content of the diet correlates well with the recommended level of 0.33%. The higher calcium content meant that the Calcium: Phosphorus ratio has been increased to between 2.15-2.44. Consequently, with increasing levels of calcium in the diet, phosphorus absorption efficiency begins to decrease. Phosphorus is an integral mineral in microbial cellulose digestion and protein synthesis in the rumen (Burroughs *et al*., 1951; Breves & Schroder, 1991). Stevens *et al.* (1971), however, reported no differences in dairy cattle performance when feeding different diets ranging in Ca:P ratio from 1.5:1 to 3:1.

The crude protein content (CP) of the diet was seen to be slightly under the requirement of 16.1%. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) values across diets fell in the correct ranges of 25-33% and 17-21%, respectively. It is important to note that the diet NDF

content was in line with that of a standard dairy TMR diet and did not exceed 35%. However, the recommended non-fibrous carbohydrate content (NFC) range of 36-44% was exceeded by up to 3% across treatments (NRC, 2001). In order to challenge the rumen, higher NFC concentration in the diet was required. A challenged rumen with fluctuating pH's created the ideal environment to evaluate the efficacy of the exogenous buffers. The NFC content is only slightly higher than the recommendation, and still considered normal in terms of a commercial TMR diet fed in industry. When formulating feed to identify the effects of buffers on rumen fermentation parameters, the diet must still meet the NRC (2001) requirements. Pushing a cow out of these ranges may skew results. In the Cruywagen *et al.* (2015) study; The CMA-based buffer treatment analysis (% of DM) had a neutral detergent fibre (NDF) value of 26.2%, and acid detergent fibre value of 20.5% (ADF) and a non-fibrous carbohydrate value (NFC) of 47.1%. The NRC (2001) states that the NDF and ADF values of the diet should fall within the range of 25-33% and 17-21%, respectively. The NFC value should be within the range of 36-47%. The integral role of starch in the acidotic diet has been discussed, however Cruywagen *et al.* (2015) did not declare the starch level of the TMR diet. Whilst this diet's NDF content does fall within the range, it is on the lower side. This, in conjunction with the NFC content, which is above the NRC (2001) recommendation, will result in an acidotic diet. One must consider that the outcome may not be a true reflection of industry standard rations fed to dairy cows, and this should be kept in mind when comparing results between studies.

Grain processing of maize silage is seen to have major influence on variation between silage sources. Furthermore, it typically does not consist of sufficient long particles which contribute towards the roughage portion of TMR (Krause & Oetzel, 2005). Thus, long chopped straw may typically be added to TMR's with a high maize silage content. This was advised after analysis of our maize silage had been conducted. However, according to Krause & Oetzel (2005), diets consisting of excessive long particles of fibre are unpalatable and subsequently sorted out of the TMR which in turn increases the risk for SARA.

5.1.2 Proximate analysis of refusals

Proximate analysis of daily refusals per treatment revealed that trial animals had sorting tendencies observed between treatments as well as overall (Table 5-1). A consistent inflation of OM for all treatments must be noted. Furthermore, reduced starch content of refusals was seen. This indicates that the experimental animal's actual intake of starch was higher than what was formulated for. An important find is that the difference in starch content between feed (269 g/kg) and refusals (253 g/kg) was the smallest on treatment 4, suggesting that less sorting of high starch

feed components occurred when animals were fed this diet. This was found to correlate with the NFC content of treatment diets. In turn, this may have influenced the rumen parameter results. Furthermore, T4 has a significantly smaller difference between both NDF and ADF of the feed on offer and refusals collected (286 g/kg versus 299 g/kg and 181 g/kg versus 193 g/kg) whilst NDF and ADF values from T1, T2 and T3 suggests that animals selected against the roughage in the diet. An interesting reduction in the calcium and phosphorus content in the refusals of T2 indicates selection for these particular minerals. This may be linked to possible associations between sodium bicarbonate and these minerals, further highlighting the need to investigate the association further to ensure lactating dairy cattle are not underfed Ca and P when diets are supplemented with sodium bicarbonate as an exogenous buffer.

Table 5-1 Each experimental treatment total mixed ration and collected refusals chemical composition (g/kg DM) over the experimental period (n = 16)

	Treatment[1]							
	T1		T2		T3		T4	
Nutrient:	Feed	Refusals	Feed	Refusals	Feed	Refusals	Feed	Refusals
DM (g/kg)	922	916	925	923	926	919	932	927
Ash (g/kg DM)	62.0	60.0	64.3	63.3	64.9	63.6	64.4	62.0
OM (g/kg DM)[2]	938	940	936	936	935	936	935	938
Starch (g/kg DM)	266	236	269	209	264	239	269	253
CP (g/kg DM)	141	137	146	123	149	135	147	146
aNDF (g/kg DM)	282	320	284	369	274	320	286	299
$aNDF_{OM}$ (g/kg DM)[4]	220	260	219	305	209	256	222	237
ADF (g/kg DM)	180	202	183	243	175	207	181	193
NFC (g/kg DM)[5]	474	445	467	411	473	443	462	454
EE (g/kg DM)	39.9	37.7	38.3	34.1	38.7	37.8	40.1	38.5
Ca (g/kg DM)	7.47	7.22	7.12	6.40	8.42	8.52	8.12	8.05
P (g/kg DM)	3.47	3.3	3.22	2.8	3.45	3.2	3.45	3.3
Ca:P	2.15	2.2	2.21	2.3	2.44	2.6	2.35	2.4

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rate.
[2] OM (g/kg DM) = 1000 – Ash(g/kg).
[3] ME (MJ/kg DM) = 0.82 x (GE x IVOMD) (Robinson *et al*., 2004).
[4] $aNDF_{OM}$ (g/kg DM) = aNDF – Ash.
[5] NFC (g/kg DM) = 100 – (NDF % + CP % + EE % + Ash %) (NRC, 2001).

5.1.3 Dietary mineral and anion-cation difference analysis

The pH of each dietary treatment was determined. The non-buffered diet, treatment 1, had the lowest pH of 4.95 and differed from treatment 2 which exhibited the highest average pH value of 5.19 (P<0.05). This is in agreement with Harrison *et al.* (1989), who reported that a control diet had a pH value of 4.94 and then sodium bicarbonate treatment diet had a pH of 5.19. The pH values of treatment 3 and 4 were 5.05 and 5.04, respectively, and did not differ from each other. Treatment 1, 3 and 4 had a similar sodium content (0.13-0.14%), whilst treatment 2 had a value of 0.26%, which is almost double when compared to the other treatments. Xu *et al.* (1994) similarly observed an increase in sodium content of the diet when sodium bicarbonate was used as an endogenous buffer. It should be noted that the buffering capacity of a forage source may modulate the effect of buffers on ruminal pH (Kennelly *et al.,* 1999).

The copper content of the treatment 3 was higher than that of treatment 2 (P<0.05). This could be due to the fact that the Lithothamnium becomes a source of the environment in which it calcified. It is possible that Lith A was mined from an environment with a higher copper content. Copper is an essential mineral as it is a co-factor to numerous enzymatic reactions in the body, such as energy metabolism and production, oxidative cell damage protection, red cell production, collagen synthesis as well as plays a role in hormone function (Lopez-Alonso & Miranda, 2020). Differences were observed between treatments for manganese content, though they all fell in the correct range (P<0.1).

The dietary cation-anion balance (DCAD) of a diet may largely explain the inherent buffering capacity of the diet (Krause & Oetzel, 2005). Low DCAD diets often require the addition of endogenous buffers to the diet that are high in sodium or potassium as the DCAD calculation is as follows: (Na + K) – (Cl + S). Higher DCAD values support increased ruminal pH's, milk yields and dry matter intake (DMI) (Block & Sanchez, 2000). Mid-lactation cows perform optimally with DCAD values of between +27.5 to +40 mequiv. /100 g. Lower or negative DCAD values are associated with diets composed of a high fermentable carbohydrate content (Krause & Oetzel, 2005). The DCAD values of the respective treatment diets were as follows; T1=7.81 mequiv. /100 g, T2=13.6 mequiv. /100 g, T3=9.11 mequiv. /100 g, T4=9.80 mequiv. /100 g (Table 5-2). All treatment diets DCAD values were determined to be below that of the optimal range, which is supported by increased time spent below rumen pH thresholds. The control diet had a lower DCAD value compared to the $NaHCO_3$ treatment (P<0.05). This was expected because of the contribution of the sodium when calculating DCAD differences.

Table 5-2 Each experimental treatment total mixed ration mineral composition, pH and DCAD values over the experimental period (n = 16)

Parameter	Treatment[1]				SEM[2]
	T1	T2	T3	T4	
TMR pH	4.95[b]	5.19[a]	5.05[ab]	5.04[ab]	0.0421
Sodium (%)	0.14[b]	0.26[a]	0.14[b]	0.13[b]	0.0026
Potassium (%)	0.96	0.95	0.98	1.02	0.0677
Sulphur (%)	0.19	0.19	0.20	0.19	0.0091
Chloride (%)	0.39	0.36	0.35	0.36	0.0542
Copper (mg/kg DM)	5.20[ab]	4.53[b]	6.57[a]	5.60[ab]	0.1569
Iron (mg/kg DM)	232	224	247	226	2.5387
Magnesium (%)	0.23	0.20	0.23	0.23	0.0084
Manganese (mg/kg DM)	57.3[c]	44.6[d]	47.1[d]	44.3[d]	6.6589
DCAD[3]	+7.81[b]	+13.6[a]	+9.11[ab]	+9.80[ab]	3.8852

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.
[3] DCAD (mequiv./100g) = [(Na % x 435 + K % x 256) – (Cl % x 282 + S % x 624)] / 10

5.1.4 Individual nutrient intakes

Individual nutrient intakes showed both a trend and significant differences across most feed parameters. Cows on treatment 4 and 1 consumed significantly less ash daily (1.27 kg DM) compared to 2 and 3 (1.31 and 1.32 kg DM respectively). Furthermore, a tendency was observed when comparing ash intakes on treatment 1 to treatment 3 and 4. Intakes of organic matter was significantly higher for cattle on treatment 1 compared to treatment 3 and 4, 19.2 versus 18.9 and 18.4 kg/d DM respectively. Furthermore, a tendency for higher organic matter was seen for cows consuming treatment 3 comparing to treatment 4. There were no statistical differences between starch intake across all TMR diets. Crude protein (CP) intakes were significantly lower for animals on treatment 4 compared to 2 and 3. This correlates with milk protein production being significantly lower in treatment 4. On average, cows on treatment 4 ate 150 g less crude protein on a dry matter basis daily.

Although there were no statistical differences between treatments for intakes of aNDF (in a range of 5.51-5.71 kg/d DM), aNDFom (aNDF – Ash) did tend to show differences, with treatment 3 having the lowest intake (4.20 kg/d DM) and treatment 1 having the highest (4.44 kg/d DM). This agreed with the daily ADF intakes of treatment 1 and 2 being the highest (3.65

and 3,64 kg DM) compared to treatment 3 (3.52kg DM). One must note the tendency for cows consuming treatment 1 to consume higher levels of ADF than for treatments containing lithothamnium-based buffers, treatment 3 and 4. Diets low in fibre are generally associated with acidosis, reduced saliva secretion, fibre digestion and rumination time (Kennelly *et al.,* 1999). This may be linked with results found in this study on ruminal pH. Non-fibrous carbohydrates were calculated to show a tendency to differ between treatment intakes, with treatment 4 having 640 g DM less than treatment 1. Fat content of treatments, although numerically different, did not differ significantly (Table 5-3); with treatment 1 to 4's values as follows; 820, 785, 777, 788 g/d DM. Calcium intakes differed significantly between treatment 2 and 3, 146 versus 169 g DM daily. Whilst phosphorus intakes differed significantly between treatment 1 and 2, 71.3 compared to 66.3 g/d DM.

Table 5-3 Individual nutrient intakes over entire experimental period for treatments (n=16)

	Treatment[1]				**SEM[2]**	***P***		
Nutrient	T1	T2	T3	T4		T1 vs T2	T1 vs T3+4	T3 vs T4
Ash (kg/d DM)	1.27^{b}	1.31^{a}	1.32^{a}	1.27^{b}	0.0084	0.01	0.07	0.01
OM (kg/d DM)[3]	19.2^{a}	19.1^{ab}	18.9^{b}	18.4^{b}	0.1902	0.11	0.02	0.06
Starch (kg/d DM)	5.51	5.57	5.36	5.31	0.1253	0.27	0.3	0.81
CP (kg/d DM)	2.89^{ab}	3.02^{a}	3.02^{a}	2.87^{b}	0.0426	0.07	0.34	0.04
aNDF (kg/d DM)	5.71	5.66	5.51	5.60	0.0902	0.66	0.19	0.53
aNDF$_{OM}$ (kg/d DM)[4]	4.44^{c}	4.34^{cd}	4.20^{d}	4.32^{cd}	0.0897	0.46	0.15	0.35
ADF (kg/d DM)	3.65^{c}	3.64^{c}	3.52^{d}	3.54^{cd}	0.0475	0.88	0.08	0.7
NFC (kg/d DM)[5]	9.76^{c}	9.60^{cd}	9.60^{cd}	9.12^{d}	0.1931	0.57	0.13	0.12
EE (g/d DM)	820	785	777	788	19.161	0.25	0.16	0.72
Ca (g/d DM)	154^{ab}	146^{b}	169^{a}	160^{ab}	4.8326	0.33	0.11	0.72

P (g/d DM)	71.3[a]	66.3[b]	69.7[ab]	68.0[ab]	1.2292	0.03	0.15	0.37

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.
[3] OM (g/kg DM) = 100 – Ash.
[4] $aNDF_{OM}$ (g/kg DM) = aNDF – Ash.
[5] NFC (g/kg DM) = 100 – (NDF % + CP % + EE % + Ash %) (NRC, 2001).
[a, b] Means within a row with different superscripts differ significantly ($P<0.05$).
[c, d] Means within a row with different superscripts show a tendency to differ ($0.05<P>0.1$).

5.1.5 Animal monitoring

Although experimental animals body weights were numerically different, they did not differ significantly with regards to their average body weights when fed treatment 1 to 4 TMR diets; 406, 404, 412 and 411 kg respectively. Even though cows gained weight throughout the experimental period, all cows gained weight at a constant rate, thus negating the effect between treatments (T1=16.9 kg, T2=19.1 kg, T3=28.7 kg, T4=14.9 kg). The mean body condition score (BCS) across treatments were as follows: 2.28 for treatment 1 and 2, and 2.31 for treatment 3 and 4. There were no differences between the effect of treatments on mean cow BCS and mean cow BCS change whilst on each respective diet (T1=0.06, T2=0.18, T3=0.12, T4=0.12) seen in Table 5-4 ($P>0.05$).

Body condition scoring and body weight records must be interpreted with caution due to the relatively short treatment periods in this study of 21 days. Furthermore, no incidences of lameness or SARA were observed over the trial experimental period. Yet again, the experimental periods were too short to evaluate the possible long-term effects of different experimental treatments on animal health. Kennelly *et al.* (1999) Found the same and recommended against feeding a similar ration for extended periods of time, supporting our observation that these types of studies may be limiting in terms of experimental period length.

Table 5-4 Body weight and body condition score of experimental cows over the experimental period when fed four different experimental total mixed rations (n = 16)

	Treatment[1]				SEM[2]	*P*		
	T1	T2	T3	T4		T1 vs T2	T1 vs T3+4	T3 vs T4
Body weight								
At start (kg)	398[cd]	394[d]	397[cd]	404[c]	3.0369	0.49	0.43	0.18
At End (kg)	414	414	426	419	8.9288	0.95	0.49	0.58
Mean (kg)	406	404	412	411	5.1618	0.79	0.41	0.95
Body weight change (kg/23-d period)	16.9	19.1	28.7	14.9	8.4449	0.86	0.65	0.29
Body condition score								
At start (units)	2.25	2.19	2.25	2.25	0.0786	0.59	1.00	1.00
At end (units)	2.31	2.37	2.37	2.37	0.0312	0.21	0.15	1.00
Mean (units)	2.28	2.28	2.31	2.31	0.0442	1.00	0.58	1.00
Change (units/23-d period)	0.06	0.18	0.12	0.12	0.0807	0.31	0.55	1.00

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.
[a, b] Means within a row with different superscripts differ significantly ($P<0.05$).
[c, d] Means within a row with different superscripts show a tendency to differ ($0.05<P>0.1$).

5.1.6 Dry matter intake and feed digestibility

Mean dry matter intake (DMI), shown in Table 5-5, did not differ and varied from 19.9 kg DM/d to 20.5 kg DM/d for treatments 4 and 1 respectively ($P>0.05$). Significant results have been reported by other researchers in TMR supplemented diets (Khorasani & Kennelly, 2001; Kennelly *et al.,* 1999) although, Xu *et al.* (1994) reported a tendency for increased intake of buffer supplemented diets. This was also seen when calculating DMI as a percent of body weight. Due to this interaction, DMI was then looked at as a percentage of body weight and metabolic weight to gain some further insight into feed intake.

Dry matter intake as a percent of both afore mentioned parameters showed significant differences. When diets are offered *ad libitum* throughout the day, animals are able to regulate their meal size and intake frequency over a 24-hour period. According to Kennelly *et al.* (1999), differences in this could account for the overall differences in fermentation patterns observed throughout the day. This will be discussed later. For both DMI parameters, treatment 4 for was significantly lower than treatment 1 and 2 (DMI as percent of BW: T1=5.18%, T2=5.17%, T3=5.08%, T4=4.96%; DMI as percent of metabolic BW: T1=22.6%, T2=22.9%, T3=22.2%, T4=21.5%). There was, however, a tendency for cows fed treatment 1 to have a higher dry matter intake as a percent of their body weight compared to treatments 3 and 4, this was mirrored for dry matter intakes as a percent of their metabolic body weight, although this time statistically significant. It is to be noted that these measurements do not fully reveal treatment differences between studies although it would appear that the removal of a buffer from the diet may help cows consume larger quantities of feed. Or perhaps that the addition of a Lithothamnium-based buffer may hinder intakes.

Titanium dioxide was used as the exogenous marker to aid in faecal output estimation. A faecal output estimation is required to determine the digestibility of individual nutrients in the feed. Dry matter digestibility was lower for treatment 1 and 4 compared to 2 and 3, 71.9 and 71.5% versus 73.4 and 74.1% respectively ($P<0.05$, Table 5-5). Furthermore, dry matter digestibility was treatment 1 was significantly lower than when compared directly to lithothamnium-based buffers, treatment 3 and 4. In agreement with these results, crude protein digestibility showed the same differences between treatments, with 69.7 and 70.6% versus 73.2 and 74.1% respectively, as well as for the significant differences observed between treatment 1 and lithothamnium-based buffers, treatments 3 and 4. Both NDF and starch digestibility's were not affected by treatment (Table 5-5). Whilst DM and CP digestibility was previously reported to be improved in cattle fed a Lithothamnium source and sodium bicarbonate (Calitz, 2009), no differences were found between NDF and starch digestibility which support our results. Khorasani & Kennelly (2001), attribute this to the degree of grain processing and NDF content in the diets. They further observed that there were no differences in the digestibility parameters between buffered and non-buffered treatment diets (Khorasani & Kennelly, 2001).

Table 5-5 Feed intake and digestibility of treatment total mixed rations over the experimental period (n = 16)

	Treatment[1]				SEM[2]	*P*		
	T1	T2	T3	T4		T1 vs T2	T1 vs T3+4	T3 vs T4
Feed intake:								
Dry matter intake (DMI) (kg/d)	20.5	20.3	20.1	19.9	0.3044	0.45	0.27	0.38
DMI as percent of BW (%)	5.18^{c}	5.17^{c}	5.08^{cd}	4.96^{d}	0.0776	0.65	0.09	0.20
DMI as percent of metabolic BW (%)[4]	22.6^{a}	22.9^{a}	22.2^{ab}	21.5^{b}	0.3311	0.60	0.05	0.25
Digestibility:								
DM Digestibility (%)[3]	71.9^{b}	73.4^{a}	74.1^{a}	71.5^{b}	0.4329	0.02	0.03	0.02
CP Digestibility (%)	69.7^{b}	73.2^{a}	74.1^{a}	70.6^{b}	0.7466	0.02	0.03	0.02
NDF Digestibility (%)	47.7	50.8	50.1	47.2	1.4109	0.17	0.59	0.19
Starch Digestibility (%)	97.7	97.7	98.2	98.2	0.2612	0.92	0.17	0.82

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.
[3] DM Digestibility (%) = [((Dry matter intake, g – Faecal output, g) / Dry matter intake, g)) × 100].
Faecal output estimation using titanium dioxide as a marker (g) = [TiO_2 consumed (g/d) / TiO2 concentration in faeces (g/g DM)].
[4] Metabolic BW (BW) = Liveweight$^{0.75}$.
[a, b] Means within a row with different superscripts differ significantly (P<0.05).

5.2 Milk production parameters

5.2.1 Milk Production

Milk production data is shown in Table 5-6 and were higher for the control treatment when compared to the Lithothamnium containing buffers. Seymour *et al.* (2005) reported that milk yield is directly correlated to dry matter intake (r^2=0.69). This may help to explain the milk production differences between treatments where, although differences between treatments were not observed (*P*>0.05), numerical differences were seen between treatments which mirror the milk yield results (Treatment 1 having the highest and 4 having the lowest as seen in Table 5-5). Xu *et al.* (1994), Harrison *et al.* (1989) and Khorasani & Kennelly (2001) found no differences in milk yield between buffered and non-buffered treatment diets in contrast to Kennelly *et al.* (1999), who reported increased milk yields by cows fed sodium bicarbonate. When comparing the two Lithothamnium containing diets, Lith A included in the TMR resulted in significantly higher milk yield than Lith B, by 0.7 kg (*P*<0.05).

Fat corrected milk (4%) (4% FCM) and energy corrected milk (ECM) yield were not different between treatments (*P*>0.05). This contradicts literature (Harrison *et al.,* 1989 and Kennelly *et al.,* 1999) that have reported increased 4% FCM yield and energy corrected milk yield for cows fed buffered versus non-buffered diets. It is to be noted that as lactation progresses, milk yield decreases whilst milk fat increases in comparison to cows early in lactation (Khorasani & Kennelly, 2001). Due to the nature of the 4 x 4 Latin square design, lactation progression may affect the statistically significant differences that were detected between treatments for milk yield and fat content. Furthermore, Khorasani & Kennelly (2001) reported that late-lactation cows tolerate higher starch diets better. This is due to the fact that there are more metabolic stressors in early lactation, whilst late-lactation cows are in a stable state with a longer adaptation period to increased starch content in the diet.

5.2.2 Milk composition

Although fat content in in milk was not significantly (*P*>0.05) nor tending to be different (*P*<0.1), substantial numerical differences were seen. Cows on treatment 4 had a fat content of 5.10%, followed by 3 with 5.04% and treatment 1 and 2 with 4.85 and 4.82% fat respectively. The fat content had a direct impact on the FCM yield (Table 5-6). It has previously been reported that the responses in milk fat decreased significantly when buffers are added to diets consisting of more than 30% forage (Erdman *et al.*, 1982). The high content of wheat straw in the experimental TMR diet may have attributed to this as many previous studies have seen improved daily milk fat yields with the addition of exogenous buffers to the diet (Xu *et al.,* 1994; Kennelly *et al.,* 1999;

Khorasani & Kennelly, 2001). Khorasani & Kennelly (2001) prevented milk fat depression with the addition of buffers to the diet, consistent with literature, and attributed the success to the ability of a buffer to maintain a more stable rumen pH for bacteria as a key factor.

Milk protein percent of cows on treatment 2 and 3 (3.91 and 3.89% respectively) were higher than cows on treatment 1 and 4 (3.74 and 3.79% respectively) (P<0.05; Table 5-6). Regardless of the changes brought about to milk protein in our study, it is generally accepted that buffers do not consistently alter the protein content of milk (Xu *et al.,* 1994). Dietary treatment had a direct impact on lactose composition in milk, with treatment 1 yielding significantly higher lactose percent than treatment 2 and 3 (5.06% versus 4.99 and 4.94% respectively). A correlation between lactose content in milk and rumen propionate content has been previously observed (Khorasani & Kennelly, 2001). It is hypothesised that increased propionate concentration in the rumen results in an increase in gluconeogenic substrate for milk synthesis which, in turn, spares amino acids from catabolism for milk synthesis, thus resulting in lowered milk lactose content. It can be concluded that the effect of dietary buffers on milk protein content is not as well defined as the effect on milk fat content (Sharma *et al.,* 2018).

Considerably low somatic cell counts are attributed to clean living areas. Although milk somatic cell counts (x 1000 cells/mL milk) were low overall for this study, significant differences were observed. Treatment 2 and 4 had the significantly lowest counts, 24.1 and 23.4 (P<0.05) respectively, whilst treatment 3 yielded the highest counts at 46.1 (x 1000 cells/mL milk). Somatic cell counts (SCC) of more than 200 (x 1000 cells/mL milk) can be used to classify a cow as having clinical mastitis, however cows with mammary infections have exhibited SCC as low as 75 (x 1000 cells/mL milk). Thus, a reduction from 46 to 23 (x 1000 cells/mL milk) may be an indication of improved udder health (Nyman *et el*., 2016). A reduction in SCC is likely linked to the immune system responding optimally to inflammation in the mammary gland (Tewoldebrhan *et al*., 2017). Should this be the case, Lithothamnium buffer source Lith B and sodium bicarbonate led to significantly lowered SCC compared to source Lith A. Treatment 2, 3 and 4 had significantly higher MUN content in milk samples compared to treatment 1 and ranged between 13.5 and 15.3 mg/dl. Although these were recorded to be in the higher side, no literature could be found correlating exogenous dietary buffers to MUN. Milk urea nitrogen levels between 10-12 mg/dl usually indicate that a well-balanced energy-protein diet is fed (Kohn, 2007). Levels higher than 14 mg/dl may indicate some wastage of CP. The MUN levels in our study were close to the upper range but comparable to many other TMR based dairy studies (Claassen *et al.,* 2016; Farmer *et al.,* 2014). No differences were seen between treatment diets for milk efficiency (P<0.05; Table 5-6).

When comparing a ration lacking a buffer, treatment 1, to treatment rations containing lithothamnium-based buffers no statistical differences nor tendencies were seen (Table 5-6). Literature is divided with regards to results supporting this.

Seymour *et al.* (2005) observed weak- to moderate correlations between volatile fatty acid contents in rumen fluid and the composition of milk. Milk fat was determined to have a strong positive association between the acetate to propionate ratio (A:P), whilst protein has a negative association with rumen pH. Lowered production of milk fat precursors in the rumen (acetate and butyrate) will result in reduced milk fat percent (Kennelly *et al.*, 1999).

Table 5-6 Milk production and composition parameters from the duration of the experimental period according to treatment total mixed ration received

	Treatment[1]				SEM[2]	*P*		
	T1	T2	T3	T4		T1 vs T2	T1 vs T3+4	T3 vs T4
Production (kg/d):								
Milk yield	26.9	26.5	26.0	25.3	0.2377	0.60	0.17	0.46
4% FCM[3]	29.1	27.8	29.1	28.2	1.1100	0.58	0.55	0.65
ECM[4]	29.0	29.1	29.3	28.0	1.0653	0.94	0.81	0.41
Composition (%):								
Fat	4.85	4.82	5.04	5.10	0.1346	0.53	0.56	0.45
Protein	3.74^{d}	3.91^{c}	3.89^{c}	3.79^{d}	0.0258	0.09	0.31	0.08
Lactose	5.06^{a}	4.99^{b}	4.94^{b}	5.01^{ab}	0.0216	0.04	0.25	0.43
SCC (x 1 000 cells/ml)	31.4^{ab}	24.1^{b}	46.1^{a}	23.4^{b}	4.3132	0.65	0.59	0.05
MUN (mg/dL)	13.5^{b}	14.9^{a}	14.7^{a}	15.3^{a}	0.3735	0.03	0.16	0.37
Milk efficiency[5]	1.29	1.29	1.25	1.29	0.0289	0.47	0.48	0.39
Milk efficiency 4% FCM[6]	1.42	1.33	1.44	1.45	0.0666	0.55	0.67	0.60
Milk efficiency ECM[7]	1.43	1.45	1.46	1.44	0.0514	0.82	0.78	0.87

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.
[3] 4 % FCM (fat corrected milk) (kg) = (0.4 x milk yield (kg)) + (15 x milk fat yield (kg)) (NRC, 2001).
[4] ECM (Energy corrected milk) (kg) = Milk production (kg) x (383 x fat % +242 x protein % + 783.2)/3140 (Sjaunja *et al.,* 1990).
[5] Milk efficiency = Milk yield (kg/d) / DMI (kg).
[6] Milk efficiency 4% FCM = 4% FCM yield (kg/d) / DMI (kg).
[7] Milk efficiency ECM = ECM yield (kg) / DMI (kg).
[a, b, c] Means within a row with different superscripts differ significantly (P<0.05).

5.3 Rumen pH monitoring:

5.3.1 Hand-held pH meter measurements

Microbial growth in the rumen relies heavily on energy obtained from the products of fibre hydrolysis, thus highlighting the importance of the understanding of fibre degradation at low ruminal pH. The pH of the rumen environment was measured upon rumen fluid collection every three hours over a 24-hour period using a portable pH meter. Rumen pH was observed to follow the typical pH curve over the 24-hour period. Whilst pH shifts of 0.5- to 1 pH unit in the rumen is common through the day (Krause & Oetzel, 2005), no differences in pH between treatments were identified at 3h00, 6h00, 9h00, 15h00, and 21h00 (P>0.05). However as shown in Table 5-7, pH differences between treatments were observed at 12h00, 18h00, and 24h00. At 12h00; animals on treatment 4 were identified to have the lowest rumen pH of 5.46 (P<0.05). Treatment 2 presented the highest pH of 5.88. At 18h00; animals fed treatment 2 and 3 had higher rumen pH's than treatment 1 recording as 5.74, 5.76 and 5.46 respectively (P<0.05). Treatment 2 outperformed treatment 3 at 24h00 when comparing rumen pH. Animals fed treatment 3 had a lower rumen pH of 5.48 compared to that of treatment 2 at pH 5.82 (P<0.05). Xu *et al.* (1994) reported no significant pH differences throughout the day between treatments, which was supported by previous research (Kennelly *et al.,* 1999). This was attributed to the many external factors that come into play during rumen fluid sampling.

Table 5-7 Ruminal pH's recorded using a portable pH meter during rumen fluid collection every three-hours for a 24-hour period for various treatment total mixed rations over experimental period

	Treatment[1]				SEM[2]
	T1	T2	T3	T4	
Time:					
03h00	5.81	5.72	5.80	5.70	0.0900
06h00	6.11	6.12	5.9	6.02	0.0964
09h00	5.99	6.03	6.07	5.75	0.1405
12h00	5.70[a]	5.88[a]	5.74[a]	5.46[b]	0.0692
15h00	5.90	6.09	5.86	5.91	0.0956
18h00	5.46[b]	5.74[a]	5.76[a]	5.58[ab]	0.0757
21h00	5.46	5.52	5.44	5.48	0.0646
24h00	5.71[ab]	5.82[a]	5.48[b]	5.74[ab]	0.0982

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.
[a, b] Means within a row with different superscripts differ significantly (P<0.05).

This information may be transformed into a graph (Figure 5-1) in order to calculate the average time that the rumen environment spent under critical pH values. The maximum ruminal pH values did not differ significantly between treatments and fell in a range of between 6.17-6.28. Similarly, the minimum ruminal pH readings did not significantly differ and varied between 5.35-5.51. Although the exact pH threshold for intake depression in dairy cattle has not yet been determined, low ruminal pH aids in increasing the osmolarity of ruminal contents which, in turn, inhibits feed intake and causes inflammation of ruminal epithelium (Krause & Oetzel, 2005). Additionally, a ruminal pH of 5.35 of animals fed treatment 1 was the lowest rumen pH that was identified during the rumen pH measurement period.

The change in pH between treatments was determined to be not significantly different and ranged between 0,78- and 0.84 pH units, on par with the report of Krause & Oetzel (2005) mentioned above. The mean pH calculated per treatment per period was however different (P<0.05). Animals fed treatment 4's diet exhibited a lower mean rumen pH than treatment 2 (sodium bicarbonate), 5.71 and 5.86 respectively (P<0.05). Erdman *et al.* (1982) reported a similar increase in average daily rumen pH from 6.13 to 6.43 with 1% DM sodium bicarbonate inclusion rate above the control treatment. Significant differences were identified for time spent under the

pH of 5.8 in the rumen between treatments 4 and 2. Animals fed treatment 2 spent, on average, 8.06 hours below pH 5.8, whilst animals on treatment 4 spent 14.6 hours below the critical pH point.

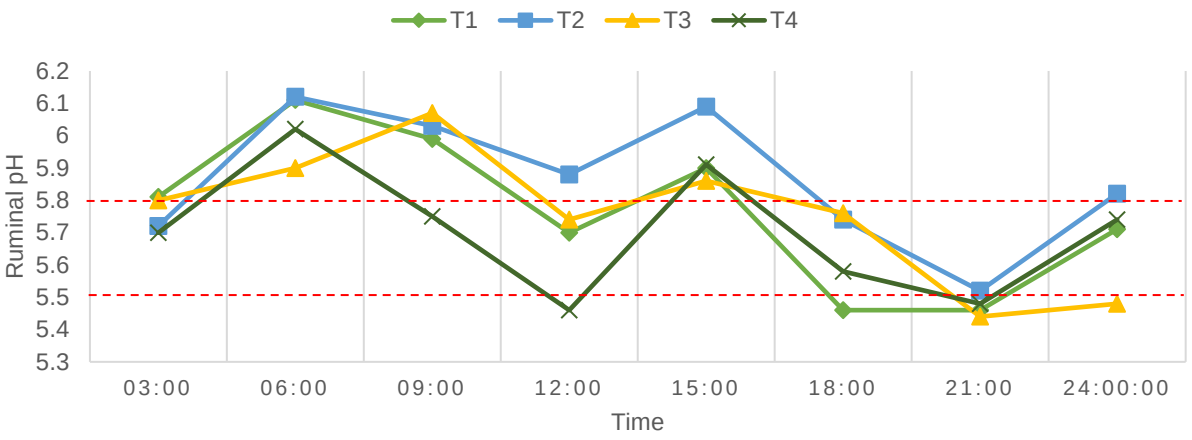

Figure 5-1 Graph depicting various treatment diets (total mixed ration: T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rates influence on ruminal pH over a 24-hour period, measured during rumen fluid collection with a pH meter at three-hour intervals (n = 16).

A ruminal pH of less than 6 depresses cellulolytic activity, resulting in lowered fibre digestion (Kennelly *et al.,* 1999). As this was the case in this study, it is possible that the lowered daily milk yields seen for cows fed Lithothamnium source B buffer were due to lowered fibre digestion in the rumen. This was seen with lowered dry matter digestibility for treatment-rations containing lithothamium-based buffers compared to the control. There were no differences found between treatments for time spent below a pH of 5.5 in the rumen, although the time ranged from 1.37-4.37 hours (P>0.05). However, a tendency for cows fed Lithothamnium-based buffers (treatments 3 and 4), was observed with regards to more time spent below pH of 5.8 in the rumen compared to the control ration, treatment 1. Woodford & Murphy (1988) reported that although different diets may result in similar daily rumen mean pH's, they will differ in the total time measured under a certain pH value, as seen in the graph above for pH point 5.8 and 5.5 (Figure 5-6). The rumen fluid profile over the 24-hour period follows a similar trend. The highest pH points were observed at 06h00 and 15h00, whilst the lowest pH's were measured at 21h00.

Table 5-8 Maximum, minimum, mean and change in pH, as well as total hours spent below 5.8 (reduction of fibrolytic bacteria onset) and 5.5 (defining pH of acidosis) calculated using data recorded from graph above

	Treatment[1]				SEM[2]	*P*		
	T1	T2	T3	T4		T1 vs T2	T1 vs T3+4	T3 vs T4
Max pH	6.19	6.28	6.19	6.17	0.0574	0.27	0.24	0.54
Mean pH	5.77^{ab}	5.86^{a}	5.75^{ab}	5.71^{b}	0.0404	0.31	0.12	0.24
Min pH	5.35	5.51	5.36	5.37	0.0594	0.44	0.44	0.75
"Change in pH"	0.84	0.78	0.83	0.80	0.0615	0.97	0.98	0.51
"Hours spent below 5.8"	9.62^{cd}	8.06^{d}	10.3^{cd}	14.6^{c}	1.6027	0.35	0.09	0.51
"Hours spent below 5.5"	4.12	1.37	3.62	4.37	1.3769	0.21	1.00	1.00

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.
[a, b] Means within a row with different superscripts differ significantly (P<0.05).

5.3.2 SmaXtec bolus data

Rumen sensors are useful in providing ruminal pH data which aid in relating rumen functioning to animal health disorders and on-farm management making decisions (Dijkstra *et al*., 2020). The sensor used in this study, SmaXtec pH Plus bolus (SmaXtec, Belgiergasse 38020 Graz, Austria), allowed for continual monitoring of reticular pH and diurnal pH variation. The SmaXtex pH Plus bolus was inserted into each cow's reticulum for the duration of the trial to continually record reticular pH every 10 minutes. In contrary to handheld pH measurements, differences were seen in maximum pH measurements, with treatment 3 exhibiting the highest pH of 6.74 and treatment 2 exhibiting a pH of 6.6 (P<0.05; Table 5-9). No differences were observed for the minimum, mean, nor change in pH of the reticulum. The change in reticular pH over time was very similar to the change in ruminal pH over time. It is advised to integrate both duration and extent of pH depression through looking at the area under the pH vs time curve in order to get a better understanding of the pH variation in relation to acidosis (Dijkstra *et al*., 2020).

A statistical tendency was seen between treatment 1 and 4 for the hours spent below pH 5.8; 13- and 54-minutes, respectively (0.05<*P*>0.1). Neubauer *et al.* (2018) reported research results in which multiple commercially available pH boli were evaluated against ruminal pH that a pH of 5.8 in the rumen, a point in which the fibrolytic bacteria population begins to dwindle. It was determined that a pH of 5.8 in the rumen correlates with a reticular bolus pH of 6.0. Table 5-8 and 5-9 highlight the differences between ruminal and reticular pH over time. It was determined that the average difference between the mean pH in the rumen and reticulum was 0.52 pH units, more than double of that found in previous studies. In a study whereby eBolus sensors (eBolus, eCow Ltd., Exeter, UK) were placed in the reticulum for comparison to daily ruminal pH fluctuations, the recorded reticular pH values were determined to be 0.24 pH units above the ruminal pH average (Falk *et al.,* 2016).

It is universally accepted that the type of diet on offer to animals will directly influence the pH difference magnitude between the reticulum and rumen, with high-starch, low-fibre diets exhibiting the largest fluctuations (Neubauer *et al.,* 2018). Thus, the diet on offer in this study would be expected to have a larger difference between ruminal and reticular pH. Furthermore, less variations in terms of pH fluctuations were seen throughout the day. Falk *et al*. (2016), concluded that due to the variation in the differences between ruminal and reticular pH across week of lactation, no fixed conversion factor may be calculated to make live comparisons between ruminal and reticular pH values. Unfortunately, the difficulty in ensuring correct calibration of these non-retrievable sensors may limit their application in research (Dijkstra *et al*., 2020).

Table 5-9 Reticulum pH's recorded using a SmaXtec pH plus bolus (SmaXtec, Belgiergasse 38020 Graz, Austria) which remains in each experimental animal's reticulum for the duration of the trial and continually uploads recorded pH's every 10 minutes over total experimental period

	Treatment[1]				SEM[2]
	T1	T2	T3	T4	
Max pH	6.66^{ab}	6.60^{b}	6.74^{a}	6.70^{ab}	0.0382
Mean pH	6.24	6.21	6.24	6.22	0.0154
Min pH	5.80	5.73	5.84	5.87	0.0573
"Change in pH"	0.86	0.86	0.89	0.83	0.0514
"Hours spent below 5.8"	0.22^{d}	0.55^{cd}	0.67^{cd}	0.9^{c}	0.2282
"Hours spent below 5.5"	0	0.02	0	0	0.0125

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.

[a, b] Means within a row with different superscripts differ significantly (P<0.05).
[c, d] Means within a row with different superscripts show a tendency to differ (0.05<P>0.1).

5.4 Rumen Volatile fatty acids

5.4.1 Average volatile fatty acids and lactic acids

The volatile fatty acid (VFA) and lactic acid (LA) content were analysed from the rumen fluid collected every 3 hours for a 24-hour period for the various treatment diets. According to van Soest (1982), VFA's are accountable for 60- to 70% of the metabolic energy available to ruminants. Furthermore, he determined that observing the actual concentration of respective VFA's in rumen fluid is preferable to looking into the molar proportions as significance may be determined on this level. Relating the concentration of VFA's in the rumen to milk production and composition aids in determining which metabolic pathways may have been altered by alternative buffer treatments.

The mean acetate and propionate concentrations are shown in Table 5-10 were not different between treatments, ranging from 38.18-38.30 mmol/L and 29.28-31.24 mmol/L, respectively, butyrate concentrations however, differed between treatments (P<0.05). Butyrate has the highest energy value per mole when compared to acetate and propionate and is the most extensively metabolised in the epithelium of the rumen wall (Baldwin & Mcleod, 2000). Rumen fluid butyrate levels from cows on treatment 3 was 18.64 mmol/L whilst 17.05 mmol/L was from cows on treatment 1 (P<0.05). Butyrate directly contributes to the hepatic energy pool and acetate production which, in turn, stimulates gluconeogenesis (van Soest, 1982). This results in more energy available for milk production and thus an improved milk efficiency for energy corrected milk (ECM). Therefore, an explanation may be reached as to why Lithothamnium source A has the numerically highest milk efficiency ECM of 1.46 alongside the significantly highest butyrate content, however, it is to be noted that this is a speculation as no significant differences were observed. Furthermore, when comparing the control, treatment 1, to Lithothamnium-based buffer treatments, 3 and 4, a tendency was observed for Lithothamnium-based buffered rations to result in increased butyrate production in the rumen. This was seen in agreement with Cruywagen *et al*. (2015), Kennelly *et al.* (1999) and Khorasani and Kennelly (2001), although these authors mentioned observed statistically significant differences. No differences were seen in total volatile fatty acid (VFA) content of rumen fluid between treatments. The volatile fatty acid concentration was converted from mmol/L to molar percentage; similarly, treatments did not differ significantly in molar percent for acetate and propionate. Once more, average butyrate followed the similar

trend as before where treatment 3 exhibited higher butyrate molar % compared to treatment 1 (P<0.05).

The mean lactic acid content of rumen fluid additionally did not differ between treatments although it followed in a similar treatment trend to A:P ratio with treatment 2 and 3 reaching levels of 0.715- and 0.702 mmol/L respectively and treatment 1 and 4 at 0.661- and 0.699 mmol/L respectively. The variation in in VFA profile is most commonly associated with variations in milk parameter production and feed nutrient partitioning. Changes in ruminal pH has been reported to shift the fermentation pathways of many microbial species while continuing to produce the same substrate (Dijkstra *et al.,* 2012). At ruminal pH's below 5.5, it can be expected that VFA absorption capacity is reduced due to increases in lactate production as *Strepococcus bovis* ferments glucose to lactate, forgoing VFA production. However, this was not observed in this study as no differences were observed between different treatments lactic acid content nor shifts in total VFA content (Oetzel, 2007).

Table 5-10 Average volatile fatty acid and lactic acid results as analysed from rumen fluid collected over a 24-hour period for various treatment total mixed rations over the entire experimental period

	Treatment[1]				SEM[2]	*P*		
	T1	T2	T3	T4		T1 vs T2	T1 vs T3+4	T3 vs T4
Volatile Fatty Acids (mmol/L):								
Acetate (A)	38.18	38.30	38.30	38.31	0.7190	0.91	0.90	0.97
Propionate (P)	30.83	29.28	29.44	31.24	1.3480	0.45	0.78	0.38
Butyrate	17.05^{b}	17.82^{ab}	18.64^{a}	17.66^{ab}	0.4540	0.27	0.09	0.18
Total VFA	86.06	85.40	86.35	87.21	1.6406	0.79	0.73	0.72
Volatile Fatty Acids (Molar %):								
Acetate	44.38	45.10	44.53	43.89	0.9474	0.61	0.88	0.65
Propionate	35.86	34.08	33.97	35.90	0.9874	0.25	0.47	0.21
Butyrate	19.75^{b}	20.82^{ab}	21.50^{a}	20.21^{ab}	0.4756	0.16	0.11	0.11

A:P ratio	1.251	1.333	1.316	1.249	0.05938	0.36	0.68	0.46
Lactic Acid (mmol/L)	0.661	0.715	0.702	0.699	0.02543	0.19	0.25	0.94

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.
[a, b] Means within a row with different superscripts differ significantly ($P<0.05$).

5.4.2 Total volatile fatty acids

All treatments followed a similar trend over the full 24-hour period, when measured three-hourly, in terms of total volatile fatty acid content. The maximum concentration of total VFA was measured at 21h00 (82.31-103.5 mmol/L) which was measured 5-hours after afternoon feeding whilst the minimum points were measured at 6h00 and 15h00 (60.88-71.31 mmol/L), an hour before afternoon and morning feeding. The increase in total VFA content after feeding agrees with Evans *et al.* (1975), who reported the same trend in cattle and sheep. Total volatile fatty acid content (mmol/L) did not differ between treatments nor for collection times over the 24-hour periods ($P>0.05$). The only exception of at 6h00, where treatment 3 and 4 had a tendency ($0.05<P>0.1$) for increased total VFA production (Table 5-11). Kennelly *et al.* (1999) and Khorasani & Kennelly (2001) both reported total VFA content elevation with the addition of an exogenous buffer to the diet. This contradicts Xu *et al.* (1994) and our results shown in Table 5-10, which exhibited no consistent effect of buffers on VFA content or composition. Differences between Lithothamnium buffer sources were not observed ($P>0.05$).

Table 5-11 Total volatile fatty acid concentration (mmol/L) as analysed from rumen fluid collected every three-hours for a 24-hour period for various treatment total mixed rations over the entire experimental period

	Treatment[1]				SEM[2]
	T1	T2	T3	T4	
Time:					
03h00	72.35	78.22	83.78	79.37	4.3670
06h00	63.40^{cd}	60.88^{d}	69.25^{c}	71.31^{c}	3.0572
09h00	76.87	77.45	80.29	85.22	5.1234
12h00	80.49	70.12	78.37	84.16	5.6943
15h00	68.20	64.12	66.47	67.35	3.2356
18h00	79.88	71.50	76.13	80.92	3.5621
21h00	99.25	100.2	82.31	103.5	11.542
24h00	87.38	88.27	83.49	77.75	6.4065

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.
[c, d] Means within a row with different superscripts show a tendency to differ ($0.05<P>0.1$).

5.4.3 Total acetate concentration

Table 5-12 and Figure 5-2 shows that total acetate rumen fluid concentration (mmol/L) measured 3-hourly, did not differ between treatments, except for at 12h00, where there was a difference ($P<0.05$) between treatment 3 (32.22 mmol/L) and treatment 4 (37.76 mmol/L). The maximum acetate concentration was measured at 09h00 (38.79-42.15 mmol/L) and 21h00 (41.71-44.44 mmol/L). This contradicts previous buffer studies where sodium bicarbonate and a Lithothamnium source were used for comparison. Cruywagen *et al*. (2015) reported both an increase in total acetate content and molar percentage of rumen fluid when a combination of sodium bicarbonate and Lithothamnium source were fed in a TMR. The relatively low inclusion level of Lithothamnium sources may account for the lack of significant differences between Lith A and B sources, as well as between other treatments.

Table 5-12 Total acetate concentration (mmol/L) as analysed from rumen fluid collected every three-hours for a 24-hour period for various treatment total mixed rations over the entire experimental period

	Treatment[1]				SEM[2]
	T1	T2	T3	T4	
Time:					
03h00	33.80	39.86	39.63	38.87	2.0738
06h00	35.47	34.63	37.85	36.92	1.3076
09h00	38.79	39.29	40.81	42.15	2.5602
12h00	35.96[ab]	32.33[b]	35.31[ab]	37.76[a]	1.3181
15h00	36.13	34.82	34.32	34.57	1.5192
18h00	40.18	42.11	37.91	38.33	3.0513
21h00	44.12	43.81	44.44	41.71	1.0852
24h00	39.65	39.57	35.96	36.19	3.1868

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.
[a, b] Means within a row with different superscripts differ significantly (P<0.05).

Whilst differences were not determined between treatments at any time point other than 12h00, it is to be noted that the total acetate concentration follows a very similar curve over the 24-hours to the total VFA content (Figure 5-2). This is because Acetate is the most abundant volatile fatty acid being produced in the rumen (Hutjens, 2008). This is to be expected as the peak-acetate production occurs between 4-5 hours after scheduled TMR feeding in the morning and evening (Evans *et al.,* 1975).

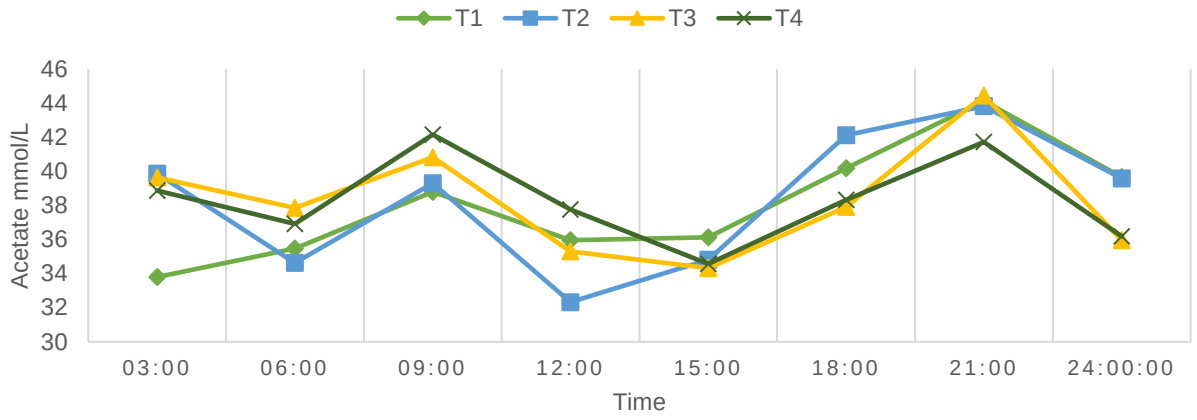

Figure 5-2 Graph depicting various treatment diets (total mixed ration: T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rates influence on rumen fluid acetate content over a 24-hour period (n=16).

5.4.4 Total propionate concentration

Propionate content in the rumen is second to acetate and is produced when non-structural carbohydrates are fermented (Hutjens, 2008). Due to the high NSC content of the experimental TMR, it was expected that the propionate concentration in the rumen would be high, as seen in Table 5-14. Propionate concentration measured three-hourly in the rumen between time periods was lowest at 06h00 and 15h00 (22.6-27.6 mmol/L) and highest at 21h00 (29.76-39.69 mmol/L). The propionate content (mmol/L) of collected rumen fluid differed between treatments at 6h00 and tended to differ at 18h00 (P<0.10). Animals fed treatment 4 had a significantly higher total propionate of 27.6 mmol/L compared to treatment 2's 22.6 mmol/L. At 18h00 (P<0.10), cows on treatment 4 tended to show a higher propionate content of 32.29 mmol/L when compared to 26.75 mmol/L of cows on treatment 2. The molar percentage of propionate in the rumen traditionally decreases upon the addition of a dietary buffer to a concentrate rich TMR (Erdman *et al.,* 1982). This effect was not observed in Table 5-13 and Figure 5-3. Lithothamnium has previously shown to exert the same effect on ruminal fluid propionate content (Cruywagen *et al.,* 2015). However, Mubiayi Beya (2007) and de Vos (2019) results with the use of Lithothamnium sources, showed no impact on rumen propionate content.

Table 5-13 Propionate concentration (mmol/L) as analysed from rumen fluid collected every three-hours for a 24-hour period for various treatment total mixed rations over the entire experimental period

	Treatment[1]				SEM[2]
	T1	T2	T3	T4	
Time:					
03h00	30.15	29.90	32.71	30.79	1.7161
06h00	24.00^{ab}	22.60^{b}	25.96^{ab}	27.60^{a}	1.3423
09h00	29.18	29.73	30.00	32.36	1.7796
12h00	32.02	27.05	30.99	32.91	2.5547
15h00	26.16	23.84	25.00	26.10	1.6068
18h00	30.75^{cd}	26.75^{d}	28.66^{cd}	32.29^{c}	1.6163
21h00	39.52	39.69	29.76	37.46	5.3444
24h00	34.86	34.69	32.43	30.37	2.8661

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcaerious marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcaerious marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.
[a, b] Means within a row with different superscripts differ significantly (P<0.05).
[c, d] Means within a row with different superscripts show a tendency to differ (0.05<P>0.1).

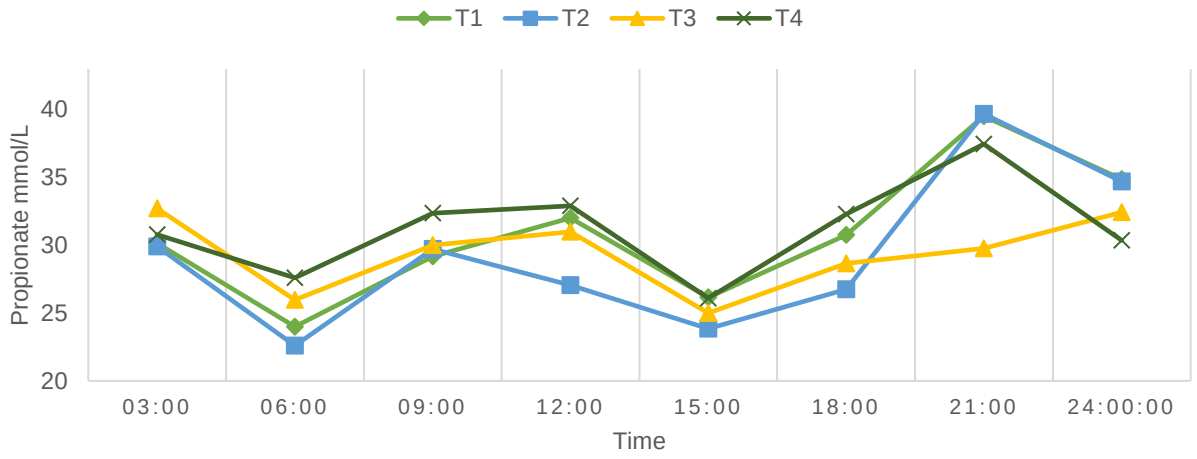

Figure 5-3 Graph depicting various treatment diets (total mixed ration: T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rates influence on the propionate content of rumen fluid over a 24-hour period (n=16).

5.4.5 Acetate to propionate ratio

Reductions in the acetate to propionate ratio, i.e. the shift in VFA patterns, ultimately leads to increased energy available to the cow (Kennelly *et al.,* 1999). Whilst all treatments followed a similar trend over the 24-hour period, differences were observed for the acetate to propionate ratio (A:P) at specific time points when measured three-hourly in the rumen. The widest ratios were observed at 06h00 and 18h00 (1.97-2.56) whilst the smallest ratios were seen at 12h00 and 9 pm (1.81-2.04) (Table 5-14; Figure 5-4). The acetate to propionate ratio was calculated and plotted according to the time that rumen fluid was collected. A difference in ratio was observed at 18h00, with treatment 2 exhibiting a ratio of 2.56 and treatment 4 ratio of 1.97 (P<0.05). Statistical tendencies (0.05<P>0.10) were observed at 6h00 and 15h00, where treatment 4 collectively had the smallest ratio. Treatment 1 and 2 exhibited ratios of 2.25 and 2.52 respectively whilst 4 presented with 2.24 at 6h00. Similarly, at 15h00, treatment 2's ratio was 2.48 whilst 4's was 2.15. The results of this study agree with Mubiayi Beya (2007). Kennelly *et al.* (1999) observed an increase in acetate and decrease in propionate content in cattle fed a buffer dietary treatment which attributed to a wider A:P ratio, which is further supported by Erdman *et al.* (1982), although was not exhibited in this trial. Erdman furthermore accredits this wider ratio to changes in milk fat production.

Table 5-14 Acetate to propionate ratio calculated from rumen fluid collected every three-hours for a 24-hour period for various treatment total mixed rations over the entire experimental period

	Treatment[1]				SEM[2]
	T1	T2	T3	T4	
Time:					
03h00	1.91	2.22	2.01	2.11	0.1830
06h00	2.45^{c}	2.54^{c}	2.43^{cd}	2.24^{d}	0.0604
09h00	1.99	2.16	2.21	2.14	0.1049
12h00	1.87	2.02	1.86	1.90	0.1166
15h00	2.25^{cd}	2.48^{c}	2.30^{cd}	2.15^{d}	0.1050
18h00	2.12	2.56^{a}	2.18	1.97^{b}	0.1546
21h00	1.83	1.81	2.04	1.84	0.6166
24h00	1.89	1.92	1.93	1.97	0.0786

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcaerious marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcaerious marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.
[a, b] Means within a row with different superscripts differ significantly (P<0.05).

[c, d] Means within a row with different superscripts show a tendency to differ (0.05<*P*>0.1).

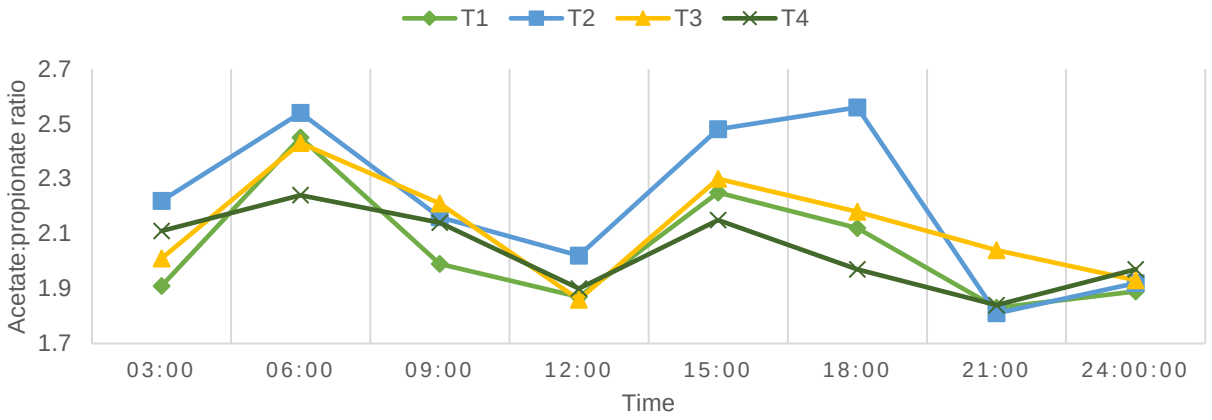

Figure 5-4 Graph depicting various treatment diets (total mixed ration: T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rates influence on the ratio between acetate and propionate of ruminal fluid over a 24-hour period (n=16).

5.4.6 Total butyrate concentration

Butyrate abundance comprises between 5- to 15% of total VFA in the rumen, making it the third most abundant VFA (McDonald *et al.,* 2011). Mammary fatty acid synthesis directly involves butyrate, coining it the term lipogenic VFA (Ishler *et al.,* 1996). The butyrate concentration (mmol/L) of rumen fluid per treatment was measured for each sample collection time point, three-hourly. No differences were observed in rumen fluid butyrate concentration from time slots between 9h00-12h00 and 18h00-24h00 (*P*>0.05). Concentrations in sampled rumen fluid differed significantly (*P*<0.05) for treatment 1 to treatment 2, 3 and 4 at 3h00 (T1=12.05 mmol/L, T2=18.43 mmol/L, T3=18.37 mmol/L, T4=17.80 mmol/L) and 15h00 for treatment 4 to treatment 2 and 3 (T4=15.15 mmol/L, T2=16.45 mmol/L, T3=16.48 mmol/L). At 21h00, butyrate content (20.09-22.79 mmol/L) was observed to be at the highest concentration, whilst a second spike (less pronounced) was seen at 9h00 (18-20.49 mmol/L). Butyrate concentration was at its lowest at 15h00 in a range of between 15.15 to 16.48 mmol/L. Although the buffer supplementation appeared to increase the butyrate concentration, only treatment 3 showed a significant increase above the control treatment (Table 5-15; Figure 5-5). This was in agreement with Cruywagen *et al.* (2015), who observed an increase in butyrate concentration as well as a tendency towards increased molar percentages when supplementing a TMR with a Lithothamnium source. Increases in butyric acid content in the rumen may increase the supply of propionate to the liver

and acetate to tissue supply by decreasing the rate of oxidation of acetate and propionate by rumen epithelium (Seymour *et al.,* 2005).

Table 5-15 Butyrate concentration (mmol/L) as analysed from rumen fluid collected every three-hours for a 24-hour period for various treatment total mixed rations over the entire experimental period

	Treatment[1]				SEM[2]
	T1	T2	T3	T4	
Time:					
03h00	12.05[b]	18.43[a]	18.37[a]	17.80[a]	1.0851
06h00	15.38[d]	15.67[d]	17.34[c]	16.01[cd]	0.5440
09h00	18.51	18.00	20.29	20.49	1.6586
12h00	16.45	16.02	16.39	18.34	0.9365
15h00	15.87[ab]	16.45[a]	16.48[a]	15.15[b]	0.3566
18h00	18.37	18.31	18.80	16.33	1.1401
21h00	20.21	20.84	22.79	20.09	1.2302
24h00	17.65	18.89	18.63	16.99	0.8239

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcaerious marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcaerious marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.
[a, b] Means within a row with different superscripts differ significantly (P<0.05).
[c, d] Means within a row with different superscripts show a tendency to differ (0.05<P>0.1).

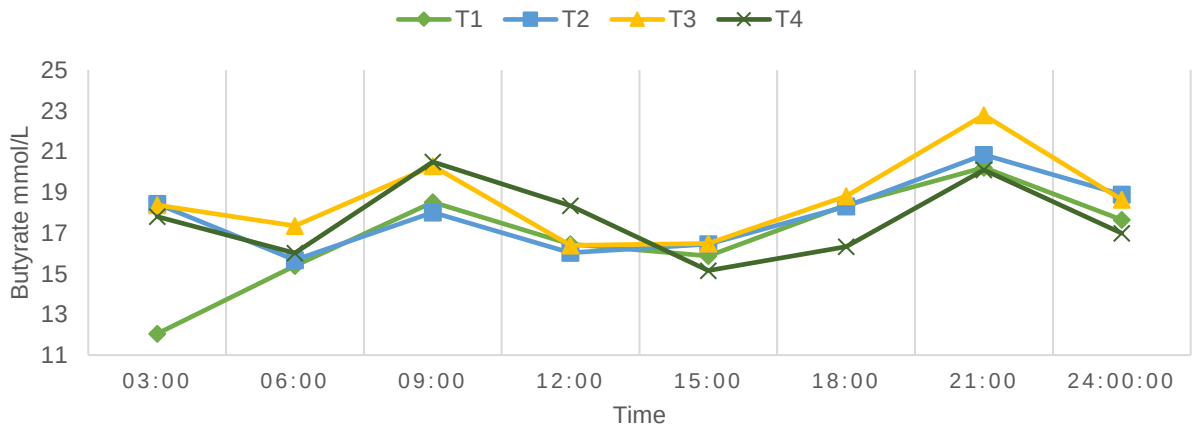

Figure 5-5 Graph depicting various treatment diets (total mixed ration: T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rates influence on rumen fluid butyrate content over a 24-hour period (n=16).

5.4.7 Lactate concentration

At pH's below 5.5, VFA absorption is affected by lactate production via the microbes, *Streptococcus bovis,* which will further decrease ruminal pH and create an environment which favours lactate production, thus entering the cycle of pH depression (Krause & Oetzel, 2005). Kennelly *et al.* (1999) and Khorasani & Kennelly (2001) reported no differences in lactate concentration nor diurnal patterns between buffer and non-buffer treatments which agrees with our results. At 24h00, a tendency (0.05<*P*>0.1) for lactate content measured three-hourly in rumen fluid was observed between treatments 1 and 2 to treatment 3; 0.72, 0.753, and 0.565 mmol/L respectively (Table 5-16). No significant differences were observed otherwise. At 3h00 and 12h00, the numerically lowest lactate (0.532-0.573 mmol/L) concentration was measured in rumen fluid whilst numerically the highest concentration were seen at 9h00 and 21h00 (0.845-0.920 mmol/L). Khorasani & Kennelly (2001) also observed the lactate content peaks in the morning and evening, in-line with Figure 5-6. This has been attributed to the eating behaviour of animals (Khorasani & Kennelly, 2001).

Table 5-16 Lactate concentration (mmol/L) as analysed from rumen fluid collected every three-hours for a 24-hour period for various treatment total mixed rations over the entire experimental period

	Treatment[1]				SEM[2]
	T1	T2	T3	T4	
Time:					
03h00	0.545	0.573	0.559	0.546	0.01563
06h00	0.599	0.715	0.931	0.842	0.17032
09h00	0.845	0.872	0.858	0.906	0.05373
12h00	0.553	0.554	0.553	0.532	0.02065
15h00	0.530	0.556	0.567	0.596	0.01041
18h00	0.596	0.583	0.676	0.804	0.11905
21h00	0.857	0.882	0.920	0.854	0.03516
24h00	0.720[c]	0.753[c]	0.565[d]	0.566[cd]	0.05422

[1] T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcaerious marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rate.
[2] SEM = Standard error of the mean.
[c, d] Means within a row with different superscripts show a tendency to differ (0.05<*P*>0.1).

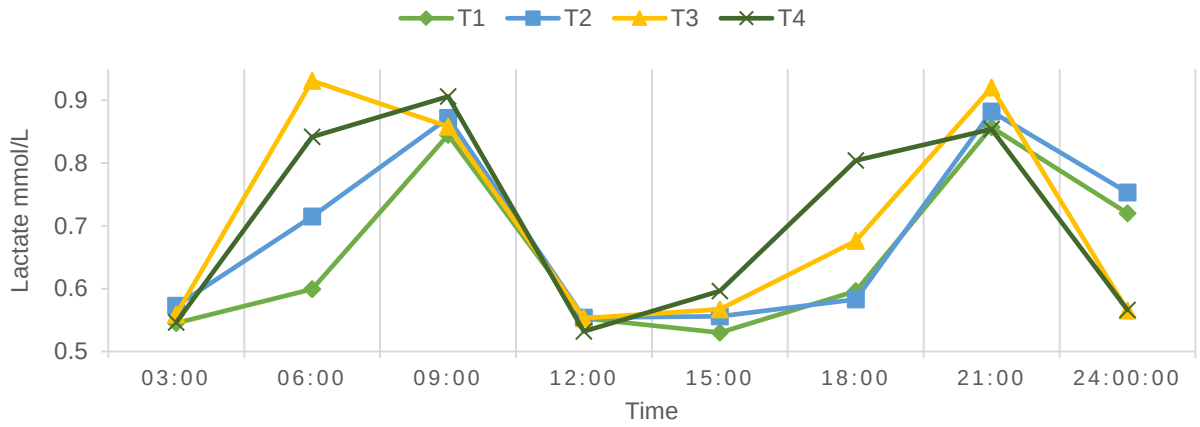

Figure 5-6 Graph depicting various treatment diets (total mixed ration: T1 = Control diet; T2 = Basal diet with 0.8% sodium bicarbonate inclusion rate; T3 = Basal diet with 0.4% calcareous marine source A (Lith A) inclusion rate; T4: Basal diet with 0.4% calcareous marine source B (Lith B) inclusion rates influence on the lactate content of ruminal fluid over a 24-hour period (n=16).

Chapter 6:

Conclusion

Improving prediction models on the effects of ruminal pH on metabolism and VFA production will aid in optimization of ruminant nutrition. Results from the *in vitro* studies revealed that $NaHCO_3$ had the highest *in-vitro* buffering efficacy, followed by Lith sources and then the control, which had the lowest buffering ability. The buffer value index provided the most complete evaluation of buffer efficacy in the rumen according to the *in vitro* results. The *in vitro* buffer value index was the highest for the $NaHCO_3$ treatment compared to the other treatments, while the Lith sources did not differ from each other. *In vitro* results suggest that $NaHCO_3$ has a higher buffering capacity when compared to the control and two different Lith sources. Further research to evaluate the respective buffers *in-vivo* was warranted to determine effects on rumen fermentation and milk production parameters. However, according to the results highlighted in the *in vivo* study, the true buffer, along with the source of calcified marine algae effects, in the rumen is unclear. One single value cannot possibly dictate how a buffer source will react with an animal as there are so many different measures of production.

Milk production was higher for the control and $NaHCO_3$ treatments when compared to Lith B but the Lith treatments did not differ. When expressed as 4% FCM or 3.5% ECM, there were no differences in milk production. Milk fat percent was not affected by treatment, but milk protein percent was higher for the $NaHCO_3$ and Lith A treatments when compared to the control and Lith B treatments. Milk urea nitrogen varied between 13.5 and 15.3 mg/dl and was lowest for the control treatment although all values were still within an acceptable range.

Dry matter intake, body weight and body condition were not affected by buffer supplementation. Digestibility of dry matter for the $NaHCO_3$ and Lith A treatments were higher when compared to the control and Lith B treatments, suggesting a healthier rumen environment. This, however, was not reflected in better NDF digestibility, higher rumen pH, higher total VFA production or lower rumen lactic acid concentration for any of the treatments as these parameters did not differ between treatments. Although the $NaHCO_3$ buffer appeared to be the most promising based on *in vitro* results, it was not supported by *in vivo* results.

A well-balanced diet, with sufficient effective fibre, less than 28% starch, excellent mixing and feed bunk management, as well as individual feeding without group pressure all contributed to a lack of response. Latin square design studies with limited cow numbers are more suited to rumen fermentation studies than prediction-oriented studies where numbers are needed.

High concentrate, low fibre diets fed to cows daily lead to a ruminal pH reduction and onset of sub-acute ruminal acidosis, which could further develop into acidosis. However, the question remains, are these effects caused by low pH or the type of diet that is on offer (Calsamiglia *et al*., 2012)? Should the effects be pH dependent, then the use of buffers is justified. On the other hand, should the effects be due to the nature of the diet, buffers may have a limited effect in the rumen. James & Chow's (1993) alternative theory towards the action of buffers in the rumen must not be discarded. These authors propose that carbonate-based exogenous dietary buffers increase water intake, rumen fluid dilution rate and flow of undegraded starch out of the rumen whilst reducing propionate production. We see that buffers do in turn help to alleviate low pH problems in the rumen, however, alternative strategies which investigate fermentation pathway control may be more successful in combating bouts of subacute ruminal acidosis in the long-term.

Chapter 7:

Critical evaluation and future research

In experiment 1.3, buffers were mixed into 70 ml of rumen fluid and then 30ml dispensed for acid titrations and repeated for base titrations. Thus, 10ml of solution remained in the flask. Bicarbonate has a quick dissolution time. Therefore, the buffer was evenly distributed in solution and resulted in more buffer being present for titrations. However, due to the slow solubilisation of Lithothamnium, treatment 3 and 4 remain undissolved at the bottom of the flask. The smaller particles move into solution, but due to this property very little of the buffer treatments were observed to be dispensed into the 100ml beakers for titration, even after swirling 5 times. This makes it difficult to critically compare and evaluate its efficacy to treatment 1, bicarbonate. For future research, a pipette should be used to get a better representative sample for buffer evaluation.

Experiment 2 made use of four mid-lactation dairy cows that were in their third lactation. Furthermore, these cows were removed from their natural pasture-grazing system and kept intensively in a pen for the entirety of the experiment and fed a total mixed ration (TMR) rather than grazing and being fed a concentrate in the milking parlour. It could be argued that due to previously being adapted to their slug-feeding regime, which amounts to sudden pH drops in the rumen post-consumption, the more controlled TMR diet available throughout the day may not have caused such an impact towards ruminal health as what was expected. Furthermore, the most stressful time in a cow's life is calving and shortly thereafter when the diets are changed accordingly. By the time mid-lactation is reached, these cows are fully adapted to their new diets and no new stressors are to come about, meaning that the animal is better equipped to deal with low ruminal pH without exhibiting major changes towards production. Future research should take this into account when selecting cows to use in studies that identify rumen and milk parameters.

Research into indwelling pH probed versus rumen pH boluses are abundant, along with the comparison between ruminal- and reticular pH. However, the impact of the diet on ruminal-reticular pH fluctuations throughout the day is not fully understood. This complicates the extrapolation of data when comparing SmaXtec pH data to the rumen pH, as well as between treatments. The reliability and accuracy over time of these bolus products are to be brought into question as it is only calibrated once before being inserted into the reticulum, where it remains for the totality of the trial. One should consider comparing ruminal pH through ruminal fluid collection to reticular pH using the SmaXtec pH Plus bolus throughout the 5-month active period of the

bolus. The bolus should be removed once a week to test if the accuracy has declined since calibration by comparing between boluses in a flask of distilled water. This could help researchers identify possible weaknesses in these kinds of tools whilst still gaining important knowledge on how the rumen and reticular pH differ throughout the day and when animals are fed different diets.

Applications that need to be further explored include bringing into question the affect that SARA has on the potency or efficacy of dietary feed additives or medication. Should the unstable rumen environment show to reduce or alter performance additives, further losses and wastes will be incurred.

An interesting reduction in the calcium and phosphorus content in the refusals of cows fed sodium bicarbonate indicates selection for these minerals in this trial. This may be linked to possible associations between sodium bicarbonate and calcium and phosphorus, further highlighting the need to investigate the association further to ensure lactating dairy cattle are not underfed Ca and P when diets are supplemented with sodium bicarbonate as an exogenous buffer.

Future research into comparing Lithothamnium sources are needed to be conducted in comparing the quality variation between and within sources. This will not only improve animal health and production on farm, but it will also aid ruminant nutritionists when evaluating dietary buffers to use in rations according to variation within products and performance differences between them.

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Appendix

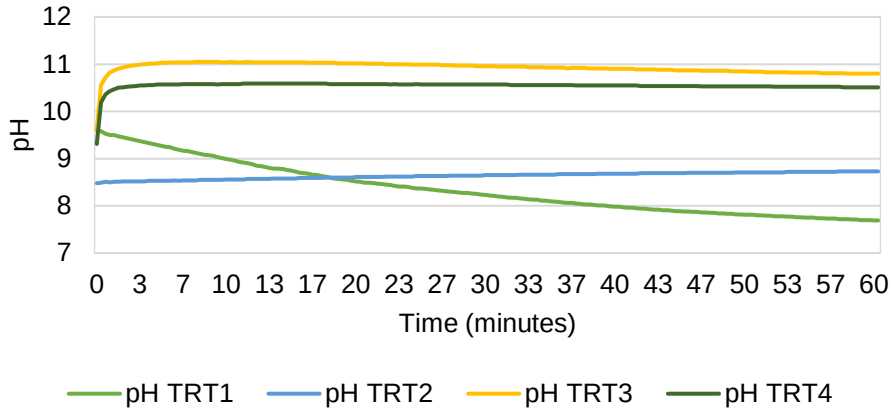

Figure A.1 Effect of buffer on pH of distilled, deionized water over 60 minutes. The pH of solution was measured every 20 seconds for the following treatments: T1= Control, T2= $NaHCO_3$, T3= Lith A, T4= Lith B.

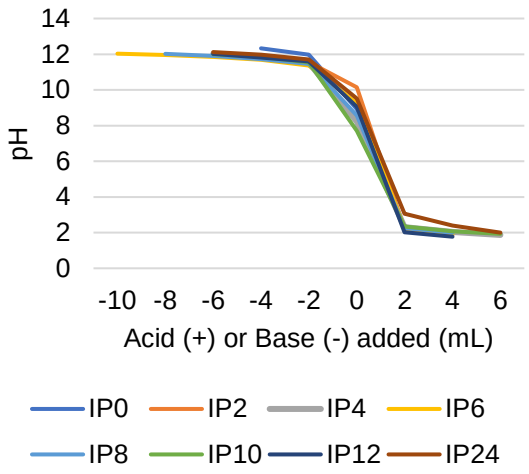

Figure A.2 Titration curve after 0, 2, 4, 6, 8, 10 and 12 hours of incubation of water in distilled, deionized water.

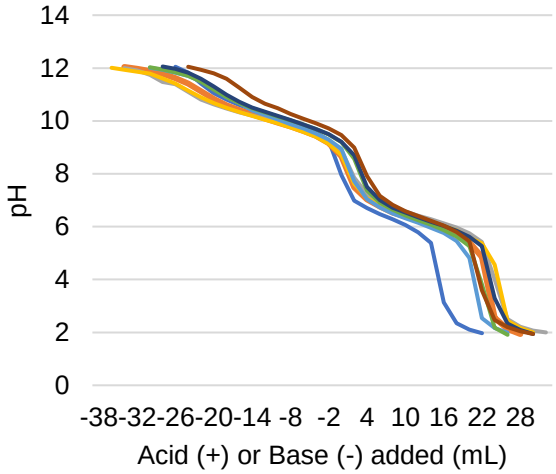

Figure A.3 Titration curve after 0, 2, 4, 6, 8, 10 and 12 hours of incubation of 0.5 g $NaHCO_3$ in distilled, deionized water.

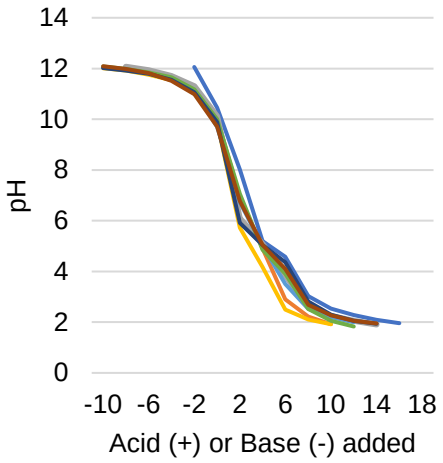

Figure A.4 Titration curve after 0, 2, 4, 6, 8, 10 and 12 hours of incubation of 0.5 g Lith A in distilled, deionized water.

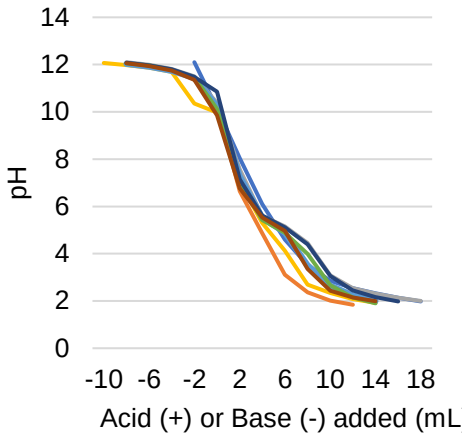

Figure A.5 Titration curve after 0, 2, 4, 6, 8, 10 and 12 hours of incubation of 0.5 g Lith B in distilled, deionized water.