The role of Calcareous Marine Algae in Reducing the Negative Health and Production Consequences of Disrupted Rumen Fermentation in Dairy Cows

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Declaration

I declare that this thesis has not previously been submitted as an exercise for a degree at the National University of Ireland, or any other University, and I further declare that the work reported herein is my own.

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Enda Neville, 7th January 2022

Collaborations

Prof. Finbar Mulligan, UCD - Experimental design, data interpretation, nutritional advice for the cows used during the experiments, management of animal studies and assistance with the writing of this thesis.

Prof. Alan Fahey- Experimental design, statistical analysis, data interpretation and assistance with the writing of this thesis.

Prof. Kieran Meade, UCD – Laboratory analysis, data interpretation and assistance with the writing of chapter 3.

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Dr. Stephen Taylor, Marigot – Experimental design, advice on experimental diets, and assistance in writing of chapter 4.

Dr. Shane O'Connell, ITT – Experimental design, laboratory analysis, and data interpretation of chapter 5

Prof Kees Plaizier, Univ. of Manitoba, Canada – Experimental design and data interpretation of chapter 5

Dr. Leluo Guan, Univ. of Alberta, Canada - Experimental design and data interpretation of chapter 5

Dedication

I would like to dedicate this thesis to Sally. This thesis would not have been possible without her support.

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List of Abbreviations

ADF	Acid detergent fibre
BCS	Body condition score
BHBA	Beta Hydroxy butyric acid
CP	Crude protein
d	Day
DIM	Days in milk
DM	Dry Matter
DMI	Dry matter intake
ECM	Energy corrected milk
EE	Ether extract
eNDF	Effective neutral detergent fibre
FCM	Fat corrected milk
g	Gram
h	Hour
Hp	Haptoglobin
IE	Ireland
kg	Kilogram
L	Litre
LPS	Lipopolysaccharides
LBP	Lipopolysaccharide binding protein
Mcal	Megacalorie
ME	Metabolisable energy
MFD	Milk fat depression

MgO	Magnesium oxide
MJ	Mega Joules
ml	millilitre
mm	Millimetre
mmol	Millimoles
NDF	Neutral detergent fibre
NDF^{forage}	Forage associated neutral detergent fibre
NEFA	Non-esterified fatty acids
NE_L	Net energy for lactation
NIR	Near infrared
NRC	National Research Council
NSC	Non-structural carbohydrates
OM	Organic matter
PDIE	Protein truly digestible in the small intestine where energy limits protein
PDIN	Protein truly digestible in the small intestine where nitrogen limits microbial protein
PeNDF	Physically effective neutral detergent fibre
PFAD	Palm fatty acid distillate
PSPS	Penn state particle separator
SAA	Serum amyloid A
SARA	Sub-acute ruminal acidosis
SCC	Somatic cell count
SCS	Somatic cell score

SE	Standard error
SEM	Standard error of the mean
TMR	Total mixed ration
Trt	Treatment
UCD	University College Dublin
UFL	Unit of energy for lactation
US	United States
VFA	Volatile fatty acids
WSC	Water soluble carbohydrate

Abstract

The objective of the thesis was to investigate the effects of calcareous marine algae (CMA) on rumen pH and fermentation, digestion, feed intake, milk production, and inflammation in dairy cows during a dietary challenge. Different types of dietary challenges were used to achieve our objective. The dietary challenges included: the transition period; a high starch total mixed ration (TMR), a grain (GR) induced sub-acute ruminal acidosis (SARA); and a ryegrass (RY) induced SARA. Calcareous marine algae was compared to a control (CON) diet, containing no dietary additive, in chapter 3, 5, and 6. In chapter 4, CMA was compared to a combination of CMA and marine magnesium oxide (MM), sodium bicarbonate (SB) and a CON treatment. Chapter 3 investigated the effects of CMA on feed intake, milk production, energy balance, serum mineral metabolites and inflammatory markers in 32 cows during the transition period compared to a CON treatment. The CMA treatment had higher prepartum dry matter intake (DMI), postpartum DMI, milk fat concentration, fat corrected milk yield, and reduced plasma serum amyloid A (SAA) compared to the CON treatment. Chapter 4 investigated the effects of CMA, with or without MM, and SB on rumen pH parameters, volatile fatty acid (VFA) production, apparent total tract digestion, and the kinetics of digestion using 4 cannulated cows in a 4 × 4 Latin square design. The CMA and CMA+MM increased mean, median and minimum rumen pH, and reduced time spent below rumen pH 5.6 and 5.4 compared to the CON treatment. There was less variation in rumen pH with the CMA treatment compared to the SB treatment. Acetate: propionate ratio was reduced, and the molar proportion of propionate increased with CMA+MM compared to CON, CMA, and SB. The CMA+MM treatment increased neutral detergent fibre (NDF) digestibility compared to CON. There were no differences in rumen fluid and particulate outflow rates, rumen retention time, or rumen *in-sacco* degradability between treatments. Chapter 5 determined the effects of CMA on rumen pH, rumen fermentation, and plasma inflammatory markers during a GR and RY induced SARA challenge using eight ruminally cannulated cows in a 2 × 2 split-plot crossover design. The experiment consisted of three phases: acclimatisation; challenge; and recovery. Both GR and RY diets successfully induced SARA temporarily. The CMA treatment reduced the minutes below rumen pH 5.4, 5.6, and 5.8 during the ACC phase, and minutes below rumen pH 5.6 and 5.8 during the REC phase. The RY diet had a lower concentration of rumen lipopolysaccharide compared to the GR diet during the challenge. There was a greater reduction in SAA with the CMA treatment compared to the CON during the recovery phase. Chapter 6 investigated the effects of CMA on feeding behaviour, rumen fermentation products,

milk fatty acid profiles, and total tract digestibility of dry matter (DM) and organic matter (OM) in 32 early lactation dairy cows compared to a CON treatment. The CMA treatment reduced the proportion of *trans*-9 18:1 fatty acid and the omega-6 to omega-3 fatty acid ratio in milk compared to the CON treatment. Daily TMR intake and feeding rate were increased in cows fed the CMA treatment compared to cows fed the CON treatment. These findings demonstrate the benefits of CMA on reducing rumen pH depressions and increasing digestion of DM and OM. Calcareous marine algae can be an effective tool for preventing rumen fermentation disruptions during a dietary change and for increasing DMI and energy balance during the transition period.

Chapter 1.

General Introduction and Theis Outline

1.1 Introduction

Dairy production is an important part of the global economy and an important segment of food production systems in many countries worldwide. Billions of people around the world consume milk and dairy products each day (Gaworski and Leola, 2014). The consumption of dairy products has been growing for the past number of years and is expected to grow further, at a rate of approximately 2% per annum for the foreseeable future (IFCN, 2021). There is growing pressure on dairy producers to supply a more sustainable product as they compete with other agricultural sectors for resources, while also adhering to increased regulatory pressures around antibiotic usage and other essential inputs. As a result, increasing the efficiency and reducing the environmental footprint of dairy production and its products while also maintaining optimum health and welfare standards among the dairy livestock, as is now demanded by consumers, will become a major focus of dairy farmers in the years ahead.

The ruminant digestive system has evolved over time to enable dairy cows to convert β-linked polysaccharides, indigestible by mammalian enzymes, into nutritious products for human consumption (Van Soest, 1994). This unique digestive system has allowed the dairy cow to be an extremely important part of the human food production process by converting nutrients, that humans cannot breakdown or utilise, into edible products for human consumption. Improving the conversion of feed into dairy produce through enhanced feed efficiency will lead to a more sustainable growth of dairy production systems. In the last 20 years, both improved genetics and nutrition have improved the feed efficiency of the lactating dairy cow (Sauer and Latacz-Lohmann, 2015). The improvements in genetics have resulted in cows with potential to produce large volumes of milk per day and the advances in dairy cow nutrition have allowed these dairy cows to realise their genetic potential for milk production. Efficient dairy cows should produce more dairy product per unit of carbon footprint. High producing dairy cows require highly fermentable diets to meet their nutrient demands. Such fermentable diets lead to increased concentrations of volatile fatty acids (VFA) in the rumen, which can accumulate leading to periods of reduced rumen pH (Whelan, et al., 2013). Formulating diets to provide adequate energy levels while also supplementing sufficient physically effective fibre to prevent rumen pH depressions is extremely difficult and, in many cases, unsuccessful. Furthermore, in some regions of the world access to good quality forage is limited due to the long distance that forage and fibre sources may have to be transported before their use in dairy cow diets adding to the economic cost and carbon footprint of dairy production.

The transition period (21 days prepartum to 21 days postpartum) is one of the most challenging times during the dairy cow's production cycle. During the transition period, cows experience a significant dietary change and rapid increase in Ca requirements to satisfy the demands of lactation. The transition period is responsible for most production diseases and illnesses experienced by the dairy throughout her lactation (Bradford, 2017). Inflammation is an emerging aspect of transition cow biology and the root cause of many problems associated with the transition period (Bradford et al., 2015). The abrupt dietary change from dry to lactating diet leads to prolonged rumen pH depressions during the early postpartum period (Penner et al., 2007). These periods of low rumen pH can predispose cows to inflammation. Another major challenge of the early postpartum period is reduced feed intake. Dietary strategies for the transition cow period should focus on avoiding extended rumen pH depressions, reduce the extent of inflammation, and increase feed intake. Strategies to prevent transition cow diseases will become more important in future as we try to reduce antibiotic usage where possible.

Rumen pH regulating feed additives provide a solution to help maintain rumen function in dairy cows and subsequently increase feed intake and the production of milk or milk components whilst also protecting the health of the dairy cow. Such feed additives may also reduce the need for antibiotics for treating illness in dairy cows by preventing problems relating to dietary-induced inflammation. Calcareous marine algae (CMA; Acid Buf or Calmin, Celtic Sea Minerals, Ireland) is a feed additive produced from *Lithothamnion sp.*, harvested off the coast of Iceland. Calcareous marine algae has been supplemented to both humans and animals for many years now with proven benefits on rumen pH and fermentation (Cruywagen et al., 2015), and milk production in dairy cows (Neville et al., 2019).

1.2 Thesis Outline

This thesis is outlined as follows:

Chapter 2

The purpose of this chapter is to review the available literature on rumen fermentation and digestion, low rumen pH, sub-acute ruminal acidosis (SARA), dietary induced inflammation, the transition period, and the use of dietary feed additives to positively alter the rumen environment. The literature reviewed in this chapter will also discuss the consequences of low rumen pH as a predisposition to diet-induced inflammation and cow health, milk production, milk components, and milk production efficiency. The literature review focuses on the transition period as an inevitable abrupt dietary change experienced by all dairy cows and how rumen fermentation disruption and inflammation can cause other health issues within this period. The review is divided into 7 different sections: the basics of rumen function; rumen microflora; microbial fermentation products; ruminal pH; dietary induced inflammation; the transition period; and rumen modifying dietary feed additives.

Chapter 3

The objective of this chapter is to compare the effects of a CMA supplemented diet to a limestone-based control diet on feed intake, milk production, energy balance, serum mineral metabolites and inflammatory markers in transition dairy cows. Thirty-two dairy are assigned to two treatments from 25 days (d) before expected parturition until 42 d postpartum.

Chapter 4

This chapter investigates the effects of dietary inclusion of CMA, with or without marine magnesium oxide, and sodium bicarbonate on rumen pH parameters, VFA production, apparent total tract digestion, and the kinetics of digestion. Four rumen cannulated dairy cows are utilised in a 4 × 4 Latin square design experiment lasting for 100 d and consisting of four separate periods of 25 d.

Chapter 5

The primary objective of chapter 5 is to determine the effects of CMA on rumen pH, rumen fermentation, and plasma inflammatory markers during a grain and ryegrass induced SARA challenge. Eight ruminally cannulated cows are assigned to four treatments in a 2 × 2 split-plot crossover design. The main plot is diet during the SARA challenge. The experiment contains three phases: acclimatisation; challenge; and recovery. This is likely the first time that a ryegrass-based SARA challenge has been compared to a grain-based SARA challenge.

Chapter 6

This chapter investigates the effects of CMA on feeding behaviour, rumen fermentation products, milk fatty acid profiles, and total tract digestibility of dry matter and organic matter in early lactation dairy cows. Thirty-two dairy cows are assigned to two treatments from 25 d before expected parturition until 42 d postpartum.

Chapter 7

In chapter 7, experiments from chapter 3, 4, 5, and 6 are discussed. An overall summary and discussion on the effects of including CMA in the diet of lactating dairy cows consuming feed ingredients typical of the northern European region is provided. This chapter also highlights the novel aspects of this research and discusses the implications of the research in this thesis for dairy cow nutrition programmes and provides suggestions for future work in this area.

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Chapter 2.

Literature Review

2.1 The basics of rumen function

2.1.1 Introduction

The ruminant digestive system has evolved over time to facilitate the breakdown of fibrous forages, which consist mainly of β-linked polysaccharides (such as cellulose and hemi-cellulose) (McDonald et al., 2011). The anatomical adaptations of the ruminant digestive system have allowed it to exploit fibrous food resources much more efficiently than other common herbivores that rely on lower gut fermentation below the main site of digestion for many nutrient in their food (Van Soest, 1994). Exposure to microbial activity in the rumen is essential for the breakdown of these β-linked polysaccharides as enzymes of mammalian origin are unable to break them down (McDonald et al., 2011). In addition, the ruminant animal has potential advantages of a large forestomach which does not inhibit their food intake as much as other foregut fermenters, which are commonly faced with the challenge that a large food intake compromises digestive efficiency (Clauss et al., 2010).

The ruminant animal has formed a symbiotic relationship with rumen microorganisms by providing them with a habitat for their growth, known as the reticulo-rumen, while the microorganisms then provide the ruminant animal with fermentation acids, microbial protein and vitamins (Russell and Mantovani, 2002). Ruminants tend to ingest feed rapidly and ruminate it later. Ruminants use their reticulo-ruminal sorting mechanism to retain food requiring further digestion while clearing the forestomach of already digested material, leaving room for new material to be ingested (Clauss et al., 2010). The process of rumination involves the regurgitation of ingesta followed by mastication, reforming the bolus by mixing with saliva and re-swallowing. This process is then repeated many times (Van Soest, 1994). One of the major factors inducing the animal to ruminate is the tactile stimulation of the epithelium of the anterior rumen. Rumination induces saliva production which contains phosphate and bicarbonate buffers to keep rumen pH in a desirable range (McDonald et al., 2011).

2.1.2 Requirements for optimum rumen function

Effective dietary fibre is very important for stimulating saliva production and ensuring optimum rumen function (Humer et al., 2018). Dietary fibre refers to substances of plant cell wall origin, cellulose, hemi-cellulose, and lignin, that are resistant to mammalian digestive enzymes (Van Soest, 1994). Mertens (1997) defined fibre as the slowly digestible or

indigestible component of feeds that occupies space in the gastrointestinal tract of animals. Effective dietary fibre stimulates saliva production and dietary fibre provides other properties of nutritional significance such as bulk density, hydration capacity, binding properties and fermentability (Van-Soest, 1978). The neutral detergent fibre (NDF) method, developed by Van Soest, has very much replaced the crude fibre method as a means of accurate fibre determination in many fields of animal nutrition (Hans-Joachim, 1997).

Forage based diets contain high levels of plant cell wall or fibre, NDF and acid detergent fibre (ADF), and it has been suggested that feeding forage-based diets is the best way to maintain rumen function and animal health (Jung and Allen, 1999). Sufficient levels of NDF are required in dairy cow diets to maintain rumen function. Providing adequate fibre in the diet of high producing dairy cows can be difficult. Too much fibre in a ruminant diet can lead to a reduction in energy density and intake. Conversely, too little fibre in a ruminant diet can disrupt normal rumen fermentation and lead to low pH and possibly rumen acidosis (Mertens, 1997). The target NDF level in dairy cow diets should be at least 25% with most of that NDF coming from forage (Allen, 1997).

2.1.3 Rumination

Typical ruminant behaviour involves the rapid ingestion of feed followed by rumination later. Rumination is the postprandial regurgitation of ingesta followed by mastication, reforming the bolus and re-swallowing (Van Soest, 1994). Rumination is a key factor in particle size reduction for long fibrous particles, which can congest the rumen if allowed to accumulate. Rumination is stimulated by tactile means or by the pressure, from coarse material, applied against sensors in the rumen wall. This can also be referred to as the ‘scratch factor’ (Van Soest, 1994). The contents of the rumen are not of uniform composition. They are in the form of stratified layers with longer particles making up the fibre mat floating on a pool of liquid containing smaller particles and digested cell contents. One of the primary functions of the stratification process is to encourage rumination by physical stimulation of the rumen wall (Zebeli et al., 2012). Large food particles, irregular in shape, with low specific gravity are retained in the top stratum of the rumen contents to allow further particle size reduction and microbial degradation while the smaller and more digested particles descend in the rumen liquor and can be washed out (McDonald et al., 2011).

2.1.4 Rumen epithelium and papillae

The rumen papillae are small finger-like projections on the internal surface of the rumen wall that provide increased surface area for the absorption of nutrients (McDonald et al., 2011). Their growth is stimulated by increasing the presence of volatile fatty acid (VFA), particularly butyric acid (Baldwin Vi et al., 2004). The rumen epithelium is the surface layer of the papillae and rumen wall, and is responsible for VFA absorption, transport, and protection of the host animal (Baldwin Vi et al., 2004). The rumen papillae and epithelium are critical to the energy supply of ruminant animals because VFA absorption, facilitated by the papillae and epithelium, account for 75% of the total metabolizable energy (ME) supply (Penner, 2019). Under normal circumstances, the rumen epithelium is covered by a protective barrier consisting of keratinised cells (Plaizier et al., 2012). Excessive concentrations of fermentation acids, particularly lactic acid, results in low pH causing the epithelium to become damaged and may lead to parts of the epithelium being sloughed away after prolonged exposure to acidic conditions (Van Soest, 1994). Steele et al. (2011) demonstrated that the structural integrity of the rumen epithelium was compromised during an experimentally induced SARA (mean pH = 5.9 and pH < 5.6 for 4.6 h/d). Breakdown of the keratinous layer of the rumen epithelium, caused by extended periods of pH depression, may lead to translocation of rumen bacteria into the bloodstream (Plaizier et al., 2012). However, it has been documented that individual cows differ in the adaptations of their rumen epithelia to high grain feeding, and this variation may be somewhat caused by genetics (Plaizier et al., 2014).

One of the greatest rates of rumen epithelium change occurs during the transition from dry to early lactation in dairy cows. During this period, cellular proliferation within the rumen epithelium is accelerated to increase the surface area for VFA absorption in order to deal with the increased VFA concentration within the rumen (Steele et al., 2015). Dieho et al. (2016) showed that increasing the level of fermentable organic matter (OM) in the diet of early lactation cows, through increased provision of concentrates, caused a more rapid growth in papillae surface area compared to a diet with lower levels of fermentable OM. That same study found that papillae surface area reduced over the 8 week (wk) dry period but had returned to the level at the start of the dry period within the first 2 wk of lactation and continued to increase up to wk 9 of lactation (Dieho et al., 2016).

2.1.5 Digesta passage kinetics

An understanding of digesta passage kinetics is necessary to calculate nutrient supply to the dairy cow in order to optimise performance and maintain dairy cow health (Warner et al., 2014). The stratification of rumen contents allows larger particles to be retained for further digestion and is central to the overall digesta passage process (Zebeli et al., 2012). Digesta passage has a big influence on the net energy extracted from a feedstuff (Krämer et al., 2013b). According to Warner et al. (2014), the potential of fibre to be digested in the rumen is governed by the microbial degradation of feed particles along with the digesta passage from the rumen. Rumen digestion can be improved by allowing potentially degradable material sufficient time in the rumen to become completely degraded and products of digestion (together with undegraded material) being allowed to pass out of the ruminant forestomach, either by transit to the lower gut or by absorption through the rumen wall (McDonald et al., 2011). However, prolonged retention time in the rumen may also decrease feed intake (Krämer et al., 2013a). According to Warner et al. (2014), the movement of feed particles through the reticulorumen via the reticulo-omasal orifice is dictated by particle size, specific gravity and effective particle density (buoyancy). Studies have shown that digesta particles must be approximately 3-4 mm or less to exit the rumen in cattle and typical rumen retention time can vary between 30-80 hours (McDonald et al., 2011) for forage particles. Rate of passage is increased when more concentrates and less forages are included in the diet due to larger size and lower specific gravity associated with forage (Krämer et al., 2013a). Within rumen digesta, concentrate particles have a higher rumen outflow rate than forages (Owens and Goetsch, 1986).

2.1.6 Rumen osmolality

Osmolality is defined as a measurement of the number of particles in a kg or L of fluid (Khajuria and Krahn, 2005). In the rumen, osmolality is usually dictated by the concentration of substances like VFA, glucose, and lactate (Owens et al., 1998). Increased osmolality is commonly associated with periods of low pH in the rumen due to the accumulation of VFA (Kent-Dennis et al., 2020). According to Kleen et al. (2003), increased osmolality will reduce dry matter intake (DMI) because of increased water flow to the rumen to counteract the increased osmolality of the rumen fluid. Furthermore, Allen (2000) suggested that rumen osmolality is highly variable and increased osmolality is associated with numerous physiological responses that may affect feeding behaviour and daily feed intake. Enemark

(2009) reported that rumen osmolality concentrations in excess of 300 milliosmole per litre (mOsm/L) are responsible for reduced DMI, along with reduced bacterial fermentation of starch and fibre. However, these effects of increased rumen osmolality on reduced DMI are not conclusive. Khafipour et al. (2009b) reported reduced rumen pH and increased osmolality but no effect on DMI when they induced SARA using alfalfa pellets. An increase in rumen osmolality can also lead to swelling and rupture of rumen papillae, subsequently compromising the barrier function of the rumen (Khafipour et al., 2009a).

2.2 The Rumen Microflora

The rumen microbiota consists mainly of; bacteria, protozoa, and fungi and they form an essential link between the ruminant animal and their diet (Weimer et al., 1999). For the last 30 years, or more, the rumen micro-biome has been investigated in detail. This has been greatly aided by the development of DNA sequencing and associated technology, and several recent publications have improved our knowledge of this area (Shokun et al., 2017; Zhou et al., 2018).

2.2.1 Importance of the rumen microflora

The ruminant animal can utilize a wide variety of feeds due to the efficiency of a highly diversified rumen microbial ecosystem containing bacteria, fungi, and protozoa (Kamra, 2005). The ruminant digestive system allows it to extract nutrients from fibrous feeds and therefore fibre or plant cell wall digesting organisms are extremely important to the overall rumen digestive process (Shokun et al., 2017). Because cellulose is one of the most abundant constituent of plant cell walls, rumen cellulolytic microorganisms perform a key role in the nutrition of ruminant animals (Weimer, 1996). The rumen microbial processes have the ability to transform fibrous feeds and low-quality protein, as well as non-protein-nitrogen, into valuable nutrients for the ruminant animal (Dewhurst et al., 2000).

2.2.2 Classification of the different microflora

2.2.2.1 Bacteria

Bacteria are the most predominant group of rumen microbes (Cammack et al., 2017). The rumen microbial ecosystem contains at least 30 predominant bacterial species at an estimated total concentration of 10^{10} to 10^{11} per ml of rumen fluid (Miron et al., 2001). Many rumen bacteria are capable of using several different carbohydrate sources as growth substrates (Weimer, 1996). Rumen bacteria that can hydrolyse a range of substrates are known as 'generalists', examples include *Butyrivibrio fibrisolvens*, and rumen bacteria that can only ferment specific substrates are called 'specialists', examples include *Fibrobacter succinogenes* which utilize cellulose only and *Ruminobacter amylophilus* which only utilizes starch (Stewart and Bryant, 1997). Most bacteria in the rumen are gram-negative with lesser numbers of gram-positive, the ratio of gram-negative to gram-positive can change based on the amount of starch added to the diet (Kamra, 2005). Rumen bacteria, together with fungi, account for about 80% of the digestive activity of rumen contents (Wang and McAllister, 2002). Bacteria living in the rumen have been divided into five groups depending on their environmental existence; 1) free-living bacteria associated with rumen liquid phase, 2) bacteria loosely associated with feed particles, 3) bacteria firmly adhered to feed particles, 4) bacteria associated with rumen epithelium and 5) bacteria attached to the surface of protozoa or fungal sporangia (Miron et al., 2001). That same study also stated that bacteria associated with feed particles (group 2 & 3) are numerically predominant and can occupy up to 75% of the total microbial population in the rumen.

2.2.2.2 Protozoa

A typical rumen microbiome can contain as much as 40 different species of protozoa at a concentration of 10^5 to 10^7 /ml of rumen fluid (Miron et al., 2001). The largest, most apparent and most significant protozoa are the ciliates, of which there are two groups: holotrich protozoa and oligotrich (previously known as entodiniomorphid) protozoa (Williams and Coleman, 1997). The oligotrich protozoa are well acclimatised to the rumen environment and utilize particulate rather than soluble food materials, whereas the holotrich protozoa can utilize soluble food particles and are more aerotolerant (Williams and Coleman, 2012). The protozoa directly affect the productivity, health and overall environmental impact of the host ruminant animal,

both through their metabolic actions and as a consequence of the post-rumen degradation and utilization of the protozoal cellular constituents (Williams and Coleman, 1997). Rumen protozoa can also account for up to 20% of the total digestive activity of rumen contents (Wang and McAllister, 2002). Despite the fact that ciliate protozoa in the rumen may feed off and engulf beneficial rumen bacteria, it has been noted that defaunation (process of removing protozoa) of the rumen may lead to reduced cellulose and protein digestion (Williams and Coleman, 2012).

2.2.2.3 Fungi

Fungi are an important part of the rumen microbial ecosystem. There are five different, known, species of fungi existing at a concentration of 10^5 /ml of rumen fluid (Miron et al., 2001). Rumen fungi may not be essential to overall rumen digestion, as they exist in very low numbers in animals fed a low-fibre diet, but they may play an important role in the breakdown of plant cell walls (Orpin and Joblin, 1997). According to Kamra (2005), the positive role performed by fungi in rumen fibre degradation is evidenced by the presence of fibre degrading enzymes produced by the fungi. Wang and McAllister (2002) suggested that fungi, along with bacteria, were responsible for 80% of the digestion of plant cell walls in the rumen.

2.2.3 Cellulolytic digestion

Cellulolytic digestion refers to the breakdown of cellulose, in the rumen, by rumen microbes. Cellulolytic digestion by rumen microorganisms is regarded as one of the most important features of the ruminant digestion process because cellulose is the most abundant component of plant cell walls (Weimer, 1996). According to Williams and Coleman (1997), some oligotrich protozoa, like *Eudiplodinium maggi,* are capable of fermenting cellulolytic material in the rumen and will even digest cellulose particles up to 25 times faster than starch particles. Up to 20% of plant cell wall breakdown can be attributed to protozoa (Wang and McAllister, 2002). Even though Orpin and Joblin (1997) documented that some rumen fungi, like *Neocallimastix frontalis,* are capable of digesting cellulose, among other polysaccharides, little is actually known about the extent to which fungi are involved in cellulolytic digestion in the rumen (Wang and McAllister, 2002). However, most scientists will agree that cellulolysis in the rumen is primarily due to the actions of the rumen cellulolytic bacteria (Weimer, 1996). Numerous authors have identified three predominant species of bacteria responsible for rumen

cellulolytic digestion and they include *Fibrobacter (formerly Bacteroides) succinogenes, Ruminococcus flavefaciens, and Ruminococcus albus* (Forsberg et al., 1981, Weimer, 1996, Weimer et al., 1999, Miron et al., 2001, Wang and McAllister, 2002, Sung et al., 2007). One of the most important characteristics of these three rumen cellulolytic bacteria, is that they are almost entirely restricted to cellulose and its hydrolytic products as growth substrates (Weimer, 1996). This nutritional specialisation enables them to break down cellulose rapidly and leads to more effective cellulose digestion (Weimer, 1996). These specialised rumen cellulolytic bacteria break down their substrates, cellulose, through the process of adhesion which can be divided into 4 phases: 1) transport of the non-motile bacteria to the substrate; 2) initial non-specific adhesion of bacteria to unprotected sites of the substrate; 3) specific adhesion with the substrate; 4) proliferation of the attached bacteria on potentially digestible tissues of the substrate (Miron et al., 2001). Enzymatic activity of the rumen cellulolytic bacteria is also essential for the optimal digestion of cellulose in the rumen (Wang and McAllister, 2002).

2.2.4 Starch digestion

In addition to cellulose digestion, rumen microbiota can also ferment non-fibrous materials such as starch and sugars which increase the rate of fermentation and the production of the ruminant animal (Russell and Rychlik, 2001). When starch granules become exposed in the rumen, the capacity to utilise starch as a source of carbon is prevalent amongst various strains of bacteria, protozoa and fungi located in the rumen (Chesson and Forsberg, 1997). The main starch digesting bacteria include: *Ruminobacter amylophilus, Prevotella ruminicola, Streptococcus bovis, Succinimonas amylolytica,* many strains of *Selenomonas ruminantium, Butyrivibrio fibrisolvens, Eubacterium ruminantium* and *Clostridium spp.* which are responsible for most of the microbial starch digestion, as well as nearly all of the larger oligotrich protozoa and all of the chytrid fungi, in the rumen (Chesson and Forsberg, 1997). Despite the fact that some studies have indicated that rumen protozoa are not essential to the nutritional status of their host animal, it has also been reported that many protozoa are able to take up and store starch granules, leading to changes in the rate of fermentation and in some cases prevention of rumen acidosis (Russell and Rychlik, 2001).

2.2.5 Lactic acid production and utilization

The rumen microbes are the key determinants influencing the production and utilization of lactic acid (Mackie and Gilchrist, 1979). The rumen microflora contains a group of lactic acid producers and a second group of lactic acid utilizers that ferment lactic acid into VFA's (Mills et al., 2014). The main rumen bacteria responsible for lactic acid production include *Streptococcus bovis, Lactobacillus species,* and *Mitsuokella multiacidus* while the bacteria responsible for the utilization of lactic acid in the rumen include: *Megasphaera elsdenii, Selenomonas ruminantium*, and *Anaerovibrio lipolytica* (Stewart and *Bryant, 1997)*. According to Counotte et al. (1981) *Megasphaera elsdenii* is the most effective rumen bacteria at utilizing lactate as it ferments lactic acid via the acrylate pathway, unlike other lactic acid utilizing bacteria who may utilize other substrates, like glucose, before lactic acid. The balance of growth between the lactic acid utilizing and lactic acid producing bacteria, in most cases, will keep the concentration of lactic acid low (Carpinelli et al., 2021). However, lactic acid concentrations can accumulate as the level of starch and soluble sugars increase, in the rumen, and the lactic acid producing bacteria exceeds the capacity of the lactic acid utilizing bacteria (Mills et al., 2014). Mackie and Gilchrist (1979) reported an increase in the growth of lactic acid utilizing bacteria, in response to increased production of lactic acid producing bacteria, which prevented a build-up of lactic acid in the rumen of sheep during a stepwise introduction to a high starch diet.

As lactic acid production increases, the rumen pH decreases and further encourages the growth of acid tolerant bacteria, like *Streptococcus bovis*, surpassing the capacity of the more acid sensitive lactic acid utilizing bacteria, like *Megasphaera elsdenii*, resulting in a spiralling reduction of pH and higher lactic acid concentrations (Mills et al., 2014). An accelerated drop in rumen pH and an overgrowth of *Streptococcus bovis* can lead to rumen acidosis which can be detrimental to the health of the ruminant animal (Russell and Hino, 1985).

2.2.6 pH sensitivity of rumen microflora

According to Chamberlain and Wilkinson (1996), optimum rumen pH is one of the requirements for microbial growth. Most of the literature dealing with this subject, except for Russell and Wilson (1996) who stated that rumen cellulolytic protozoa and fungi may be sensitive to low rumen pH, has focused solely on rumen bacteria so this section will be based

mainly on rumen bacterial interactions with pH. The majority of rumen bacteria prefer pH near neutrality (pH 6.0 – 7.0) for optimum growth, although some species, like *Streptococcus bovis* (*S. bovis*) and *Prevotella ruminicola*, can grow at a lower pH range of pH 5.0 – 6.0 (Weimer, 1996). Cellulolytic bacteria are particularly sensitive to low pH and growth can be totally inhibited when pH drops to less than pH 6.0 which means that the feeding of diets high in starch or soluble sugars, which is common in most parts of the world nowadays, will reduce cellulose digestion due to a lowering of rumen pH (Russell and Wilson, 1996).

Differences in intracellular pH regulation dictate the pH sensitivity of rumen bacteria. Acid tolerant bacteria, like *S. bovis*, allow their intracellular pH decline in response to a decreasing extracellular pH protecting them from the influx and accumulation of fermentation acid anions, whereas acid sensitive bacteria maintain a stable intracellular pH subsequently allowing their extracellular pH to decline leading to an influx of fermentation acid anions (Russell and Rychlik, 2001).

The effect of pH on bacterial adhesion, one of the main mechanisms behind how bacteria breakdown their substrates, has been examined with some studies showing that *Fibrobacter succinogens* have an optimal level of adhesion to cellulose between pH 6.0 and 7.0 (Miron et al., 2001). A rumen pH of 5.7 was associated with reduced cellulolytic bacterial attachment to fibre substrates in an *in-vitro* study carried out by Sung et al. (2007). The negative effect of lactic acid production, from lactic acid producing bacteria, on rumen pH is twofold as only these lactic acid producing bacteria can survive the more acidic environment and lactic acid becomes the main substrate produced (Mills et al., 2014).

2.2.7 The effect of lipids on rumen microflora

Lipids can be defined as organic compounds that are insoluble in water but soluble in organic solvents and are classified as either glycerol-based or non-glycerol-based. Non-glycerol-based lipids provide an indigestible, impervious barrier to the exterior of plant surfaces and include waxes and cutins while glycerol-based lipids contain fatty acids or, in biochemistry terms, chains of carbons that end in an acid or carboxyl group (Jenkins and Harvatine, 2014). There is no significant evidence to suggest that long chain fatty acids that are hydrolysed in the rumen can be absorbed from or digested in the rumen, the primary site of lipid absorption in the ruminant animal is the small intestine (Annison and Bryden, 1998). However, the structure of

both lipids and fatty acids can be changed in the rumen. Transformation of lipids entering the rumen by the rumen microbial population involves two major processes, lipolysis and biohydrogenation. Lipolysis is the hydrolyzation of ester linkages in complex lipids by microbial lipases causing the release of free fatty acids into the rumen, biohydrogenation then converts unsaturated fatty acids to saturated fatty acids via isomerisation of *trans* fatty acid intermediates, followed by the hydrogenation of the double bonds (Jenkins et al., 2008). The evolutionary function of biohydrogenation, as a microbial pathway in the rumen, is not fully understood but it does serve as a protection mechanism for microbes against the toxic effects of unsaturated fatty acids (Jenkins, 1993). The most active rumen microorganism involved in lipolysis is the bacterial species *Anaerovibrio lipolytica*, while some of the *Butyrivibrio*-like bacterial species are also involved but are less active (Lourenço et al., 2010). Rumen bacteria also play a primary role in fatty acid biohydrogenation and the *Butyrivibrio* species is thought to be of principal importance in the overall process (Jenkins et al., 2008). While lipolysis is completed almost entirely by rumen bacteria (Annison and Bryden, 1998), biohydrogenation in the rumen is achieved by both the microbial bacteria and microbial protozoa with the bacterial species being the most important (Lourenço et al., 2010).

Within the biohydrogenation process, there exists a number of steps, outlined in Figure 1.1. Firstly, an isomerization reaction converting the cis-12 double bond in unsaturated fatty acids to a trans-11 isomer takes place. Once the trans-11 bond is formed, then hydrogenation of the cis-9 bonds in $C_{18:2}$ occurs by microbial reductase and finally the trans-11 $C_{18:1}$ is hydrogenated to $C_{18:0}$ by microbial reductase (Jenkins, 1993). An altered microbial population may cause a shift in the intermediates produced from the biohydrogenation process, as outlined in Figure 1.2, leading to milk fat depression (MFD) in dairy cows (Jenkins and Harvatine, 2014). The cause of this shift in production of biohydrogenation intermediates has been investigated by a number of studies over the last decade. Fuentes et al. (2009) investigated the effect of reducing the pH from 6.4 to 5.6 compared to increasing the level of concentrate in the diet from 30 to 70% on the production of the biohydrogenation intermediates, *trans-10, cis-12* CLA, and found that the reduced pH had a much greater effect on the production of these intermediates compared to increasing the level of concentrate in the diet. Van Nevel and Demeyer (1996) discovered that when the pH dropped below 6.0 both lipolysis and biohydrogenation were greatly reduced and concluded by stating that lipolysis was much more sensitive to low pH than biohydrogenation.

In summary, lipid metabolism in the rumen is a complex process involving lipolysis and biohydrogenation. The rumen microbiome contains particular species that have an essential role in both the production of lipase for lipolysis and the hydrogenation of fatty acids in biohydrogenation. The effect of an altered rumen environment by means of a reduced pH can reduce the efficacy of both these processes.

2.2.8 Protein digestion and microbial protein synthesis

Microbial protein is created in the rumen following the breakdown of feed protein to amino acids and ammonia (Tamminga, 1979). The primary proteolytic microorganisms involved in rumen protein digestion are *Prevotella ruminicola*, *Peptostreptococci* species and the protozoa (McDonald et al., 2011). The microbial protein synthesised in the rumen is then absorbed, subsequently supplying amino acids for milk production as well as the growth and repair of tissues. An increase in rumen available energy can result in a greater microbial protein synthesis and subsequent amino acid supply to the rumen, which may improve milk protein secretion in lactating dairy cows.

Microbial protein synthesis is also sensitive to changes in rumen pH. It has been demonstrated that the efficiency of microbial protein synthesis decreases as rumen pH is reduced from pH 7.0 to 6.0 (Strobel and Russell, 1986). According to Sinclair et al. (1995), the consequence of lower rumen pH on microbial protein synthesis is due to an increased energy reliant discharge of protons at lower pH and a diversion of energy to non-growth activities. Hoover and Stokes (1991) also reported work to demonstrate the importance of rumen pH for optimum microbial protein synthesis and demonstrated that a drop in rumen pH from pH 6.5 to pH 5.5 reduced the effectiveness of microbial protein synthesis.

2.3 Microbial Fermentation Products

2.3.1 Volatile fatty acid production

Volatile fatty acids (VFA), primarily acetic, propionic and butyric acids and to a lesser extent isobutyric, valeric and isovaleric acid (Aluwong et al., 2013), are end products of microbial fermentation (Nagaraja et al., 1997). Acetic, propionic and butyric acids account for

approximately 95% of the total VFA in rumen fluid (Bannink et al., 2006). These VFA, formed in the rumen, are the primary energy source for the ruminant animal (Penner, 2019). Concentrations of different VFA, in the rumen, are measured in an effort to illustrate the general nature of rumen fermentation (Seymour et al., 2005). It has been understood that the molar proportions of the concentrations of VFA can change in response to a large variety of dietary manipulations (Sutton et al., 2003). According to Bramley et al. (2008), optimum levels of total VFA concentration should be greater than 95 mmol per litre of rumen fluid.

The utilisation of absorbed nutrients in dairy cow metabolism can be significantly affected by both the total yield of VFA and the type of VFA formed (Dijkstra et al., 1993). VFA production in the rumen is dependent on the type of substrates consumed by the ruminant animal and their relative utilisation rates (Whelan et al., 2013). Microbial species present in the rumen will have a major influence on the type of VFA produced due to the fact that many rumen microbes are substrate specific and have characteristic metabolic pathways (Bannink et al., 2008). For example, most amylolytic bacteria will ferment starch producing propionic acid while cellulolytic bacteria will ferment cellulose to produce acetic acid (Stewart and Bryant, 1997). When Sutton et al. (2003) investigated the effects of feeding a normal diet (7.8kg DM/d concentrates and 5.1kg DM/d hay) compared to a low roughage diet (11.5kg DM/d concentrates and 1.2kg DM/d hay), they found that the net production of total VFA increased from 89.5 MJ/d (total VFA concentration of 86.6 mmol/litre) to 109.1 MJ/d (total VFA concentration of 92.8 mmol/litre). This increase can be attributed to a significant increase in the net production of propionic acid, from 25.8 MJ/d (16.4 mmol/litre) to 55.6 MJ/d (34.3 mmol/litre) for the normal and low roughage diet respectively, while the net production of acetic acid and butyric acid remained the same. One of the primary functions of acetic and butyric acid is to act as precursors for long-chain fatty acid synthesis in the mammary glands while propionic acid is a substrate for gluconeogenesis in the liver and the main source of glucose in the animal (Bannink et al., 2006). Therefore, different VFA may influence the partitioning of energy into milk or body tissues (Morvay et al., 2011). A classical study examining isocaloric infusion of acetic acid compared to propionic acid found that acetic acid infusion resulted in more energy secreted as milk while propionic acid infusion favoured more energy deposited in body tissue (Ørskov et al., 1969).

The removal of VFA from the rumen is due to passage of the rumen fluid to the omasum, or by absorption through the rumen wall (Dijkstra et al., 1993). The rate at which VFA are cleared

or absorbed from the rumen depends on a number of factors, including VFA concentration, rumen pH, volume of rumen fluid and fluid outflow rates (Bannink et al., 2008). The rate of absorption of VFA from the rumen also depends on papillae surface area (Dieho et al., 2017).

Volatile fatty acid production in the rumen influences rumen pH (Humer et al., 2018). The ratios of VFA produced also influence the rumen pH with a higher acetate: propionate ratio associated with a greater rumen pH (Bramley et al., 2008). Kolver and de Veth (2002) also documented a negative relationship between total VFA concentration and rumen pH while acetic acid had a positive relationship with rumen pH. Subsequently, rumen pH can affect the rate of VFA absorption through the rumen wall with a low rumen pH having a stimulatory effect on total VFA absorption (Gäbel et al., 2002). Studies have shown that the fractional absorption rate of propionic and butyric acid increased from 0.35/h to 0.65/h and 0.28/h to 0.85/h as rumen pH decreased from 7.2 to 4.5, whereas the fractional absorption rate of acetic acid remained unchanged as pH declined (Allen, 1997).

The relationship between VFA production and milk production parameters in lactating dairy cows was investigated by Seymour et al. (2005) who revealed that the concentration of butyric and propionic acid in rumen fluid were most highly related to milk yield whereas the concentration of acetic acid or total VFA in rumen fluid had little or no relationship with milk yield. Sutton et al. (2003) discovered that changing from a normal diet to a low roughage diet induced MFD and the only observed change in VFA profile was a significant increase in propionic acid production.

To conclude, the type of VFA produced is substrate dependant, influenced by changes in the rumen microflora, and are the main source of energy for the lactating dairy cow. Individual VFA, e.g., acetic, propionic and butyric acid, have been reported to influence energy partitioning in the dairy cow and can affect both milk production and milk composition. The total VFA concentration in the rumen may have a negative relationship with rumen pH and both VFA clearance and absorption are essential to maintain rumen pH within a desirable range.

2.3.2 Methane production

Methanogens are present in the rumen in large quantities fluctuating from 10^7 to 10^9 cells/ml of rumen fluid depending on the type of diet fed (Kamra, 2005). Methanogens are a specialised

group of secondary fermenting anaerobic microbes, belonging to the domain archaea, who utilise primarily hydrogen (H_2) and carbon dioxide (CO_2) as substrates to create methane from the process of methanogenesis (Stefenoni et al., 2021). Methane production is closely related to VFA production in the rumen, for example the main source of H_2 comes from the fermentation of plant cell wall carbohydrates into acetate and butyrate whereas the fermentation of starch and sugars into propionate does not result in the production of H_2 (Aguerre et al., 2011). Methanogens play a key role in promoting a continuous rumen fermentation process by scavenging the rumen for molecular H_2 (Kamra, 2005). However, there is also a negative aspect to the production of CH_4 in the rumen due to the fact that CH_4 production, from ruminant livestock production systems, is considered a major contributor to greenhouse gas emissions in many countries worldwide (Stefenoni et al., 2021). A low rumen pH reduces the ability of methanogens to consume H_2 leading to an increase in free H_2 during the gas phase of rumen fermentation leading to reduced methanogenesis and a lower production of methane (Moss et al., 2000).

Ruminant diet manipulation can change the rate of methane production from the rumen. Aguerre et al. (2011) investigated the effect of forage: concentrate (F:C) ratio on methane production and found that increasing the F:C ratio from 47:53 to 68:32 increased CH_4 production by 110 g per cow/d and by 3.6 g/kg of ECM produced. This can be explained by the fact that starch digesting bacteria have a competitive relationship with methanogens by producing large amounts of propionate, and also consume H_2, until the pH drops under 5.3 and propionate production is drastically reduced and H_2 begins to accumulate in the rumen (Moss et al., 2000). The role of specific strains of rumen protozoa in methane production was discussed by Morgavi et al. (2010) where the effects of defaunation, summarised from different datasets, showed a reduction in methane production of 10.5%. However, individual studies within the dataset yielded quite variable results illustrating the inconsistent role that defaunation may play in reducing methane production.

2.4 Rumen pH

2.4.1 Regulation of rumen pH

Rumen pH is regulated by the equilibrium between acid production created from fermentation of carbohydrates and the removal, absorption or neutralisation of these acids (Humer et al., 2018). The rumen pH of a dairy cow is extremely responsive to meals and chewing behaviour. Rumen pH decreases following meals and increases during bouts of rumination which induces saliva production (Allen, 1997). Cows produce a large amount of buffers in saliva (Van Soest, 1994). Considerable variation in rumen pH is experienced during the day and it is not uncommon to achieve values below pH 6.0 when cows consume large amounts of concentrate (Cerrato-Sánchez et al., 2007). According to Krause and Oetzel (2006), shifts in pH units of 0.5 – 1.0 during a 24 h period is common, this equates to a 5-to-10-fold change in the ion concentration of rumen fluid. Figure 1.4 illustrates a typical pattern of diurnal rumen pH variation. The decline in rumen pH is more rapid following a meal, as meal size increases and as dietary NDF concentration decreases (Allen, 1997). It is estimated that the nadir rumen pH occurs 6 – 8 h post feeding in total mixed ration (TMR) fed cows and 2 – 4 h post feeding in component fed cows, this nadir rumen pH value is more responsive to diet composition than the daily mean rumen pH (Krause and Oetzel, 2006).

2.4.2 Physically effective fibre

The concept of physically effective fibre or physically effective neutral detergent fibre (peNDF) was developed by Mertens (1997) and refers to the physical characteristics of fibre (primarily particle size) that are responsible for chewing activity and the biphasic nature of rumen contents (mat of large particles floating on a pool of fluid and minor particles). Physically effective fibre is the portion of feed that stimulates chewing activity and rumination, and subsequent saliva production (Allen, 1997). Mertens (1997) also proposed effective neutral detergent fibre (eNDF) as a measurement of fibre in ruminant diets. Effective NDF is the ability of a feed to substitute forage or roughage in a dairy cow diet so that milk fat concentration is successfully maintained (Mertens, 1997). Correct formulation of dairy cow diets should consider the balance between the acid produced from fermentation in the rumen and adequate saliva production from peNDF induced chewing (Khorrami et al., 2021). The proportion of OM fermented in the rumen can vary considerably with diet which subsequently effects the

quantity of fermentation acids formed and the requirement for peNDF (Allen, 1997). A consequence of inadequate dietary peNDF is reduced chewing activity, leading to less salivary buffer secretion, which in turn leads to lower rumen pH and a subsequently altered rumen fermentation process and microflora population (Zebeli et al., 2012). This altered rumen fermentation with lower pH may induce low ratios of acetate to propionate in the rumen VFA (Mertens, 1997).

The peNDF content of a diet can be calculated by multiplying the NDF content of a diet by its physical effectiveness factor (pef). Lammers et al. (1996) developed the Penn State Particle Separator (PSPS) to provide a practical, on-farm, technique for calculating the physical effectiveness of fibre in TMR by determining the variation in particle size of a particular ration. Particle distribution can be established by using the PSPS to determine three fractions: proportion of particles retained on the 19.0-mm sieve, proportion of particles that pass through the 19.0-mm sieve but are retained on the 8.0-mm sieve, and proportion of particles that pass through the 8.0-mm sieve (Lammers et al., 1996). Yang and Beauchemin (2005) agreed that particle size could be used to determine peNDF.

It has been shown that a peNDF value of 22% of total diet DM was required to maintain an average rumen pH of 6.0 and a peNDF value of 20% of total diet DM was needed to maintain milk fat concentration at 3.4%, for early to mid-lactation Holstein cows (Mertens, 1997). Increased chewing time, as a result of more peNDF in the diet, may improve pH and subsequent fibre digestibility in low forage diets (Yang and Beauchemin, 2005). Accurate ration formulation for dairy cows can be achieved by adjusting NDF for effectiveness. Physically effective NDF can be used to determine the lower limit of forage: concentrate (F:C) ratio in the ration (Mertens, 1997). Therefore, peNDF can be used as a parameter in dairy cow diet formulation to ensure sufficient saliva production while maintaining adequate energy levels.

Yang and Beauchemin (2005) observed a positive effect on the digestibility of DM, OM and NDF of 2.6%, 2.5% and 9.3% respectively, with a high peNDF maize silage diet compared to the exact same diet with a low peNDF content. However, there was no effect on milk production or DMI in that same trial. A separate study that looked at the relationship between the level of peNDF in the diet and the effects it had on chewing activity, rumination and rate of passage found that peNDF was responsible for increased chewing time. However, there was no significant effect of treatment on rumen pH or rate of passage and the lowest level of peNDF

had the highest total concentration of VFA's (Beauchemin and Yang, 2005). The lack of significant effect of peNDF on pH in this trial was unusual and is in disagreement with most studies in this area (Allen, 1997, Mertens, 1997). Beauchemin and Yang (2005) stated that peNDF alone may not be accurate in predicting rumen pH and other parameters like DMI and fermentability of feeds may need to be taken into account.

The basal forages used may also be of high significance when considering optimal peNDF. Yang and Beauchemin (2006) examined different levels of peNDF using barley silage-based diets and found that increasing levels of peNDF decreased total tract fibre digestibility and reduced microbial efficiency in the rumen. This was due to decreased intestinal digestion rather than reduced rumen digestion according to the authors. Predicting rumen pH based on diet nutrient composition, using variables such as eNDF or peNDF is somewhat reliable with TMR. Using eNDF or peNDF to predict rumen pH in ryegrass-based diets is much less accurate compared to TMR because the measurement of eNDF or peNDF based on the particle size method is not applicable to ryegrass (Kolver and de Veth, 2002). However, Kolver et al. (1998) assigned an eNDF of between 40 to 50% for ryegrass pasture, depending on the ryegrass quality, based on its effects on rumen pH.

2.4.3 Feeding behaviour and rumen pH

Feeding behaviour has a big influence on rumen pH, rumen fermentation and digestion (DeVries, 2017). Cows typically divide their feeding time into a sequence of meals separated by nonfeeding intervals (DeVries et al., 2003). Consuming smaller and more frequent meals helps cows to avoid sharp drops in rumen pH (Owens et al., 1998). Penner (2009) demonstrated that fasting followed by gorging of feed will induce periods of rumen pH depression. In addition to the intrinsic buffering provided by the consumption of the feed particles, subsequent rumination of that feed will have an even bigger impact on the rumen pH (DeVries et al., 2009). Therefore, some researchers have proposed rumination behaviour or time spent ruminating as an important indicator of rumen pH and rumen health (Krause and Oetzel, 2006). DeVries et al. (2009) found that cows at high-risk of prolonged rumen pH depressions had similar feeding and lying behaviour to cows that were at low-risk of prolonged rumen pH depressions, but rumination behaviour of the high-risk cows was lower compared to the low-risk cows. The sorting of feed is another key element of feeding behaviour that effects rumen pH. This leads to cows consuming diets that are different to the formulated diet, due to cows selecting against

larger feed particles in favour of smaller feed particles and causes a reduction in rumen pH (Plaizier et al., 2014). Both higher forage and high DM diets are sorted to a greater degree (DeVries et al., 2009).

2.4.4 Consequences of low rumen pH

2.4.4.1 Fibre digestion

Low rumen pH can have a negative effect on fibre digestion due to the inhibition of cellulolysis at low pH values (Mould et al., 1983). Both *in-vitro* and *in-vivo* experiments have reported that cellulose digestion can be severely impeded by even moderate depressions in rumen pH (Russell and Wilson, 1996). According to Plaizier et al. (2008), the 24 and 48 hour *in-situ* NDF degradability of forages was reduced by 20.5% and 24.8%, respectively, when incubated in the rumen of lactating dairy cows fed a pH reducing, acidotic diet. A separate study, directed by Cerrato-Sánchez et al. (2007), reported that the negative effects of low rumen pH on the rumen fermentation of fibre was initiated when the pH declined to suboptimal values (< 5.5). According to Mulligan et al. (2002), total tract digestibility of NDF and organic matter (OM) in cattle were positively related to rumen pH for diets based on grass silage and soya hulls. This provides further evidence to validate the importance of rumen pH for optimum fibre digestibility in dairy cows.

2.4.4.2 Milk yield

Krause and Oetzel (2005) described how milk yield was reduced from 35.3kg/d to 31.7kg/d during an episode of low rumen pH (increase in hour below pH < 5.6 of 7.16 h/d and reduction in mean rumen pH of 0.46) and despite the pH recovering after a period of time, cows did not return to base milk yield (>35kg/d). Stone (1999) established that milk production in cows suffering from prolonged episodes of low rumen pH was decreased by 2.7kg/d during a clinical study carried out on a large-scale dairy farm in the U.S. However, studies investigating low rumen pH in grazing cows did not report any negatives effects on milk yield (Bramley et al., 2008, O'Grady et al., 2008). Thus, there is a lack of evidence that milk yield is a certain consequence of low rumen pH in lactating dairy cows.

2.4.4.3 Milk fat depression

Milk fat depression is one of the symptoms that is regularly associated with low rumen pH in dairy cows (Plaizier et al., 2014). There are numerous views as to whether MFD is a significant consequence of low rumen pH or not. For example, Kleen et al. (2003) suggests that cows may start to experience episodes of low rumen pH in similar situations to where MFD is likely to occur, rather than to interpret MFD as a consequence of low rumen pH itself. There are many other important factors that can affect milk fat composition in lactating dairy cows, e.g., breed, season and stage of lactation (Oetzel, 2007). Enemark (2009) concluded that using milk fat composition, in early lactation cows, as an indicator of low rumen pH was inaccurate due to the poor relationship between rumen pH and milk fat concentration in early lactation cows. Milk fat is the component of milk solids that can be most readily influenced by dietary changes (Stockdale et al., 2003). A number of studies have shown MFD as a sequel to an alteration in the ration (Kleen et al., 2003). There have been numerous attempts to explain the link between rumen pH and milk fat synthesis and subsequent effects on MFD. The importance of fibre, and more importantly physically effective fibre, for rumen function in the dairy cow has been examined to look at its role in MFD. Enjalbert et al. (2008) found that mean rumen pH was closely related to milk fat content. Allen (1997) examined the effect of NDF on chewing time, in dairy cows, and its subsequent effect on saliva production. This study found that milk fat concentration and rumen pH were positively related, illustrated in Figure 1.3. In a study that examined the effects of sodium bicarbonate in dairy cow diets, an increase in milk fat concentration with sodium bicarbonate corresponded to improved rumen fermentation and increased rumen pH (Hu and Murphy, 2005).

Rumen pH and its effect on rumen fermentation, most notably acetic, butyric and propionic acid production, has often been associated with MFD. Hu and Murphy (2005) documented a positive relationship between milk fat concentration, the molar quantity of acetic acid produced and the acetate: propionate ratio. A survey on the prevalence and effects of low rumen pH in Australian grazing dairy herds categorized their results in three different groups of cows based on their mean rumen pH data. Cows in group one, which had the lowest mean rumen pH, had lower milk fat concentration. Also, the significantly lower concentrations of acetic and butyric acid in group one cows were notable in this research (Bramley et al., 2008). Plaizier et al. (2008) stated that reduced acetate: propionate ratio has often been linked to MFD, but also

acknowledging that there are other theories behind MFD, which include increased insulin and the production of trans-octadecenoic acids in the rumen.

Bauman and Griinari (2003) outlined three different theories behind MFD. The first theory was that a decrease in acetic and butyric acid was responsible as acetic acid was a precursor for de novo milk fat synthesis. A large bank of evidence supports this theory due to the fact that a change in VFA profile was commonly associated with reduced milk fat production across different diets. However, when cows experiencing MFD were supplemented with abomasal infusions of acetic acid, there were mixed results in terms of a milk fat response. The second theory proposed by Bauman and Griinari (2003) was the 'Glucogenic-Insulin Theory' which examined the effect of increased insulin and its positive effect on milk fat production. In the mammary gland, insulin is required for the maintenance of normal mammary cell function and similar to the previous theory, this was also disproved due to the varying results observed from infusions of insulin (Bauman and Griinari, 2003). The third theory of MFD proposed by Bauman and Griinari (2003) was the 'Rumen Biohydrogenation Theory' which the authors claimed to be the most likely cause of MFD. This theory is based on the concept that certain dietary conditions alter the pathways of rumen biohydrogenation to produce unique fatty acid intermediates, some of which are effective inhibitors of milk fat synthesis. Baumgard et al. (2000) identified *trans*-10 *cis*-12 conjugated linoleic acid (CLA) as the main inhibitor of milk fatty synthesis, effecting de novo milk fat synthesis, produced from an altered rumen biohydrogenation process. The same study showed that the CLA isomer *trans*-10 *cis*-12 caused a 42% and 44% reduction in milk fat concentration and overall milk fat yield respectively. Colman et al. (2010) was also in agreement with Baumgard et al. (2000) stating that low rumen pH affects the rumen environment, changing both the nature of the rumen microbial population and the biohydrogenation process. However, the same study found *cis*-9 *trans*-11 C18:2 to be the most effective predictor of low rumen pH and not *trans*-10 *cis*-12 C18:2 which was previously the only biohydrogenation intermediates to be associated with low rumen pH.

To conclude this section on MFD, it is evident that there is a strong link between low rumen pH and MFD. Bauman and Griinari (2003) proposed three very plausible theories that provide explanation to MFD in lactating dairy cows. However, the 'Rumen Biohydrogenation' theory remains the most convincing and most likely cause of MFD in modern dairy production systems. There still remains some questions over the exact mode of action behind the

interaction between low rumen pH and MFD and there also exists various views as to the most potent CLA isomers at depressing milk fat synthesis.

2.4.4.4 Milk protein

Milk protein content does not vary greatly and shows little response to protein levels in supplements (Bargo et al., 2003). With high levels of protein supplementation, the milk protein content did not vary whereas the milk production showed a 6 – 18 % increase (Bargo et al., 2003). Microbial protein synthesis is optimised when a carbohydrate source is supplied to the rumen microbes (Bargo et al., 2002). Experimentally induced low rumen pH can increase milk protein production. This is supported by the observation of Rafferty et al. (2019) who found increased milk protein concentration was associated with greater time at low reticulo-rumen pH in grazing cows. It is not fully understood why this happens, but one theory is that increased microbial protein synthesis in the rumen, due to an increase in rumen digestible organic matter, is responsible for the rise in protein production (Plaizier et al., 2008).

2.4.4.5 Dry matter intake

High producing lactating dairy cows require an adequate DMI to support the caloric demands of their daily lactation and maintenance requirements. Reduced DMI has been associated with low rumen pH (Kleen et al., 2003). There are many explanations used to describe the causative factors behind a decreased DMI during episodes of low rumen pH. Enemark (2009) suggested that an increase in rumen osmolality, associated with a disrupted feeding pattern during an episode of low rumen pH, may be linked to decreased DMI. Lower DMI has also been linked to reduced rumen motility triggered by certain mechanisms, like increased production of fermentation acids during periods of low rumen pH (Kleen et al., 2003). Krajcarski-Hunt et al. (2002) induced an episode of low rumen pH by replacing 25% of the TMR with cereal-based pellets and found that DMI of TMR dropped when animals were introduced onto the rumen pH reducing diet. Furthermore, Krause and Oetzel (2006) believe that inflammation of the rumen epithelium after an episode of low rumen pH may play a role in the depression of feed intake. However, an experiment conducted by Krause and Oetzel (2005), where low rumen pH was induced to investigate its effect on production by feeding 3.5 or 4.6 kg of a wheat-barley pellet, discovered that DMI was not affected by a lower rumen pH.

In summary, extensive periods of low rumen pH may lead to reduced DMI in dairy cows in certain situations. Some of the theories explaining the causative factor behind reduced DMI in an animal experiencing prolonged periods of low rumen pH include increased rumen osmolality, reduced rumen motility and inflammation of the rumen epithelium.

2.4.4.6 Laminitis and claw horn disruption lesions (CHDLs)

There are conflicting views on the association of laminitis and rumen pH. Early literature used the term 'laminitis' with an implied nutritional aetiology based on equine laminitis to describe the presence and diagnosis of CHDLs (Randall et al., 2018). Laminitis refers to inflammation of the hoof laminae (Danscher et al., 2009). Many studies, in the past, have associated laminitis and CHDLs with low rumen pH and rumen acidosis (Kleen et al., 2003). The translocation of Lipopolysaccharide from the rumen to the blood stream during periods of low rumen pH has been shown to induce systemic inflammation (Khafipour et al., 2009a). This systemic inflammation is thought to induce inflammation at the hoof laminae (Plaizier et al., 2014).
Danscher et al. (2009) found that non-pregnant diary heifers, with acute ruminal acidosis (pH < 5.5) presented signs of lameness, claw pain, and joint diffusion, collectively interpreted as acute laminitis after an oligofructose overload. However, the symptoms of acute lamintis dissappeared relatively quickly when animal returned onto a normal diet. Donovan et al. (2004) reported higher hoof scores at 50 – 70 days in milk (DIM), indicative of poor hoof quality, for a group of cows that showed a higher prevalence of low rumen pH following an abrupt dietary change in early lactation but failed to detect a significant relationship between low rumen pH postpartum and high hoof scores on an individual cow basis. Bramley et al. (2005) suggested that pasture-based cows with a low rumen pH had a higher risk of developing laminitis than cows with a rumen pH within a normal physiological range. Furthermore, research from New Zealand proposed that dairy cows in grazing herds are susceptible to CHDLs, possibly due to sub-optimal rumen function from grazing very digestible pastures (Westwood et al., 2003).

Despite the many studies investigating the links between low rumen pH and laminitis, there is no conclusive evidence available within the literature to support a causal relationship between low rumen pH and CHDLs or inflammation of the laminae as a precursor to CHDLs (Randall et al., 2018). Therefore, further research in this area is required to decisively define if lowered rumen pH is causal to inflammation of the laminae and a precursor to CHDLs.

2.4.4.7 Rumen bacteria

Bacterial density in the rumen solids can be significantly lower when the ruminant animal has a low rumen pH on a high-grain diet compared to when fed a hay-based diet with a normal rumen pH (Hook et al., 2011). The diversity of bacteria present also differed significantly between animals consuming a high-grain diet with a low rumen pH, and animals fed a hay-based diet with a normal rumen pH (Hook et al., 2011). Khafipour et al. (2009) found that the main shift during an episode of low rumen pH was a reduction in gram-negative *Bacteroides*. That same study also discovered that rumen fluid taken from animals with a low rumen pH ingesting a high grain diet, analysed on real time PCR, was dominated by *Streptococcus bovis* and *Escherichia coli* (*E. coli*) as opposed to *Megasphaera elsdenii* and *Prevotella albenis* in the rumen fluid of the animals with a normal rumen pH (> 5.5) consuming a low-grain diet or a diet based on alfalfa pellets. The level to which the rumen pH dropped and the extent to which the rumen wall was inflamed were highly correlated with the abundance of *E. coli* (Khafipour et al., 2009). Fernando et al. (2010) found that populations of fibre digesting bacteria, *Butyrivibrio fibrisolvens* and *Fibrobacter succinogenes* were gradually reduced as the animals adapted to a high grain rumen pH reducing diet.

2.4.4.8 Liver abscesses

Low rumen pH has been linked to liver abscesses caused by translocation of rumen bacteria, such as *Fusobacterium necrophorum* and *Arcanobacterium pyogenes,* into the bloodstream as a result of reduced barrier function of the rumen epithelial mucosa (Plaizier et al., 2008). Rumen parakeratosis or rumenitis is the erosion and ulceration of the rumen epithelium leading to inflammation and increased risk of bacterial colonisation (Oetzel, 2007). According to Steele et al. (2011), rumen parakeratosis is the result of feeding a high grain diet causing extended periods of low rumen pH, which leads to the increased permeability and reduced thickness of the rumen epithelium. Liver abscesses can be detrimental to the health of the animal, possibly leading to peritonitis around the site of the abscess (Krause and Oetzel, 2006) and can also lead to the spread of bacteria from the liver to other organs like the lungs, heart and kidneys (Kleen et al., 2003).

2.4.4.9 Loose faecal consistency

It has been stated that faecal appearance can provide indirect evidence of low rumen pH in dairy cows (Bramley et al., 2005). Studies have shown that cows consuming low fibre diets, causing a low rumen pH, produced faeces that appeared more liquid but actually contained more DM (Plaizier et al., 2008). Enemark (2008) suggested that a lack of rumen fibre mat in low fibre diets leads to larger undigested fibre particles deposited in the faeces. Faeces from cows with a low rumen pH may appear brighter or more yellowish than unaffected cows and contain small bubbles giving the faeces a 'foamy' appearance (Plaizier et al., 2008). In a farm study on the prevalence of low rumen pH in Irish grazing dairy herds, O'Grady et al. (2008) discovered that cows in the low pH group (<5.8) had significantly lower faecal consistency than cows in the normal pH group (>5.8). Kleen et al. (2003) proposed two theories in an attempt to explain the relationship between impaired rumen function and altered faecal colour and consistency: 1) a large outflow of fermentable carbohydrates into the large intestine from the rumen inducing post-rumen fermentation; 2) increased osmolarity resulting in the binding of fluid to the faeces in the intestine.

Therefore, consistency faecal scoring may be useful in predicting low rumen pH at herd level, but results should be interpreted with caution as there may be other factors to be mindful of like high parasite burden, salmonellosis and other bacterial or viral diseases (Bramley et al., 2005). However, it is worth noting that loose faeces may also be caused by other factors, such as diets based on fresh ryegrass pasture or containing too much rapidly degradable protein (Ireland-Perry and Stallings, 1993).

2.4.5 Sub-Acute Ruminal Acidosis (SARA)

The occurrence of prolonged episodes of low rumen pH to non-physiological levels is referred to as Sub-Acute Ruminal Acidosis (SARA) (Kleen and Cannizzo, 2012). Sub-Acute Ruminal Acidosis (SARA) is a digestive disorder caused by the ingestion of large quantities of highly fermentable carbohydrates in combination with a lack of physically effective fibre. Digestion of such feeds in the rumen leads to a build-up of VFA causing a depression in rumen pH (Humer et al., 2018). Inadequate levels of physically effective fibre means that the amount of saliva produced is incapable of buffering the rumen fluid and contents within a physiological range (Khorrami et al., 2021).

2.4.5.1 Definitions of SARA

It is generally, but not universally, accepted that SARA is the occurrence of low rumen pH for a certain period of time. The exact definition of the pH thresholds applying will vary depending on the sampling procedure and the location in the rumen from where the fluid is sampled. (Garrett et al., 1999). For field studies collecting rumen fluid through rumenocentesis, Garrett et al. (1999) proposed a pH threshold of 5.5 and Duffield et al. (2004) recommended pH thresholds of 5.5, 5.8 and 5.9 for the diagnosis of SARA when rumen fluid samples collected are by rumenocentesis, through a rumen cannula from the ventral sac and using a stomach tube, respectively. The timing of rumen fluid sampling also has a large bearing on the pH of the sample taken, as rumen pH varies considerably throughout the day (Keunen et al., 2002). Therefore, interpretation of rumen pH values needs to be based on a standardised rumen fluid collection time and the threshold for SARA needs to reflect the sampling time. Gozho et al. (2005) used a threshold rumen pH depression of < 5.6 for at least 3 hours per day (h/ d), validated by the occurrence of inflammation on the days that rumen pH was below the proposed threshold. According to AlZahal et al. (2007), SARA could be defined as a daily reduction of rumen pH for 148 to 283 min/d and 284 to 475 min/d under 5.6 and 5.8, respectively. Al Ibrahim et al. (2012) reported that the most pronounced rumen pH depressions took place 2 – 3 h post feeding in early lactation pasture-fed cows.

There are other authors who believe SARA is more than just a pH related problem. According to Bramley et al. (2008), valerate concentrations in rumen fluid can be used to indicate cows at risk of SARA, due to the higher concentrations of valerate observed in the rumen fluid of cows that were at higher risk of developing SARA and were within the lowest pH category of cows examined. Valerate is a product of *Megasphaera elsdenii*, the main lactate utilizing bacteria in the rumen, and the presence of valerate in rumen fluid is indicative of lactate concentration (Bramley et al., 2008). Calsamiglia et al. (2012) suggested that SARA is not only a pH-dependent pathology, but it is also the result of changes in the microbial population secondary to the type of diet fed and proposed to re-name SARA as a “high-concentrate syndrome”.

2.4.5.2 Different types of SARA

Traditional assumptions of SARA have focused on high grain or high starch diets. The cause of SARA involves at least one or a combination of the following factors: fermentation acid generation, natural buffering through salivation, and absorption of fermentation acids (Allen, 1997). Therefore, SARA can also be induced in diets containing low levels of starch through particle size reduction or a deficiency in physically effective fibre (Beauchemin et al., 2003). Khafipour et al. (2009b) experimentally induced an alfalfa-based SARA challenge, by replacing alfalfa hay with alfalfa pellets, and effectively reduced mean rumen pH and increased time below pH 5.6. Over the last 20 years, the susceptibility of pasture-based dairy cows to SARA has been highlighted, despite consuming low to moderate levels of starch, and a forage that is not mechanically altered (e.g., pelleting) to reduce particle size (Bramley et al., 2008; O'Grady et al., 2008). From a nutrient composition perspective, pasture-based diets meet all the minimal requirements for optimum rumen function and prevention of SARA with more than adequate levels of total NDF and NDF from forage. However, Kolver et al. (1998) assigned an eNDF factor of between 40 to 50% for NDF in ryegrass pasture, depending on the ryegrass quality. Based on this methodology, the eNDF of common pasture-based diets could be as low as 16% and indicative of SARA.

2.4.5.3 Prevalence of SARA

SARA is considered as one of the most common digestive disorders effecting the modern dairy cow at herd level. The prevalence reported varies between different studies undertaken in various parts of the world. Most studies reporting the prevalence of SARA at herd level use the rumenocentesis procedure to measure rumen pH. A study carried out on 15 dairy farms in Wisconsin found that 19% of early lactation cows and 26% of mid-lactation cows had SARA (Garrett et al., 1997). A separate study in the same region indicated that 20.1% of both early and peak lactation cows were suffering from SARA (Oetzel et al., 1999). An Italian study that monitored cows between 5-60 DIM across 10 herds revealed 33% of cows were diagnosed with SARA (Morgante et al., 2007). Kleen et al. (2009) determined the prevalence of SARA to be 13.8% across 197 dairy cows, at various stages of lactation, from 18 herds in The Netherlands. Kleen et al. (2013) examined 315 dairy cows from 26 herds in Germany and diagnosed 20% of the cows as being in a state of SARA. Sub-acute rumen acidosis can also be prevalent in pasture based grazing dairy herds. In Ireland, O'Grady et al. (2008) analysed rumen fluid from 144 different cows across 12 herds and classified 11% of the cows as having

SARA. Another grazing study which was carried out in Australia, showed that 10% of cows examined had an acidotic profile, categorised by a rumen pH of 5.74, and were said to be highly at risk of developing SARA (Bramley et al., 2008). Despite the fact that prevalence levels vary between different studies, it can be agreed that SARA is a common production disease prevailing across most modern dairy cow herds throughout the world.

2.4.5.4 Economic consequences of SARA

A thorough understanding of how SARA effects dairy cows is important to both farmers and the dairy industry from an animal health and an economic perspective. The economic consequences of SARA are due to reduced efficiency of milk production, premature culling, and increased mortality (Enemark, 2008). Only few studies exist that attempt to quantify the actual economic loss due to SARA despite the large body of research that documents the disease in general. Sub-acute ruminal acidosis is estimated to cost the U.S.A dairy industry between US$ 500 million to US$ 1 billion every year (Krause and Oetzel, 2006). The production losses alone were estimated to be US$ 1.12/ cow/ day in a herd diagnosed with SARA (Krause and Oetzel, 2005). In a separate study on a 500-cow dairy herd in New York, U.S., the lost income due to SARA was thought to be in the region of US$ 400 to US$ 475/ cow/ year based on an observed decrease of 3 kg/ cow/ day in milk yield as well as a significant reduction in milk fat and protein concentration (Krause and Oetzel, 2006). However, there have also been studies reporting the prevalence of SARA in dairy herds that observed no negative economic consequences associated with cows either suffering from, or at risk of SARA (Bramley et al., 2008, O'Grady et al., 2008).

2.4.5.5 Prevention strategies

Strategies to prevent the onset of SARA in dairy cows are very important at herd level. There are a number of strategies that can be used as a tool to prevent SARA. However, it can prove very difficult to supply enough energy to the modern high yielding dairy cow, whilst also promoting adequate rumen function (Kleen et al., 2003). According to Oetzel (2007), excessive intake of rapidly fermentable carbohydrates is the most obvious cause of SARA in dairy cattle and careful attention must be paid to analysing the diets offered to dairy cows, both on paper and by examining its physical form at the feed bunk, to ensure adequate energy density and the ability to promote satisfactory saliva production. Plaizier et al. (2014) recommended

meticulous formulation of diets to ensure at least 19% NDF from forage and no more than 44% non-fibre carbohydrates (NFC) as a portion of total diet DM. Feeding rumen buffers, including sodium bicarbonate or calcareous marine algae, may also help to relieve SARA by stabilising rumen pH and increasing milk production (Enemark, 2009; Rafferty et al., 2019). The gradual adaptation to starch rich diets (Westwood et al., 2003) and increasing the number of feed deliveries to spread out the number of meals consumed by a cow during the day (Plaizier et al., 2014) will act as a preventative tool in avoiding SARA. Management practices such as ensuring cows have access to feed throughout the day and that each cow has sufficient head space at the feeding trough will help prevent against gorging and increased production of fermentation acids over a short period of time, leaving the rumen unable to remove these acids quickly enough and causing a depression in rumen pH. Strategies to help avoid SARA in grazing dairy herds can include giving cows a gradual introduction to pasture after calving (Al Ibrahim et al., 2012), offering a source of physically effective fibre (Mertens, 1997) such as straw, to supplement the grass diet, and including high NDF or ingredients with a slower rate of fermentation in the compound fed at milking time (Bramley et al., 2005). Rafferty et al. (2019) has also demonstrated that marine based rumen buffers prevent long periods of low rumen pH in grazing dairy cows. Strategies to prevent gorging on fresh grass after a period of starvation may also help to moderate rumen pH in pasture based dairy cows. Zhang et al. (2013) reported that feed restriction reduced VFA absorption, when feed was re-introduced, and may lead to the accumulation of VFA causing a depression in rumen pH.

2.4.6 Diagnostic tools for identifying SARA and low rumen pH in dairy herds

Clinical signs of SARA in a dairy herd can be difficult to identify and may be easily overlooked due to the complex nature of the different symptoms (Garrett et al., 1999). Developing a more accurate method for identifying low rumen pH may prove very beneficial to dairy farmers, veterinarians and nutritionists, allowing them to make better informed decisions and avoid production losses or health consequences associated with SARA.

2.4.6.1 Measuring rumen pH

Measuring the pH of rumen fluid in dairy cows can help to identify some of the risk factors associated with SARA (Geishauser et al., 2012). However, it is more difficult to diagnose SARA in the field or outside of experimental conditions (Gasteiner et al., 2012). The

measurement of rumen pH in a subsample of cows may be used as a method of SARA diagnosis within dairy herds or groups of dairy cows (Garrett et al., 1999). The analysis of rumen fluid is the most accurate form of measurement to assess the condition of the rumen environment, e.g., measuring microbial populations, VFA production, pH and lactic acid concentration (Gasteiner et al., 2012). Various different methods exist for the collection of rumen fluid for analysis (Garrett et al., 1999).

2.4.6.2 Rumenocentesis

Rumenocentesis is a common field technique used for the collection of rumen fluid for SARA diagnosis (Duffield et al., 2004). Rumenocentesis is the acquisition of rumen fluid via the abdominal wall and percutaneous needle aspiration from the caudoventral rumen (Garrett et al., 1999). Measuring rumen pH with the rumenocentesis method allows for measurements to be taken on-farm. Rumenocentesis is a more invasive technique, compared to other methods of rumen fluid collection like the oral stomach tube method, involving surgical preparation of the centesis site, as well as chemical and physical restraint while animals used for this procedure have increased risk of localised abscesses or peritonitis around the puncture area (Duffield et al., 2004). Work from Garrett et al. (1999) suggested that rumen fluid collected by rumenocentesis was 0.28 pH units lower than fluid collected through a rumen cannula. However, the measurement of rumen pH via rumenocentesis is reported to be superior to the use of an oral stomach tube due to the increased risk of samples from the reticulo-rumen being contaminated by saliva (Duffield et al., 2004). A subsample of 12 cows in a dairy herd can be used to diagnose SARA, if 3 cows have a pH $\leq$ 5.5 they are diagnosed as being affected by SARA (Garrett et al., 1999).

2.4.6.3 Oral stomach tube

Rumen fluid may also be sampled via the oesophagus with an oral stomach tube (Geishauser et al., 2012). The rumen scoop is a flexible metal tube with a special sampling device at one end. The tube used for this procedure is inserted into the mouth, travels along the oesophagus closed and into the reticulo-rumen where it is opened for 10 to 20 seconds to allow the head to fill with fluid. It is then closed carefully, the head unscrewed, and the rumen fluid collected (Geishauser et al., 2012). The oral stomach tube can be used for on-farm sampling of rumen fluid and has the advantage of being less invasive than rumenocentesis so it can be used more

often. However, rumen fluid samples taken with an oral stomach tube can be up to 0.5 pH units higher than corresponding samples taken via rumenocentesis because samples were contaminated with saliva (Gasteiner et al., 2012). Duffield et al. (2004) also reported rumen fluid samples obtained with an oral stomach tube having a higher pH and containing greater concentrations of bicarbonate than samples acquired with rumenocentesis or via the rumen cannula.

2.4.6.4 Rumen cannula

The use of rumen cannulated animals for the harvesting of rumen fluid is specific to research farms for experimental purposes only. The method of fitting an animal with a cannula is expensive and invasive, it involves a modified surgical technique requiring a rumen clamp (Duffield et al., 2004). The practice of rumen fluid sampling via the cannula enables the researcher to take samples from various locations within the rumen. The sample collection techniques for these sampling locations are described in more detail by Duffield et al. (2004). Internal pH probes externally linked to data loggers could be inserted into the rumen via the cannula to measure rumen pH continuously (WTW Sentix 41 Electrodes) as described by Cruywagen et al. (2015). This type of equipment is held in place by a specifically designed rumen cannula plug and allows the pH probe to remain in the centre of the rumen. One advantage of this method is that the pH probe can be recalibrated daily.

2.4.6.5 Indwelling pH probes

In more recent years, indwelling wireless pH probes placed in the reticulo-rumen have been used to continually monitor pH (Gasteiner et al., 2012; Jonsson et al., 2019). This wireless pH measurement has been proven to accurately diagnose SARA (Sato et al., 2012; Jonsson et al., 2019) and also allows for the improved monitoring of pH fluctuation throughout the day and relative to feeding times. These pH probes rest in the reticulum for the duration of the measurement period, this means pH measurements will be from the reticulum. Indwelling wireless pH measurement and data transmitting is a very useful tool for long term measurement of rumen pH in dairy cows, both on-farm and in an experimental setting (Gasteiner et al., 2012).

2.4.6.6 Rumen temperature

The measurement of rumen temperature may play a role in the accurate prediction of rumen pH and subsequent diagnosis of SARA at herd level. AlZahal et al. (2008) evaluated the potential of rumen temperature to predict low rumen pH by measuring both rumen temperature and pH continuously every minute for 4 days with the same indwelling electrode. The results showed that temperature was inversely related to pH and there was a strong correlation between time spent above 39.4 °C and time spent below pH 5.8, 5.6 & 6.0.

2.4.6.7 Milk fatty acid profile

There have been some suggestions that specific fatty acid concentrations in milk may act as a means of predicting SARA or low rumen pH. Enjalbert et al. (2008) discovered that lactating dairy cows experiencing an episode of low rumen pH had strong modifications in the milk fatty acid profile including changes in odd-chain saturated fatty acids and the *trans*-10 C18:1 / *trans*-11 C18:1 ratio. The authors therefore suggested that milk fatty acid profile could be used as an accurate non-invasive diagnostic tool for identifying low rumen pH at herd level as the *trans*-10 C18:1 fatty acid originates from rumen fermentation and is an intermediate of the biohydrogenation process in the rumen. Colman et al. (2010) also proposed the use of specific milk fatty acids to categorise cows with a low rumen pH but established that the *cis*-9, *trans*-11 C18:2 fatty acid was the main discriminatory, between the SARA and control (non-SARA) treatment, and not the *trans*-10 C18:1 fatty acid which was used by Enjalbert et al. (2008) to predict SARA. Colman et al. (2010) concluded by saying that milk fatty acid profiling can discriminate between an acidotic and non-acidotic cow, but the specific fatty acids used may vary and warrant further investigation. According to Fievez et al. (2012), odd and branched-chain fatty acids (OBCFA) in the milk of lactating dairy cows may also play an important role in the detection of low rumen pH. It was discovered that the levels of *iso* C14:0 declined while levels of C15:0, C17:0 and / or C17:1 *cis*-9 increased during an episode of low rumen pH.

2.5 Dietary induced inflammation

2.5.1 Inflammation

Inflammation is triggered by components of adaptive and innate immunity in response to stimuli associated with infection and tissue injury (Bradford et al., 2015). Inflammation can be divided into 2 categories: “acute inflammation” which involves the typical signs of redness, swelling, heat, and pain along with a rapid increase in expression of inflammatory mediators, whereas “subacute inflammation” causes more mild increases in inflammatory mediators that influence chronic changes in tissue function (Bradford et al., 2015). Acute phase proteins (APP) are produced in liver in response to inflammation and are used as markers of inflammation (Li et al., 2012). The acute phase response acts as a preventative mechanism to avoid further injury, to isolate and destroy the infective organism, to remove harmful molecules and debris, and to activate the healing processes required to return the organism to normal function (Plaizier et al., 2008). The main APP include serum amyloid A (SAA), haptoglobin (Hp), ceruloplasmin, and C-reactive protein (Bradford et al., 2015).

2.5.2 Lipopolysaccharide

Rumen bacteria, found under normal physiological conditions, do not contain virulence factors that allow them to invade and exploit epithelial tissue for nutritional benefit (Garcia et al., 2017). Rumen fermentation disruptions provides ideal conditions for the proliferation of opportunistic microbes and their products leading to pathogenic effects and subsequent inflammatory response (Devant et al., 2016). Lipopolysaccharides (LPS) are derived from the cell wall of gram-negative bacteria and are released by lysing or shedding during periods of rapid growth by these gram-negative bacteria (Chiquette et al., 2015). The translocation of LPS into the blood circulation triggers an inflammatory response (Plaizier et al., 2014). Once translocation to the blood stream has occurred, lipopolysaccharide binding protein (LBP) binds to LPS and delivers it to the liver for detoxification, hence why LBP is used as a marker of LPS translocation (Horst et al., 2019). During SARA or rumen fermentation disruptions, the rumen epithelium is damaged (Steele et al., 2011). The most likely mechanism for LPS translocation is by simple passive diffusion via physically damaged parts of the rumen epithelium (Garcia et al., 2017).

2.5.3 Rumen and hindgut acidosis, and inflammation

Experimentally induced grain-based SARA challenges are effective in reducing mean rumen pH, increasing time below pH 5.6, increasing rumen LPS concentrations, and subsequently invoking an inflammatory response through increased concentrations of inflammatory markers detected in serum or plasma (Gozho et al., 2007; Emmanuel et al., 2008; Khafipour et al., 2009a). Rumen pH can be depressed, and SARA induced in diets containing low levels of starch through particle size reduction or a deficiency in physically effective fibre (Beauchemin et al., 2003). Khafipour et al. (2009b) experimentally induced an alfalfa-based SARA challenge, by replacing alfalfa hay with alfalfa pellets, and effectively reduced rumen mean rumen pH, increased time below pH 5.6, and increased rumen LPS concentration but did not detect any increases in plasma or serum inflammatory markers. Gressley (2017) proposed that rumen events during SARA are reflected in the hindgut and that starch undigested in the rumen that passes to the large intestine leads to LPS translocation from the large intestine. Li et al. (2012) reported increased LPS concentration in the rumen and caecal digesta, and increased LBP concentration in plasma during a grain-induced SARA compared to an alfalfa-induced SARA and a control. The authors of this study concluded that LPS translocation from the hindgut was the likely cause of these effects.

2.5.4 Consequences of diet-induced inflammation

The magnitude of inflammation varies greatly between cows (Bradford, 2017) and therefore, the consequences of inflammation will be more severe in certain cows. One of the main consequences of inflammation is the increased energy cost, redirecting energy away from maintenance and production towards the activated immune system. Horst et al. (2019) reported that 1kg of glucose is used by an activated immune system over a 12-hr period. Furthermore, Bertoni et al. (2008) carried out a study looking at milk production in transition cows ranked by the degree of inflammation. Results of this study showed that cows in the highest quartile for inflammation had 20% lower milk yield compared to the cows in the lowest quartile for inflammation.

2.6 Transition period

The transition period, 21 days (d) prepartum to 21 d postpartum, is one of the most challenging periods for a dairy cow. The "transition" refers to dairy cows going from a near maintenance state in late gestation rapidly changing to a state of increased metabolic and nutrient demands required for the onset of lactation (Cardoso et al., 2020). The magnitude of these changes experienced by the dairy cow going from a non-lactating to a lactating state is what causes many of the challenges or production diseases that we associate with the transition period.

2.6.1 Feed intake and energy balance

One of the transition cow's biggest challenges is the sudden increase in nutrient requirements for colostrum and milk production while DMI, and subsequent nutrient supply, is far from sufficient (Drackley, 1999). Bell (1995) reported that the requirements for NE_L and metabolizable protein in healthy dairy cows at 4 DIM exceeded intakes by 26 and 25%, respectively. Dairy cows are extremely efficient at transferring essential nutrients to milk (Wilkens et al., 2020). It's therefore not surprising that the study by Bell (1995) found that 97% of NE_L and 83% of metabolizable protein were partitioned toward the mammary gland, leaving a significant deficit supply for maintenance needs.

The depression in DMI in the final 21 d prepartum is inevitable. Many theories have been proposed for this inevitable reduction in DMI. Forbes et al. (1977) reported that rumen volume was restricted by space constraints from the growing foetus. However, foetal growth is more gradual during the final trimester of gestation, whereas the magnitude of DMI reduction only becomes significant until in the final few days before parturition (Grummer et al., 2004). Furthermore, Park et al. (2001) established that ruminal water holding capacity was unchanged as cows transitioned from prepartum to postpartum, suggesting that physical capacity of the rumen is not the cause of prepartum DMI depression.

In smooth muscle, the excitation-contraction coupling is dependent on extracellular calcium (Ca) promoting excitability of neurons within the smooth muscle cells (Wilkens et al., 2020). Motility of the digestive tract in dairy cows is determined by smooth muscle contractions (Jorgensen et al., 1998). Therefore, one of the main causes of reduced DMI in the immediate postpartum period may be reduced gut and rumen motility due to deficient Ca supply (Goff, 2008).

Grummer et al. (1990) considered if the surge in blood oestrogen might be responsible for the reduction in DMI in late gestation. Evidence to support this theory was provided by the same authors (Grummer et al., 1990) when they found that an injection of serum oestradiol-17β reduced feed intake in lactation cows, and work by Green et al. (1994) described a significant negative correlation between feed intake and serum oestradiol-17β concentrations in late gestation ewes. However, Dewhurst et al. (2000) disagreed with Grummer et al. (1990), proposing that other factors besides oestrogen must be involved in the regulation of DMI prepartum because the reduction in DMI begins before the rise in oestrogen in advance of parturition.

More recently, Horst et al. (2021) suggested that the potent anorexic effects of inflammatory mediators, released during an immune response, are responsible for the DMI reduction experienced by many transition cows around parturition. The increased rumen VFA accumulation and reduction in rumen pH associated with the early postpartum period has been reported to reduce DMI (Penner et al., 2007). The cause of this reduction in DMI could be explained by the increase in rumen osmolality which is commonly associated with low rumen pH and VFA accumulation (Allen, 2000). Further research is required to investigate the abovementioned mechanisms and other factors that could be responsible for DMI depression in late gestation. However, it is most likely that there are numerous factors, acting simultaneously, underpinning this phenomenon (Grummer et al., 2004).

While achieving a high level of DMI in the last 21 d of the prepartum period is an important metric for successful herd-level transition programmes, the change in DMI over the 21-d close-up prepartum period may be even more crucial (Grummer et al., 2004). Grummer and co-authors collated data from three transition cow studies and used regression analysis to investigate the relationship between average DMI from d -21 to -14 prepartum or the change in DMI from d -21 to -1 prepartum with plasma NEFA and liver triglyceride at d 1 postpartum. Plasma NEFA and liver triglyceride at d 1 postpartum were used as metrics for metabolic status. There was a significant decline in liver triglyceride and plasma NEFA at d 1 postpartum as the magnitude of DMI was decreased, whereas there was no significant relationship between average DMI from d -21 to -14 prepartum and liver triglyceride and plasma NEFA on d 1 postpartum. Consequently, current feeding recommendation suggest that the provision of controlled energy diets during the close-up prepartum period can lead to a more successful transition to lactation, citing benefits such as increased DMI in the early postpartum period (Cardoso et al., 2020).

2.6.2 Mineral balance challenges

The mineral status of the transition dairy cow is crucial. One of the main challenges to mineral balance in the transition cow is parturition, which results in cows assuming the mammoth task of maintaining Ca homeostasis (Goff, 2008). Despite the significant increase in dairy cow milk yields over the last 50 years, incidence of clinical hypocalcemia remains unchanged while the incidence of sub-clinical hypocalcemia (SCH) remains a major problem (Wilkens et al., 2020). Dairy cows experience a magnitude of change in Ca demand from the late gestation to the onset of lactation that vastly outweighs that of any other species (Wilkens et al., 2020). To put this into perspective, the plasma pool of a dairy cow would have to be exchanged 10 times to produce a moderate yield (10kg) of colostrum, but only 3 times for the mineralisation of the foetal skeleton. (Wilkens et al., 2020). More specifically, the Ca requirement of a dairy cow increases 4-fold at the onset of lactation (Cardoso et al., 2020). That's why the prevalence of SCH (blood Ca < 2 mmol/L) in dairy herds worldwide remains high at 25% in primiparous cows and 60% in multiparous (Reinhardt et al., 2011). In many instances SCH is undiagnosed, but its economic impact remains significant, and it can predispose cows to other production diseases (Cardoso et al., 2020). Recent research has further developed our understanding of SCH by illustrating that not all SCH is the same. The persistence of low blood Ca or SCH diagnosis in the days following parturition is more important than the nadir of blood Ca concentration at 24 hours (McArt and Neves, 2020).

Magnesium (Mg) intake during the prepartum period plays an important role in the incidence of sub-clinical hypocalcemia. A review by Lean et al. (2006) found prepartum diet Mg content to have one of the biggest effects on the incidence of SCH during the early postpartum period. Low concentrations of Mg in blood have been shown to reduce parathyroid hormone (PTH) secretion and inhibit some of the effects of PTH, such as the synthesis of $1,25(OH)_2D_3$ (Wilkens et al., 2020). A review by Schonewille (2013) highlighted the importance of prepartum dietary Mg level in preventing SCH and also outlined the considerable variation in the bioavailability constants for typical Mg sources used in prepartum dairy cow diets. It's also worth noting that the level of potassium (K) in prepartum diets can have an antagonistic effect on Mg, subsequently reducing Mg absorption from the diet (Schonewille, 2008). Prepartum diet concentration of phosphorous (P) has a more complex relationship with SCH (Lean et al., 2006). Research has shown that reducing prepartum diet P from 0.28 to 0.15% increased Ca concentration before parturition (Wilkens et al., 2020). The review by Lean et al. (2006) cited the level of negative dietary-cation-anion-difference (DCAD) achieved by the prepartum diet

as the second main dietary variable to influence SCH in the early postpartum. Feeding negative DCAD diets induces a metabolic acidosis, evidenced by a reduction in urinary pH, and restores sensitivity of tissues to PTH (Goff et al., 2014). Feeding negative DCAD diets prepartum has been proven to increase blood Ca and prevent SCH during and immediately after parturition (Leno et al., 2017).

2.6.3 Low rumen pH in transition cows

In addition to strategies focused on increasing DMI, the use of energy dense diets, containing very fermentable ingredients, is another strategy used to increase energy consumption during the early postpartum period. As a result, the risk of SARA during the transition period is high (Mulligan and Doherty, 2008). The change from high fibre, low energy dry cow diets to energy dense, low fibre, highly fermentable lactating diet requires the rumen to change very rapidly (Penner, 2009). The increased fermentation acid load generated during the early postpartum period occurs when rumen papillae have not reached their full potential absorptive capacity for lactation (Dieho et al., 2016). As a result, SARA ensues. These suboptimal rumen conditions can lead to other problems such as laminitis, reduced DMI, abomasal displacement, and ulceration (Mulligan et al., 2006). Accurate diet formulation during the close-up prepartum period can reduce the burden of SARA during the transition by adapting the rumen to highly fermentable diets, like the diets fed in the immediate postpartum period (Cardoso et al., 2020).

2.6.4 Inflammation during the transition period

Reduced immune function has been well documented as a consequence of the transition from gestation to lactation and a cow's ability to deal with infections can be compromised (Martinez et al., 2014). In more recent times, inflammation or an activated immune system has become and emerging aspect of transition cow biology (Bradford et al., 2015). Sub-acute inflammation predisposes cows to other metabolic disorders and reduces production in the subsequent lactation (Bradford, 2017). Nearly all transition cows experience some degree of inflammation during the early postpartum period, the only difference between cows is the magnitude and persistence of the inflammation (Horst et al., 2019). Horst et al. (2020) proposed that immunoactivation or subacute inflammation was contributing to SCH and that low levels of circulating Ca in the blood was a consequence of inflammation, in some cases. A study by the

same group (Al-Qaisi et al., 2020) revealed how an oral supplement containing soluble calcium and live yeast ameliorated LPS induced hypocalcemia. There is strong likelihood that dietary induced inflammation from low rumen pH and SARA, discussed earlier in this review, are contributing to many of the inflammatory related production diseases experienced by cows during the transition period (Bradford et al., 2015).

2.7 Rumen modifying dietary feed additives

2.7.1 Yeast

Yeasts are microscopic single cell organisms, of about 5-10 microns in size, that belong to the fungi family. Live yeast culture is the most common and therefore, the following section will focus on the live yeast only. Live yeast products are thought to have beneficial effects on the rumen microbial population, rumen VFA production, immune function, and milk production parameters in lactating dairy cows (Al-Qaisi et al 2020b).

Newbold et al. (1996) examined the hypothesis behind two different modes of action for yeast products: 1.) yeast consumed oxygen in the rumen and promoted an optimum environment for anaerobic bacteria; 2.) yeast produced malic acid which stimulate the growth lactic acid utilising bacteria. This study concluded that the stimulation of rumen bacteria by live yeast could be due to its ability to consume oxygen but adding malic acid to the diet did not affect lactic acid production in the rumen. Chaucheyras-Durand et al. (2008) proposed three main effects that yeast products have on the rumen: 1.) aiding in the development of the young rumen by favouring microbial establishment; 2.) stabilisation of rumen pH and interactions with lactate-metabolising bacteria; and 3.) increasing rumen fibre degradation and interactions with plant-cell wall degrading microorganisms.

Marden et al. (2008) found that yeast increased rumen pH, VFA concentration and acetate: propionate ratio when fed to early lactation dairy cows. Al Ibrahim et al. (2012) also fed live yeast to early lactation dairy cows on both TMR and pasture-based diets and found that live yeast reduced the time spent below pH 5.8 and increased total rumen VFA concentration. Thrune et al. (2009) examined the effect of live yeast in a 60:40 forage: concentrate based diet and reported increased mean rumen pH and reduced time below the SARA threshold with the

live yeast treatment. However, VFA concentrations tended to be lower with the yeast treatment in this study (Thrune et al., 2009). Guedes et al. (2008) documented how yeast improved rumen pH, increased acetate to propionate ratio and reduced variation in rumen pH. Denev et al. (2007) also observed a reduction in rumen pH variation with the yeast treatment. The meta-analysis conducted by Desnoyers et al. (2009) described how live yeast increased rumen pH, VFA concentration, and total tract digestibility across numerous studies. Many studies have reported a reduction in rumen lactate concentration when feeding live yeast (Guedes et al., 2008; Marden et al., 2008; Desnoyers et al., 2009; Al Ibrahim et al., 2012). According to Carpinelli et al. (2020), live yeast increased the production of lactate-utilizing bacteria, *Megasphaera elsdenii*, which may lead to reduced rumen lactate concentration.

Many studies have demonstrated increased OM, NDF and ADF digestion as a result of feeding yeast (Yoon and Stern, 1996, Marden et al., 2008). Yeast may improve the digestion of low-quality maize silage by promoting a healthier rumen microbial population (Guedes et al., 2008). Improved digestion may be a subsequent effect of increased bacterial activity in the rumen (Yoon and Stern, 1996; Chaucheyras-Durand et al., 2008; Guedes et al., 2008). Marden et al. (2008) demonstrated that the difference between yeast and sodium bicarbonate was the ability of the yeast to stimulate fibrolytic bacteria leading to improved fibre digestion. Denev et al. (2007) found that autoclaved yeast was ineffective at stimulating rumen microbial bacteria suggesting that only yeast with viable live cells could affect the rumen microflora.

Work by Bach et al. (2018) reported that live yeast supplementation increased the expression of genes regulating inflammation in the rumen epithelial cells of transition cows. Al-Qaisi et al. (2020b) supplemented live yeast to cows during a heat stress challenge and reported a reduction in SAA on the live yeast treatment. A separate study by the same group, found that live yeast in combination with a soluble Ca source alleviated the effects of LPS induced hypocalcaemia in cows. Therefore, live yeast appears to have a role in reducing inflammation when fed to dairy cows.

A meta-analysis, which incorporated 36 different studies on yeast products fed to dairy cows, reported an increase of 1.18 kg/d, 1.61 kg/d and 1.65 kg/d for milk yield, 3.5% FCM and ECM, respectively, in the yeast treatments compared to the control treatment (Poppy et al., 2012). A separate meta-analysis conducted by Desnoyers et al. (2009), reported that live yeast increased milk yield by 1.2 kg/d in lactating dairy cows based on 157 different experiments. Moallem et

al. (2009) also reported an increase of 2 kg of 4% FCM per cow/ d when yeast was fed during a heat stress situation. Chaucheyras-Durand et al. (2008) found live yeast to have a beneficial effect on milk fat concentration in lactating dairy cows, possibly through improved biohydrogenation of fatty acids in the rumen. However, both (Desnoyers et al., 2009) and (Moallem et al., 2009) did not see any significant difference in milk fat concentration when yeast was included in the diet.

The effects of yeast on DMI seem to vary with some studies reporting an increase in DMI on yeast fed treatments (Desnoyers et al., 2009, Moallem et al., 2009) and other studies showing no effects on DMI (Thrune et al., 2009). The review by Poppy et al. (2012) reported an increase in DMI of 0.62 kg/d in early lactation dairy cows with live yeast but a reduction in DMI of 0.78 kg/d in late lactation dairy cows.

To conclude, yeast products have been shown to have beneficial effects on both ruminant digestion and dairy cow performance. Based on the two reviews by Desnoyers et al. (2009) and Poppy et al. (2012), live yeast can increase milk production in dairy cows.

2.7.2 Ionophores

Ionophores are feed additives that can change the microbial populations of the rumen through ion transfer across cell membranes (Duffield and Bagg, 2000). They are lipophilic compounds that are toxic to many bacteria, protozoa, fungi, and other organisms (Ipharraguerre and Clark, 2003). Ionophores selectively inhibit gram-positive bacteria and not gram-negative bacteria due to differences in bacterial cell wall structure (Duffield and Bagg, 2000). This leads to a change in rumen fermentation resulting in increased amounts of energy and nitrogen extracted from feeds, through better rumen digestion (Ipharraguerre and Clark, 2003). Therefore, energy metabolism can be improved through greater production of propionic acid among rumen volatile fatty acids with a significant reduction in methane production (McGuffey et al., 2001). One of the most popular ionophores used commercially, in animal diets worldwide, is monensin (Ipharraguerre and Clark, 2003). Monensin is approved for use in lactating dairy cow diets in several countries including Australia, Argentina, Brazil, Canada, New Zealand, South Africa, and the USA (Odongo et al., 2007). In the EU, Monensin is only permitted for use as an oral controlled release capsule as a medicinal product under veterinary instruction and there

are strict limitations on the maximum amount of monensin allowed to be administered to each animal (European Medicines Agency, 2013).

Many studies have demonstrated the benefits of feeding monensin to dairy cows on subsequent milk production (Duffield and Bagg, 2000, McGuffey et al., 2001, Duffield et al., 2008c, b). Duffield et al. (2008b) analysed the effect of monensin on production parameters in lactating dairy cows across 77 different trials. They concluded that monensin significantly increased milk yield by 0.7kg/day with a reduction in milk fat and protein concentration but no effect on total milk fat and protein kg's. That same meta-analysis found that monensin reduced DMI by 0.3kg/day leading to an improved milk production efficiency and a positive effect on both body condition and body weight. Ipharraguerre and Clark (2003), in agreement with Duffield et al. (2008b), reported an increase in milk production, milk production efficiency, DMI and reported a significant decrease in milk fat content when monensin was fed due to a shift in rumen fermentation reducing the proportion of acetic acid and increasing the proportion of propionic acid produced.

A meta-analysis conducted by Duffield et al. (2008a) looked at the impact of feeding monensin on metabolism in dairy cows and found that monensin significantly reduced blood levels of β-hydroxybutyric acid (BHBA), acetoacetate, and non-esterified fatty acids (NEFA) while levels of glucose and urea were increased in the blood. Monensin can also have an effect on the health of dairy cows, reducing the risk of ketosis, displaced abomasum and mastitis, especially in early lactation (Duffield et al., 2008c). However, monensin was found to have no effect on fertility parameters in lactating dairy cows, like conception to first service and days to pregnancy (Ipharraguerre and Clark, 2003, Duffield et al., 2008c).

Studies examining the effect of feeding ionophores on methane production in dairy cows over a 6-month period have shown positive results in terms of a reduction in both grams of methane per cow/d and g of methane/ kg of body weight produced when the ionophore Rumensin was included in the diet (Odongo et al., 2007).

Including ionophores in the daily diet of dairy cows is not approved in all parts of the world but studies have demonstrated the positive effects of feeding ionophores. Feeding ionophores to dairy cows can increase milk production, increase milk production efficiency, reduce levels

of BHBA and NEFA in the blood, increase levels of blood glucose and reduce methane production but can also depress milk fat concentration and DMI.

2.7.3 Essential oil-based products

Over the past number of years there has been increasing interest in exploiting natural products as feed additives to solve problems in animal nutrition and livestock production (Wallace et al., 2002). Essential oils are composites of secondary metabolites taken from the plant volatile fraction by steam distillation and are related to the substances that provide specific odours and smells to many plants (Calsamiglia et al., 2007). Essential oils can vary among different plants and can even vary within the same plant (Benchaar et al., 2008). Essential oils are able to interact with microbial membranes and hinder the growth of certain gram-positive and gram-negative bacteria. Similar to monensin, most compounds of essential oils are lipophilic and unable to penetrate into the membrane of gram-negative bacteria which means they are more effective at reducing the growth of gram-positive bacteria (Calsamiglia et al., 2007). However, the antibacterial activity of essential oils is not only due to one specific mode of action but involves several targets in the bacterial cell (Benchaar et al., 2008).

There are a limited number of studies that provide scientific evidence on the effect of essential oils on rumen microbial fermentation (Calsamiglia et al., 2007). The effects of essential oils on rumen fermentation are also variable. Benchaar et al. (2008) reported that essential oils have increased total VFA concentration in some *in-vitro* studies but overall, taking into account a greater number of studies, essential oils have caused either a decrease or no change in VFA concentration of rumen fluid. However, this varied response was measured over a range of experiments incorporating various types of essential oils using different dose rates. Busquet et al. (2006) incubated 12 plant extracts; anise oil, cade oil, capsicum oil, cinnamon oil, clove bud oil, dill oil, fenugreek, garlic oil, ginger oil, oregano oil, tea tree oil, yucca as well as 6 secondary plant metabolites; anethol, benzyl salicylate, carvacrol, carvone, cinnamaldehyde and eugenol in rumen fluid based on a 50:50 F:C diet for 24 hr at 4 different dosage rates; 3, 30, 300 & 3000 mg/l. Most treatments reduced the VFA concentration at the 3000 mg/l dosage rate, except for cade oil, capsicum oil, dill oil, fenugreek, ginger oil, and yucca where no effect was found. Anethol, anise oil, carvone, and tea tree oil reduced the concentration of both acetate

and propionate while garlic oil and benzyl salicylate, at a dosage rate of 300 or 3000 mg/l, reduced the production of acetate but increased the production of propionate and butyrate. Castillejos et al. (2006) also reported variable results investigating the effects of 5 essential oil compounds; eugenol, guaiacol, limonene, thymol, and vanillin on rumen fermentation at different dosage rates; 5, 50, 500 and 5000 mg/l. Most of the essential oil compounds displayed antimicrobial activity and decreased total VFA concentration at high doses, but all of the eugenol treatments and the 5 mg/l thymol treatment altered the VFA profile without reducing the total VFA concentration.

In-vitro batch culture experiments have provided some evidence that essential oils and their components have the potential to improve nitrogen and/or energy utilization in the ruminant animal (Benchaar et al., 2008). Rumen fluid, from sheep and cattle that were fed essential oils, showed decreased levels of ammonia production, yet proteinase and peptidase activities remained unchanged (Wallace et al., 2002). That same experiment found that hyper-ammonia producing bacteria were the most sensitive, of the rumen bacteria, to essential oils in pure culture. Calsamiglia et al. (2007) also looked at the relationship between essential oils and protein metabolism in the rumen when they documented an accumulation of amino acid nitrogen and a reduction in ammonia nitrogen, from the analysis of rumen fluid treated with the essential oil, thymol, during an *in-vitro* study, suggesting that thymol inhibited deamination. Calsamiglia et al. (2007) also reported studies that observed a blend of essential oils (BEO), containing primarily thymol, eugenol, vanillin, and limonene, inhibiting protein degradation, from rumen fluid *in-vitro*, and a separate study that found BEO reduced ammonia degradation in the rumen fluid of dairy cows.

It is clear that essential oils have potential to effect rumen fermentation, protein metabolism and reducing methane production from the *in-vitro* work carried out. However, there is an urgent need for future research to focus on *in-vivo* studies to gain a more accurate view as to how essential oils will affect the rumen in a live animal and to determine correct dosage rates. There also exists many different essential oil compounds, some of which have potential to positively alter rumen fermentation and others which exhibit no effect or may even have a negative effect on the rumen fermentation. This warrants further research so that we can, in future, accurately differentiate between different essential oil compounds based on *in-vivo* studies.

2.7.4 Calcareous marine algae

Calcareous marine algae [CMA] (95% ash, 30% Ca, 5.5% Mg, 0.5% P) is a natural product produced from the skeletal remains of the seaweed *Lithothamnion sp.*, harvested off the Icelandic coast (Cruywagen et al., 2015). Calcareous marine algae consist mainly of Ca carbonate, occurring in three different calcium structures, calcite (65%), aragonite (23%), and vaterite (12%). This unique polymorph structure, along with the physical honeycomb structure of CMA results in a slow release of minerals in an acid environment (Cruywagen et al., 2015).

Cruywagen et al. (2004) first examined the effect of CMA on rumen fermentation and milk production in a dose response trial using lactating dairy cows at 0.125 to 1.2% DM. This experiment suggested that the optimum dose rate for CMA should be 0.3 – 0.6% DM as VFA concentration was at its optimum at 0.6% and maximum milk production was achieved over 0.3%. Cruywagen et al. (2015) found that CMA had a positive influence on rumen pH, acetate production and total VFA production in TMR-fed dairy cows. The same study found that CMA reduced the time spent below rumen pH 5.5 when compared to both the control and a sodium bicarbonate treatment (Cruywagen et al., 2015). A further study investigating the effects of CMA on rumen pH reported a reduction in time spent below rumen pH 5.5 in cows fed a 45% forage TMR based on low DM grass silage, maize silage, and concentrates (Neville et al., 2019).

Calcareous marine algae has also been found to influence milk production in lactating dairy cows (Cruywagen et al., 2004, Bernard et al., 2014, Cruywagen et al., 2015). Both Cruywagen et al. (2004) and Cruywagen et al. (2015) found that feeding CMA increased milk fat concentration, FCM and milk yield when compared to the control treatment and a sodium bicarbonate treatment in cows a high starch TMR. Bernard et al. (2014) documented no significant differences in milk yield or composition, compared to the control treatment or sodium bicarbonate, when CMA was fed to lactating dairy cows through a TMR containing 50% forage. However, Bernard et al. (2014) discovered that CMA increased the efficiency of milk production (energy corrected milk/DMI), compared to sodium bicarbonate, as DMI was significantly higher for the sodium bicarbonate treatment in the last two weeks of the trial. This was in agreement with Cruywagen et al. (2015) who also concluded that CMA could increase efficiency of milk production. The experiment by Neville et al. (2019) demonstrated an

increase in milk fat concentration compared to the control and an increase in fat and protein yield compared to a sodium bicarbonate treatment and the control.

Both Neville et al. (2019) and Rafferty et al. (2019) examined the use of CMA in combination with marine MgO. Neville et al. (2019) reported increased milk fat concentration compared to the control, increased milk protein concentration compared to the control and sodium bicarbonate treatments, and also reduced the time spent below rumen pH 5.5, compared to the control and sodium bicarbonate treatments. Rafferty et al. (2019) tested a CMA and marine MgO combination product in pasture-based dairy cows and found increased milk yield and a reduction in time spent below reticulo-rumen pH 5.8 compared to the control treatment.

To conclude, CMA has demonstrated positive effects on rumen fermentation and milk production in lactating dairy cows. Compared to sodium bicarbonate, CMA increased the efficiency of milk production.

2.7.5 Sodium bicarbonate

Sodium bicarbonate has become one of the most commonly used buffers in the dairy industry (Hu and Murphy, 2005). The addition of sodium bicarbonate to the diets of high producing dairy cows, as a rumen buffer, has become standard procedure in numerous parts of the world (Rauch et al., 2012). Sodium bicarbonate is a chemical feed additive, characterised by an acid dissociation constant ($pK_a = 6.25$) similar to normal rumen pH, that can be used to stabilize rumen pH in cows suffering from rumen acidosis (Marden et al., 2008).

The best-known mode of action of sodium bicarbonate is to maintain rumen pH to values that promotes the growth of fibrolytic bacteria (Pérez-Ruchel et al., 2014). Russell and Chow (1993) proposed that sodium bicarbonate worked via an increased rate of rumen fluid dilution which was caused by an increase in water intake. This led to a large amount of undegraded starch leaving the rumen and reduced rumen propionic acid production. An experiment examining the effects of sodium bicarbonate on rumen fermentation in twelve male lambs, housed in metabolic cages and fed a pasture only diet, carried out by Pérez-Ruchel et al. (2014) provided evidence to support Russell and Chow (1993).

Many studies have examined the effects of sodium bicarbonate on rumen fermentation (Khorasani and Kennelly, 2001, Santra et al., 2003, Kawas et al., 2007, Marden et al., 2008, Pérez-Ruchel et al., 2014). Some studies demonstrated how sodium bicarbonate could increase rumen pH (Santra et al., 2003, Marden et al., 2008, Pérez-Ruchel et al., 2014) but Khorasani and Kennelly (2001) found that sodium bicarbonate had no influence on rumen pH in a trial evaluating the effects of a rumen buffer on late lactation dairy cows. Hu and Murphy (2005) found that sodium bicarbonate significantly increased rumen pH in lactating dairy cow diets when fed in maize silage-based diets but had no effect on rumen pH in diets that contained little or no maize silage as the main source of forage.

Studies carried out by both Marden et al. (2008) and Santra et al. (2003) showed a beneficial effect of sodium bicarbonate on total VFA production and specifically acetate production. Khorasani and Kennelly (2001) documented an increased molar proportion of acetate to propionate in the sodium bicarbonate treatment but found no change in total VFA production. This finding was in agreement with Hu and Murphy (2005). However, Kawas et al. (2007) found that sodium bicarbonate decreased the molar proportion of acetate and increased the molar proportion of propionate when they examined the rumen fluid of lambs fed a finishing diet. This finding seems to be specific to this trial only.

The effect of sodium bicarbonate on DMI has been documented in a number of studies. Both Kawas et al. (2007) and Hu and Murphy (2005) demonstrated that sodium bicarbonate increased DMI when fed to finishing lambs and lactating dairy cows respectively. This increased DMI may be due to increased rate of passage, as explained by Russell and Chow (1993). Pérez-Ruchel et al. (2014) reported that the sodium bicarbonate treatment tended to reduce mean total retention time in the digestive tract, but they reported no difference in. There are many other studies showing no change in the DMI of animals fed sodium bicarbonate compared to control diets (Khorasani and Kennelly, 2001, Santra et al., 2003, Rauch et al., 2012).

The meta-analysis of lactating dairy cows' responses to sodium bicarbonate, conducted by Hu and Murphy (2005), incorporated 27 published studies and found that sodium bicarbonate had no effect on milk production or protein concentration but did significantly improve milk fat concentration in diets where maize silage was the main forage source. Both Rauch et al. (2012) and Khorasani and Kennelly (2001) agreed with Hu & Murphy when they found that sodium

bicarbonate could prevent milk fat depression but had no effect on any other milk production parameters.

In summary, sodium bicarbonate can have a beneficial effect on rumen pH and can be used as an aid to prevent milk fat depression. The effect of sodium bicarbonate on VFA production shows an improvement in acetic acid production, but usually at the expense of propionate and total VFA production is not different. Sodium bicarbonate can increase DMI but can also reduce digestibility of ingested feed, possibly, due to increased rate of passage in the rumen.

2.7.6 Magnesium oxide

The role of magnesium oxide (MgO) in rumen fermentation and its effects on production in lactating dairy cows has been investigated and documented in published literature for some time (Thomas and Emery, 1969). Some studies have reported beneficial effects of MgO on rumen pH (Erdman et al., 1982) and on maintaining milk fat in lactating dairy cows (Teh et al., 1985) when supplemented on its own or along with sodium bicarbonate. According to Erdman (1988), MgO has an alkalising effect on the rumen by raising the pH but could not define MgO as a true buffer because it has no defined pKa and is relatively insoluble in water. Particle size in feed grade MgO may influence the efficacy of the material by aiding in the prevention of low rumen pH. Work carried out by Xin et al. (1989) provides evidence to suggest that reducing the particle size of MgO will increase its availability to the animal. Erdman (1988) summarised data from numerous peer reviewed papers examining the effects of various different rumen buffering products and found that MgO increased rumen pH by 0.15 units on average and acetate: propionate ratio was consistently increased, by an average of 0.65 units, when MgO was used as a buffer. However, work by Erdman et al. (1980) showed that supplemental MgO had no effect on rumen pH.

Erdman et al. (1982) documented a 1.8% increase in DM digestibility, a 12% increase in ADF digestibility and an improvement in nitrogen balance when MgO was fed to 24 lactating dairy cows in a trial investigating the effects of MgO and sodium bicarbonate on digestion. The use of MgO in conjunction with sodium bicarbonate has been investigated on numerous occasions. Erdman et al. (1980) demonstrated that a combination of MgO at 0.8% DM and sodium bicarbonate at 1.5% DM increased FCM by 5.6 kg and 5.8 kg per cow/day compared to a

control and MgO (0.8% DM) on its own, respectively. Kaplan et al. (2010) also documented a beneficial response to feeding MgO in conjunction with sodium bicarbonate when the treatment containing 1% sodium bicarbonate and 0.5% MgO significantly increased milk yield compared to the control diet. Bach et al. (2018) reported mixed results when they compared MgO to a control and sodium bicarbonate during a SARA challenge in dairy cows. The MgO treatment prevented a drop in DMI, and milk yield compared to the sodium bicarbonate treatment and the control but had no effects on time spent below rumen pH during 1 and 2 kg barley challenge periods (Bach et al., 2018).

In summary, MgO can increase rumen pH and increase milk yield in lactating dairy cows but may be more effective when fed in combination with sodium bicarbonate. Particle size may also determine the efficacy of MgO in the rumen. There currently exist a knowledge deficit surrounding the use of MgO in combination with or compared to CMA.

2.8 Knowledge gaps

Several areas have been identified as gaps in the literature and will be partly addressed by work in this thesis. There is limited research on the use of CMA in transition cows. The work of Wu et al. (2015) showed very little differences between the CMA and control groups. However, sodium bicarbonate was used in the control diet postpartum which may have reduced the magnitude of milk production and DMI differences between CMA and control and reduced the chances of detecting significant differences. The effects of CMA compared to a control diet with no buffer is needed to better describe the effect of CMA. Wu et al. (2015) used pre and postpartum diets with similar NDF levels, which is not typical of transition cow diets used in the field. Most transition cows experience a big change in diet, going from a high NDF dry cow diet to a low NDF lactating diet. It is important to investigate the use of CMA in such dietary regimes, which are more relevant to commercial conditions. The effect of CMA on energy balance has not been measured previously, therefore this thesis will broaden the literature around the use of CMA in dairy cows.

The effects of CMA on inflammation during a dietary challenge or during the transition period has never been investigated before. A closer look at the effects of CMA on rumen pH and fermentation while also measuring rumen LPS and blood inflammatory markers will be

addressed within this thesis and will also be useful in contributing to our knowledge around CMA in dairy cows.

There is literature demonstrating the effects of CMA on increased total milk fat production but there has been no research carried out looking at the effects of CMA on the fatty acid profile of cow's milk. This thesis will investigate this area in more detail.

Feeding behaviour in dairy cows is very important, as discussed earlier in this review, and can have important consequences for rumen pH and total feed intake. The effects of CMA on feeding behaviour in dairy cows has never been measured before and therefore, this thesis will investigate the effects of CMA on feeding behaviour in dairy cows.

There is limited knowledge around the effects of CMA on rumen degradation rates of fibrous feeds, total tract digestion, and digestion kinetics. This thesis will also address this topic and delve into possible effects of CMA on the digestion of feedstuffs.

Experimentally induced SARA using high grain diets are commonly used and well defined. However, there is no literature describing ryegrass-based SARA challenges and subsequent effects on rumen LPS and systemic inflammation. Also, the effects of CMA on rumen pH or rumen fermentation products during an experimentally induced SARA challenge have not been investigated previously. This thesis will also try to expand our knowledge around this area.

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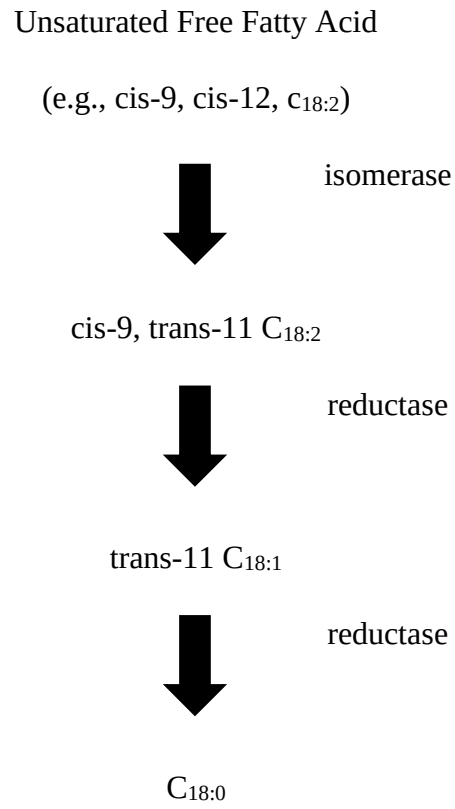

Figure 2.1 Key steps in the conversion of esterified plant lipid to saturated fatty acids by lipolysis and biohydrogenation in ruminal contents (adapted from Jenkins (1993))

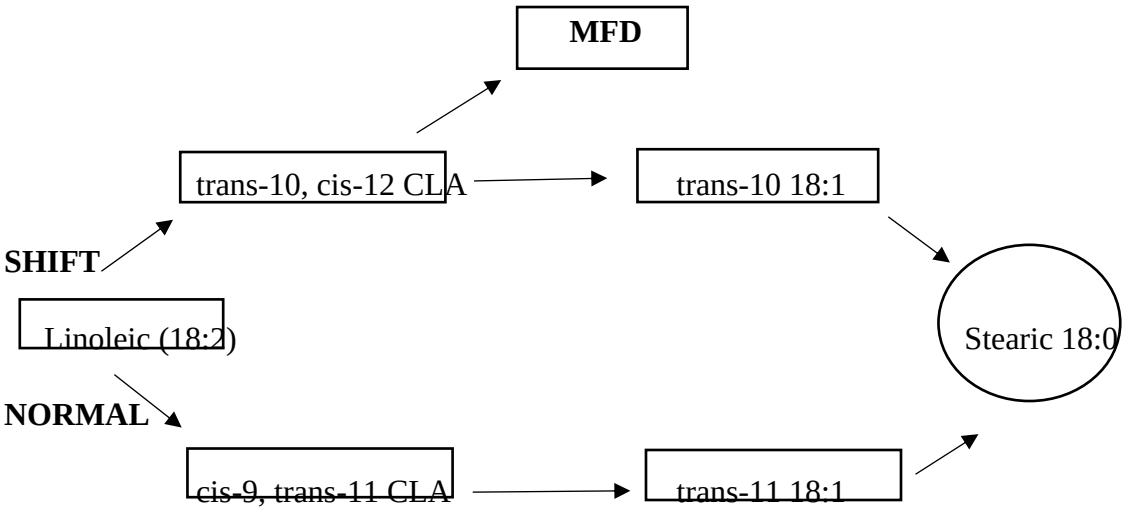

Figure 2.2 The shift in intermediates produced from biohydrogenation of linoleic acid in ruminal contents as a result of a diet-induced microbial shift. MFD, milk fat depression (adapted from Jenkins & Harvatine (2014)).

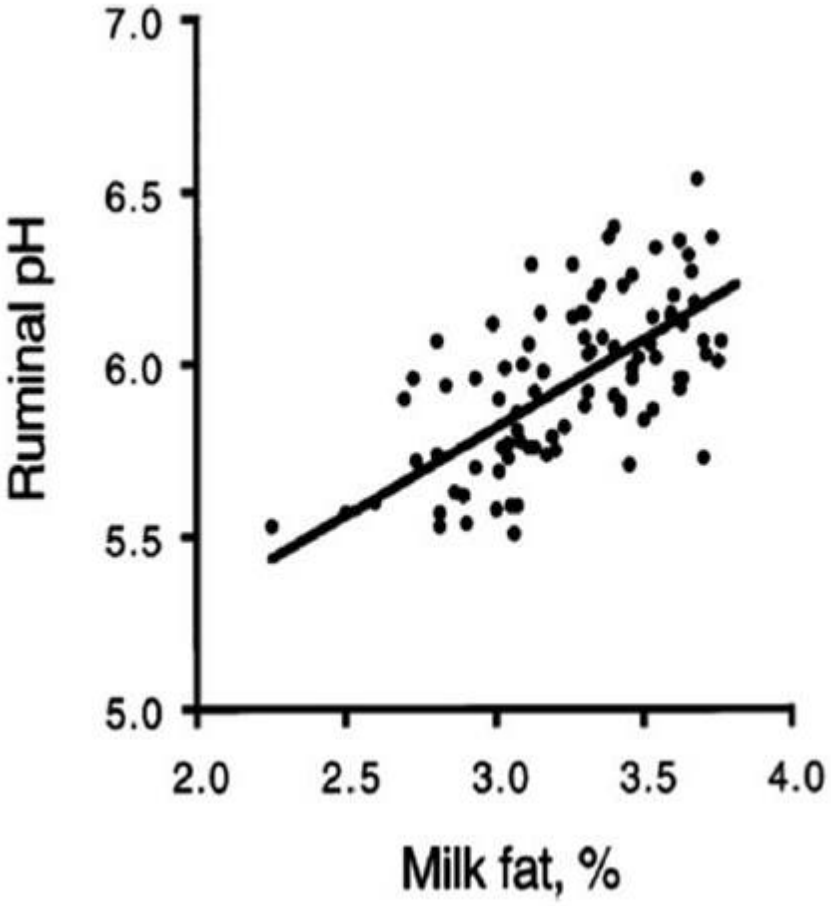

Figure 2.3. The relationship of milk fat percentage and mean ruminal pH from experiments reported in the literature using rumen cannulated, lactating dairy cows with ruminal pH reported as within-day means (adapted from Allen (1997)).

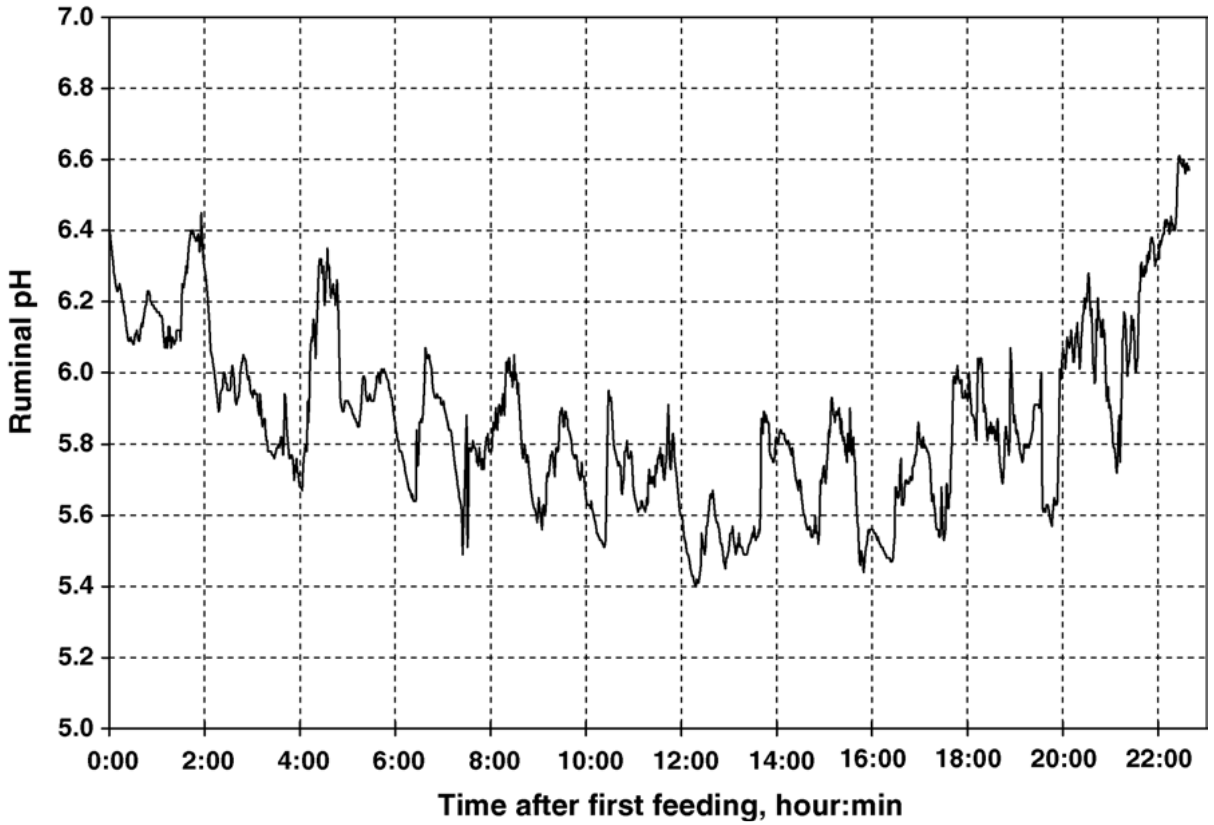

Figure 2.4 Post-feeding variations in ruminal pH over a period of 24 h. Dry matter intake of the current day was 22.7 kg. Average ruminal pH for that day was 5.87 with a standard deviation of 0.25 and a range from 5.40 to 6.61 (adapted from Krause and Oetzel (2006)).

Chapter 3.

Effects of calcareous marine algae on milk production, feed intake, energy balance, mineral status, and inflammatory markers in transition dairy cows

3.1 Abstract

The objective of this experiment was to compare the effects of calcareous marine algae (CMA; Acid Buf, Celtic Sea Minerals) with a limestone-based control on feed intake, milk production, energy balance, serum mineral metabolites and inflammatory markers in transition dairy cows. Twenty-two multiparous and 10 primiparous cows were assigned to 2 treatments from 25 days before expected parturition until 42 days postpartum. Cows were assigned to treatment according to a randomized complete block design based on parity, pre-experimental body condition score, previous 305-d milk yield and fat + protein yield for multiparous cows and predicted transmitting ability for milk yield and fat + protein yield for primiparous cows. Cows were fed a negative dietary cation-anion difference (DCAD) [-50 mEq/kg] total mixed ration (TMR) based on corn silage, grass silage, and straw during the prepartum and a 50:50 forage: concentrate TMR based on grass silage, corn silage and concentrate during the postpartum period. The 2 dietary treatments consisted of a control (CON), which contained limestone as the primary calcium source, and CMA where limestone was replaced by CMA at 0.42% and 0.47% DM for the pre- and postpartum periods, respectively. The dietary treatments were fed as 2 different concentrate pellets added to the TMR. Cows fed the CMA diet had higher dry matter intake in both the prepartum (+ 1.08 kg) and postpartum period (+ 0.94 kg) compared with cows fed the CON diet. Fat yield (+ 0.11 kg), fat concentration (+ 0.43%), and 4% fat corrected milk (+ 1.56 kg) were higher in cows fed CMA compared with cows fed CON. Concentration of plasma serum amyloid A was reduced and serum P increased on the CMA treatment compared with the CON treatment. These findings demonstrate the benefits of supplementing CMA to dairy cows during the transition period compared with a CON treatment, containing limestone as the primary Ca source.

3.2 Introduction

The transition period, 21 days prepartum to 21 days postpartum, is one of the most challenging periods for a dairy cow (Grummer, 1995). The "transition" refers to dairy cows going from a near maintenance state in late gestation and rapidly changing to a state of increased metabolic and nutrient demands required for the onset of lactation (Cardoso et al., 2020). Some of the challenges that occur during the transition period include sub-clinical hypocalcaemia (SCH), SARA, negative energy balance (NEB), and reduced immune function and inflammation (Mulligan and Doherty, 2008). Feeding highly fermentable diets in early lactation is necessary to minimise the extent of NEB but can lead to periods of low ruminal pH and subsequently induce an inflammatory response (Bradford et al., 2015). Low ruminal pH or SARA in early lactation can also reduce DMI (Penner et al., 2007). The calcium requirement of a dairy cow at parturition increases 3-fold whilst dietary intake is reduced by 30% at the same time, potentially leading to insufficient blood calcium levels and SCH (McArt and Neves, 2020). Recent research has indicated that inflammation may be partly causal to SCH incidence during the early postpartum period (Horst et al., 2020). A study by Al-Qaisi et al. (2020) revealed how an oral supplement containing soluble calcium and live yeast ameliorated lipopolysaccharide (LPS) induced hypocalcemia.

Calcareous Marine Algae (CMA; Acid Buf or Calmin, Celtic Sea Minerals) is a feed additive produced from *Lithothamnion*, harvested off the coast of Iceland. The CMA supplement contains: 95% Ash, 30% Ca, 5.5% Mg, 1% P, and 0.7% K. Calcareous marine algae has been used in humans as a Ca and Mg supplement, through research studies and as a commercially available supplement, for many years. Aslam et al. (2010) demonstrated that markers of bone mineralisation and bone strength were increased in mice supplemented with CMA compared with mice receiving a control treatment containing limestone. Research carried out on CMA in human subjects has demonstrated improvements in calcium metabolism when supplemented to premenopausal women (Zenk et al., 2017) and reduced pain when supplemented to patients suffering with knee osteoarthritis (Heffernan et al., 2020). Further research, using LPS treated human macrophage cells, discovered CMA to have an anti-inflammatory role in inhibiting nuclear factor kappa B activation through a reduction in the phosphorylation and degradation of its upstream inhibitor kappa B alpha, leading to reduced cyclo-oxygenase-2 gene expression (O Gorman et al., 2012). Calcareous marine algae has also been used extensively in ruminant animal diets, as a ruminal buffer and mineral supplement, both at farm level and in a research setting (Bernard et al., 2014; Cruywagen et al., 2015; Wu et al., 2015; Neville et al., 2019).

Previous research in dairy cows reported that CMA improved ruminal pH regulation and fermentation (Cruywagen et al., 2015), increased milk fat and protein production (Neville et al., 2019) and improved feed efficiency parameters (Bernard et al., 2014). Cruywagen et al. (2015) described CMA as sparingly soluble in water, due to its chemistry and unique physical structure derived from its marine origin, and therefore effective as ruminal buffer while also providing a soluble source of Ca in the rumen. The use of CMA in transition dairy cows is a relatively new concept and could positively impact ruminal fermentation and calcium metabolism, due to its ability to provide Ca and buffer in the rumen, at a time when the risks of SARA and SCH are high. Wu et al. (2015) previously investigated the supplementation of CMA to transition dairy cows. During this experiment, cows were either supplemented with CMA or a control diet prepartum and assigned to a diet containing sodium bicarbonate or CMA postpartum using a 2 × 2 factorial design. The outcomes of this experiment were higher protein yield for CMA cows versus control cows with no differences detected for DMI or other milk production parameters in pre- or postpartum. There has been no previous research carried out on the effects of CMA on inflammatory markers and energy balance in transition dairy cows or the effects of CMA compared with a control containing no ruminal buffer or rumen modifier during the postpartum stage of the transition period.

The objective of this experiment is to compare the effects of supplementing CMA to cows during the transition period on feed intake, milk production, energy balance, serum mineral metabolites and inflammatory markers compared with a control diet containing limestone as the primary calcium source. Based on previous experiments demonstrating the effects of CMA on ruminal pH (Cruywagen et al., 2015; Neville et al., 2019), we hypothesise that feeding CMA will increase feed intake, and subsequently improve energy balance. We also hypothesise that CMA will reduce the level of inflammatory markers compared with the control diet by avoiding periods of low ruminal pH and potential for LPS to translocate from the rumen to the bloodstream, providing a more soluble supply of Ca in the rumen, and inhibiting LPS induced nuclear factor kappa B activation.

3.3 Materials and methods

All procedures described in this experiment were approved by the Animal Research Ethics Committee (AREC) at University College Dublin (UCD) and conducted under experimental license from the Health Products Regulatory Authority (HPRA) under the European directive 2010/63/EU and S.I. No. 543 of 2012. Each person who carried out procedures on experimental animals, during this experiment, was authorized to do so by means of individual authorization from the HPRA.

3.3.1 Experimental design and feeding management

Thirty-two (22 multiparous and 10 primiparous) Holstein cows were selected from the dairy herd at UCD Lyons Farm, Co. Kildare, IE. Cows were assigned to treatment according to a randomized complete block design with repeated measures based on parity, pre-experimental BCS (multiparous: range = 2.5 – 3.75, median = 2.75; primiparous: range = 2.75 – 3.5, median = 3.25), previous 305-day milk yield (7,009 ± 1,404) kg, and fat and protein yield (556 ± 97) kg for multiparous cows and predicted transmitting ability (PTA) for milk yield (255 ± 127) kg, and fat and protein yield (22 ± 7) kg for primiparous cows. The control (CON) treatment group had a mean pre-experimental BCS of 2.8 (range: 2.5 – 3.5, median: 2.75), a mean previous 305-day milk yield of 7,154 kg (range: 4,014 – 9,293 kg, median: 6,916 kg), and a mean fat and protein yield of 552 kg (range: 349 – 700 kg, median: 518 kg) for multiparous cows and a mean pre-experimental BCS of 3.4 (range: 3.25 – 3.5, median: 3.5), a mean PTA for milk yield of 240 kg (range: 49 – 393 kg, median: 245 kg), and a mean PTA for fat and protein yield of 24 kg (range: 17 – 35 kg, median: 21 kg) for primiparous cows. The CMA treatment group had a mean pre-experimental BCS of 2.9 (range: 2.5 – 3.75, median: 3), a mean previous 305-day milk yield of 6,865 kg (range: 4,668 – 9,097 kg, median: 6,919 kg), and a mean fat and protein yield of 560 kg (range: 375 – 659 kg, median: 608 kg) for multiparous cows and a mean pre-experimental BCS of 3.2 (range: 2.75 – 3.5, median: 3.25), a mean PTA for milk yield of 270 kg (range: 76 – 449 kg, median: 284 kg), and a mean PTA for fat and protein yield of 21 kg (range: 13 – 32 kg, median: 18 kg) for primiparous cows.

Dry matter intake was the most important variable for the objective of this experiment and considered to have one of the highest levels of variation among the variables that were

measured in this experiment. A sample size calculation based on 80% power and a significance level of 0.05 based on post-partum DMI, was used to determine sample size for this experiment. A CV for post-partum DMI of 18.2% and an expected difference of 18% was estimated by averaging the results of previous studies (Wu et al., 2015; Little et al., 2017; Neville et al., 2019) and it was determined that the sample size for this experiment was n = 16.

The 2 dietary treatments consisted of a control (**CON**) and CMA (**CMA**), where limestone was replaced by CMA at 0.42% and 0.47% DM for the pre- and postpartum periods, respectively. The dietary treatments were fed as 2 different concentrate pellets added to the TMR. The ingredient composition of the 2 dietary treatments in pre- and postpartum are outlined in Table 1. The inclusion rate of the CMA additive was based on a predicted daily DMI of 11.7 kg DM for the prepartum and 21.4 kg DM for the postpartum to ensure a daily intake of 50g and 100g of CMA per cow per day in the pre- and postpartum periods, respectively. The dosage rates of CMA were determined from previous experiments (Wu et al., 2015). Limestone and MgO levels were adjusted in the CMA treatment to balance diets for calcium and magnesium.

The diets were formulated using recommendations from INRA (2018). Prepartum diets were designed to supply 100% of the energy requirements of a 650 kg non-lactating dairy cow in late gestation with a predicted DMI of 11.7 kg DM/ cow per d. Both prepartum diets were formulated to contain; 15% starch and water-soluble carbohydrates (WSC), 0.9% Ca, 0.46% Mg, and -53 meq/kg DCAD, based on corn silage, grass silage, barley straw, and soybean meal. Postpartum diets were designed to supply 95 % of the energy requirements of a 650 kg lactating dairy cow yielding 32 kg of milk/d containing 3.9 % of fat and 3.3 % of protein with a predicted DMI of 21.4 kg DM per cow per d and a feed allocation of 22.5 kg DM per cow per d, to allow for 5 % refusals. The 2 postpartum diets were formulated to contain: 49% concentrate, 29% starch and WSC, and 22% NDF from forage, based on grass silage, corn silage, and concentrate. Multiparous and primiparous cows were trained to use the computerized feeding stations prior to the experiment. All cows were introduced to the feeding stations and fed a control diet from 28 d before expected calving date as an acclimatization to the diet, feeding system and the barn. The experimental diets were introduced to the cows from 25 d prepartum.

Cows received their complete prepartum diet and most of their postpartum diet through a TMR once daily at 0900 h. Each treatment was mixed separately with a Keenan Feeder (Keenan Feeding Systems) and each dietary treatment received the same total mixing time, 10 min after all the ingredients were added to the mixer wagon. During milking, cows received a pellet (1

kg as fed at am and 1 kg as fed at pm milking) to incentivize them into the milking parlour. Parlour troughs were inspected before and after each cow entered the parlour and any refusals were recorded. Water was available ad-libitum and water troughs were cleaned daily.

The grass silage used consisted predominantly of perennial ryegrass (*Lolium perenne*). The crop was cut using a mower-conditioner during the early boot stage of vegetation (growth stage 41) (Zadoks et al., 1974), wilted for 16 h and harvested with a Krone Big X (Krone GmbH and Co) forage harvester (mean particle length 50 mm). The crop was then ensiled under a black polythene cover without the use of an additive. The corn (*Zea maiz*, variety Tekni) used for corn silage was grown with the aid of plastic film (Samco Agricultural Manufacturing Ltd). The crop was harvested at the dough stage (growth stage 85) (Lancashire et al., 1991) using a Krone Big X (Krone GmbH and Co.) precision chop forage harvester (mean particle length 25mm). The harvester was equipped with a kernel processor to improve starch digestibility. The harvested corn silage was ensiled under a black polythene cover without the use of an additive.

Prior to the experiment, fresh samples of both grass and corn silage were sent to a forage laboratory (Trouw Nutrition) for near infrared (NIR) analysis (FOSS NIR systems 5000) to provide nutrient values so that the basal TMR could be formulated. The ingredient composition of pre- and postpartum diets is presented in Table 1. The concentrate portion of the postpartum diet was fed in the form of a pellet, and added to the TMR, to aid in mixing accuracy. The concentrate pellet used in the TMR was manufactured by Brett Brothers Ltd. The milking parlour pellet was manufactured by Gain Feeds.

3.3.2 Animal care and housing

Each cow was on the experiment for a minimum of 57 d (57 – 67d), 25 d before expected calving date to 42 d postpartum. Sampling and data collection were carried out from 21 d prepartum to 42 d postpartum. Cows were housed in a free stall barn with 1.25 stalls per cow available during the pre- and postpartum period. When cows were observed to be within 24 h of calving, they were moved to a loose pen to facilitate calving while still receiving their respective diet. Cows had ad-libitum access to TMR for 22 h every day through specific computerized feeding boxes (RIC System, Insentec B.V.). There were 20 feeding boxes available for the 32 cows enrolled on this experiment (0.6 boxes/ cow), with boxes divided evenly across treatments. Cows were milked twice daily, at 0800 h and 1600 h, in a rotary

milking parlor (Dairymaster) and stalls were also cleaned, with new sawdust bedding added, twice daily.

3.3.3 Data collection, sampling procedures, and sample analyses

Samples of TMR and refusals were collected daily and dried at 104°C in a forced air oven for 16 h to establish the DM content of the TMR and refusals. Individual daily feed intakes were recorded on the computerized feeding system and used, in combination with daily TMR DM content to calculate DMI. Daily TMR allocation was 105% of the DMI from the previous d. Samples of TMR were taken from each treatment during feed-out 3 times per week, pooled into weekly samples by treatment, and stored at - 20°C until they were analyzed. Concentrate, grass silage, corn silage and straw samples were collected weekly and stored at - 20°C prior to analysis. Subsamples of the composite TMR from both pre- and postpartum periods, corn silage, grass silage, barley straw, soybean meal, and concentrate samples were dried at 55°C for 72 h. The subsequent dried samples were ground using a Norris hammer mill fitted with a 1 mm screen (Lab Mill, Christy Turner). Ash content was determined by incineration of a 5 g sample in a muffle furnace (Nabertherm GmbH, Lilienthal, Germany) at 550°C for 5.5 h. The N content of the feed was determined by combustion using a Leco 528 instrument (Leco Instruments UK). Crude protein was then calculated using N × 6.25. Neutral detergent fiber and ADF were determined according to the method of Van Soest et al. (1991) using the Ankom 220 Fiber Analyzer (Ankom™ Technology). As part of the NDF procedure, both sodium sulfite (Na_2SO_3) (Ankom Technology) and heat stable alpha-amylase (Ankom Technology) were used for the analysis of TMR, corn silage, barley straw, and concentrate subsamples while only sodium sulfite (Na_2SO_3) was used for grass silage subsamples. Starch content was determined using the Megazyme Total Starch Assay Procedure (product no: K-TSTA; Megazyme International Ltd). Briefly, 0.2 mL of aqueous ethanol was added to 100 mg of sample and mixed using a vortex. Then, 2 mL of dimethyl sulfoxide was added, and the sample incubated in a boiling water bath for 5 min. As the samples were removed from the boiling water, 3 mL of thermostable α-amylase (50 m*M*, pH 7.0) plus calcium chloride (5 m*M*) was added, followed by a further incubation step in a boiling water bath 6 min, vortexing vigorously after 2, 4 and 6 min. After 4 mL of sodium acetate buffer (200 m*M*, pH 4.5) plus calcium chloride (5 m*M*) and amyloglucosidase (0.1 mL, 3,300 U/mL) were added, samples were then incubated at 50°C for 30 min. Then, 2.0 mL of sample was centrifuged at 19,000 X *g* for 5 min, followed by

mixing 0.1 mL aliquots of each sample with 3.0 mL of glucose determination reagent and incubated at 50°C for 20 min. Absorbance of each sample was then measured at 510 nm on a spectrophotometer. Starch content was determined by the following equation:

Starch, % = absorbance of sample × conversion factor for absorbance to µg glucose × extraction volume × (sample dilution factor/ sample weight) × 0.90 (AOAC, 2005). The method outlined by Birch et al. (1974) was used for the analysis of WSC. Ether extract was measured using a Soxtec instrument (Tecator) according to the method of AOAC 107 (1970).

Milk samples were collected on 2 separate days each week during the postpartum period. Each day consisted of an a.m. and p.m. sample, pooled in proportion to the specific a.m. and p.m. yields to create 1 milk sample/ d and 2 milk samples/ cow for each week. Samples were preserved (Broad Spectrum Microtabs II, D&F Control Systems Inc.) and stored at 4°C until analyzed. Milk samples were analyzed within 48 h of collection. Daily milk yields were automatically recorded using the Weighall milk meter system (Dairymaster). Concentrations of milk fat, protein, lactose, urea, and casein were determined in a commercial milk laboratory (Progressive Genetics) using infrared analysis (CombiFoss 5000, Foss Analytical). Values for ECM and 4 % FCM were calculated using the following formulae:

ECM = (0.3273 × milk yield kg) + (7.65 × milk protein kg) + (12.97 × milk fat kg) (Tyrrell and Reid, 1965).
4% FCM = (0.4 × milk yield kg) + (15 × fat yield kg) (Gaines, 1928).

Energy balance was calculated as daily mean energy balance (unit of energy for lactation, equivalent to 1,700 kcal of net energy for lactation [UFL]/ d) and area under the curve (AUC) energy balance (UFL × wk). Total AUC for energy balance, over the 6-wk postpartum period, was calculated using the trapezoidal rule (Cardoso et al., 2011). Body condition score of each cow was assessed once per week prepartum and postpartum, and at parturition, using the 5-point scale method, as described by Edmondson et al. (1989). Each BCS assessment was performed by the same individual who was trained in the 5-point scale method described by Edmondson et al. (1989). The change in BCS over the postpartum period was reported in addition to the weekly BCS for each cow. The BCS change from wk 1 to 3 was calculated as the difference in BCS between wk 1 and 3. Similarly, BCS change from wk 4 to 6 was

calculated as the difference in BCS from wk 4 to 6. Bodyweight of each cow was recorded daily during postpartum after a.m. milking and before access to feed.

Blood samples were harvested by jugular venipuncture on d -7, < 6 h, 24 h, 48 h, and d 7, 14, 21, 28 post parturition. Samples collected on d -7, 7, 14, 21, and 28 were collected 6 h after feed delivery. Blood samples were collected into evacuated tubes (BD, Oxford, UK), which were either coated with a clot activator, fluoride oxalate or lithium heparin. Upon collection, tubes were placed on ice until processed accordingly. Samples from the tubes with clot activator were stored in 4 ^{o}C for 12 h and then centrifuged at 2,100 X *g* and 4 ^{o}C for 20 min to obtain serum and used for the determination of Ca, P, Na, K, Cl, Mg, nonesterified fatty acid (NEFA), and BHB concentration. Fluoride oxalate blood tubes were immediately centrifuged at 1,800 X *g* and 4 ^{o}C for 15 mins to obtain plasma for glucose determination. Lithium heparin blood tubes were also immediately centrifuged at 2,100 X *g* and 4 ^{o}C for 10 mins to obtain plasma for serum amyloid A (SAA) and haptoglobin (Hp) determination. Blood samples were analysed for Ca, P, Na, K, Cl, Mg, NEFA, BHB and glucose in the UCD veterinary clinical pathology laboratory (School of Veterinary Medicine) using Randox Kits according to manufacturer's instructions, with an RX IMOLA analyzer (Randox Laboratories). Serum amyloid A and Hp were measured using ELISA kits (Tridelta Diagnostics Ltd.) as described by Gozho et al. (2007).

3.3.4 Data screening and statistical analyses

Data residuals were examined for normality using the UNIVARIATE procedure of SAS (version 9.4, SAS Inst.). Following assessment of normality, outliers were removed (± 3 STD from the mean). Data for milk yield contained 8 observations which were outside ± 3 STD from the mean and deemed as outliers and removed from the data set. 5 timepoints for 5 different CON cows and 3 timepoints for 3 different CMA cows. There were no outliers removed within any of the other variables. Milk production, intake, energy balance, BCS, BW, and blood data were analyzed as repeated measures using the MIXED procedure (SAS, version 9.4). The model included fixed effects of treatment, week, block, and parity as well as treatment by week interaction with cow considered as the random effect. Repeated measures were modelled for week and the variance-covariance structures were selected based on the lowest Bayesian Information Criterion. Milk fat concentration, DMI, and Na, K, Cl, and BHB serum

concentration required a compound symmetry covariance structure. Serum Mg concentration required the first-order autoregressive covariance heterogenous structure and SAA required the toeplatz covariance structure. All other milk, BCS, BW, plasma and serum data required the first-order autoregressive covariance structure. All data presented in Tables 3 - 6 are expressed as LSM ± SEM. Statistical significance was declared at $P \leq 0.05$.

3.4 Results

3.4.1 Chemical analysis of TMR

The nutrient composition of the CON and CMA treatments in the prepartum and postpartum periods were presented in Table 3.2. Values for PDIN, PDIE, UFL and NE_L were predicted based on the INRAtion 4.07 computer program and NRC (2001).

3.4.2 Prepartum variables

Table 3.3. outlines the effect of the CMA treatment and the CON treatment on DMI, BCS, serum energy metabolites, and serum mineral concentration during the prepartum period. The CMA treatment increased DMI ($P = 0.03$) and reduced serum NEFA concentration ($P = 0.10$) compared with the CON treatment, while there was no difference in serum BHB concentration or BCS between the 2 groups during the prepartum period. There was no difference between treatments for serum concentrations of Ca, Mg, P, Na, K, and Cl. There were no treatment by week interactions detected for any of the prepartum variables.

3.4.3 Postpartum performance

Table 3.4. shows the effect of CON and CMA on DMI, energy balance, BCS, BW, and serum and plasma energy metabolites. Cows fed CMA had higher DMI compared with the cows fed CON ($P = 0.05$) during the postpartum period. Mean energy balance was similar for both treatments, while AUC energy balance (UFL × wk) was higher on the CMA treatment ($P = 0.06$) compared with the CON treatment. The mean BCS, BCS change from 1 to 3 weeks and from 4 to 6 weeks were all similar between both treatments. Mean BW and days to nadir BW were not affected by treatment. Concentrations of BHB and NEFA in serum and glucose in plasma were similar between the CMA and CON treatments. Nadir BW (CON: 584 kg, CMA: 594 kg) and d to nadir BW (CON: 12.7 d, CMA: 12.2 d) were not reported in the tables and did not differ between treatments.

The effects of treatments on milk production during the postpartum period are outlined in Table 3.5. Milk yield, protein yield, and fat + protein yield were similar between treatments. The

CMA treatment increased 4% FCM ($P = 0.03$) and fat yield ($P = 0.04$) compared with the CON treatment. Energy corrected milk yield was be higher on the CMA treatment compared with the CON treatment ($P = 0.07$). Cows fed CMA had higher milk fat concentration compared with cows fed CON ($P < 0.01$). There was no difference between treatments for milk protein, lactose, casein, and urea concentration.

The effect of the CON and CMA treatments on postpartum serum mineral concentrations plasma inflammatory markers are outlined in Table 3.6. No differences were detected between treatments for concentrations of serum Ca, Mg, Na, K, and Cl while the CMA treatment increased the concentration of serum P ($P < 0.01$) compared with the CON treatment during the postpartum period. Plasma SAA was lower in cows fed CMA ($P = 0.07$) compared with the cows fed CON, but Hp was not affected by treatment. There were no treatment by week interactions detected for any of the postpartum variables.

3.5 Discussion

3.5.1 Prepartum variables

Cows fed the CMA diet had similar serum concentrations of Ca, P, and Mg compared with cows fed the CON diet during the prepartum period. This agrees with previous work by Wu et al. (2015). Mineral related challenges in dairy cows typically occur at the onset or in the days following parturition (Goff, 2008). Therefore, differences in Ca and Mg supply between the CMA treatment and the CON treatment due to potential differences in bioavailability may not be detected during the prepartum period as the cows' mineral metabolism is not challenged to the same degree as it is during the days following parturition. Furthermore, serum mineral concentrations were all within the expected range for healthy prepartum dairy cows (Quiroz-Rocha et al., 2009).

The higher DMI observed with the CMA treatment during the prepartum period disagrees with previous work by Wu et al. (2015). Increased DMI might be related to improvements in ruminal pH and fermentation, and faster rate of passage from the rumen due to increased digestibility of nutrients. Calcium supply and status are important for rumen contractions in the early post-partum and will partly dictate how much DM can be consumed by the cow (Goff, 2008). Although there were no differences in major mineral concentrations prepartum, increased availability of Ca and Mg from the CMA treatment may have contributed to these differences in DMI prepartum. However, further research is required to investigate this.

The CMA fed cows had lower concentrations of serum NEFA postpartum compared with CON fed cows during this study. Excess concentrations of NEFA in the serum of dairy cows is commonly used as indicator of poor metabolic status and NEB during the transition period (Mulligan and Doherty, 2008). Increased DMI and subsequent improvements in energy balance with CMA during the prepartum period may have caused a reduction in NEFA concentration. However, these results must be taken in context as the level of NEFA in serum detected in the CON is well within normal levels and not indicative of energy balance issues for prepartum dairy cows. The similarity between treatments for serum BHB concentrations is in-line with results of previous research (Wu et al., 2015).

3.5.2 Postpartum performance

Increased DMI with the CMA treatment compared with the CON treatment during the prepartum period of this experiment followed through into the postpartum period. These results differ from that of Wu et al. (2015) who detected no differences in DMI with CMA during the postpartum period. However, the CMA versus CON comparison in our experiment and in the Wu et al. experiment need to be taken in the context of basal diet used. The control diet used by Wu et al. (2015) in the postpartum contained 1.26% sodium bicarbonate. The use of sodium bicarbonate has been proven to increase DMI compared with control diets (Kawas et al., 2007) and when compared with diets containing CMA in mid-lactation (Neville et al., 2019; Cruywagen et al., 2015). Therefore, diets used by Wu et al. (2015) did not allow for an accurate comparison of CMA versus limestone only on DMI during the postpartum. Changes in mineral status are difficult to relate to DMI differences in our experiment as there were no difference in all the major serum mineral concentrations, with the exception of serum P. Reduced DMI is commonly cited as a negative consequence of low ruminal pH and disrupted fermentation (Kleen et al., 2003). Therefore, the evidence of the CMA treatment's effects on ruminal fermentation within the literature (Cruywagen et al., 2015; Neville et al., 2019) is a more likely cause of DMI increases over the CON treatment. The reduction in plasma SAA concentration with CMA fed cows discovered during this experiment could also contribute to differences in DMI between the CMA and CON treatments. Links between inflammation, SARA, and DMI in early lactation have been discussed extensively in recent years (Bradford et al., 2015). The release of inflammatory mediators during an immune response can initiate a reduction in DMI around calving, due to their potent anorexic effects (Horst et al., 2021). Therefore, the tendency for differences in inflammatory mediators between the CON fed cows and CMA fed cows could be indicative of greater inflammation experienced by CON fed cows and may have contributed to the reduced DMI seen in the CON cows during this study.

Results of this experiment indicate that cows supplemented with CMA during the transition period can maintain better energy balance, measured as AUC energy balance, compared with cows fed the CON treatment. The effects of CMA on energy balance in transition dairy cows has not been measured before. Increased energy balance in cows, during early lactation, can have far-reaching consequences later in lactation, such as better fertility performance (De Vries and Veerkamp, 2000). The increased energy balance on the CMA treatment was likely caused by greater DMI throughout the prepartum and postpartum periods. These findings indicate that not all of the increase in DMI, in the CMA fed cows, was apportioned towards increased FCM

production, improved energy balance for cows fed CMA was also evident in this experiment. The improvements in energy balance did not translate into improvements in BCS, BW, or serum energy metabolites concentration during the postpartum. However, such improvements in energy balance are subtle and may not be detectable in BCS, BW, or serum energy metabolites changes over the duration of this study (6 wk).

Milk production during the postpartum stage of this study was influenced by the CMA treatment. While there were no differences detected for milk yield, 4% FCM and ECM yields were higher in cow's fed the CMA diet compared with cows fed the CON diet. Previous work by Wu et al. (2015) reported no effects of the CMA treatment on milk yield, FCM or ECM during the early postpartum period compared with the control treatment. However, the sodium bicarbonate inclusion in the control diet used by Wu et al. (2015) may have influenced milk production variables. Previous work investigating the effects of CMA in using mid-lactation dairy cows, using similar diets to the current study, also found no differences in milk yield, FCM or ECM (Neville et al., 2019). The improvements in both FCM and ECM, detected in the current study but not in previous work, are likely explained by a combination of diet type and stage of lactation. The lack of CMA effect on milk yield is consistent with previous work (Bernard et al., 2014; Neville et al., 2019)

Milk fat concentration and fat yield were higher on the CMA treatment compared with the CON treatment. The difference of 0.43% milk fat concentration in favour of CMA is highly significant and very large from a biological perspective. Both Cruywagen et al. (2015) and Neville et al. (2019) reported similar effects of CMA on milk fat concentrations, albeit with cows later in lactation. However, Wu et al. (2015) found no effect of CMA on milk fat concentration or yield. The inclusion of sodium bicarbonate in the control diet used by Wu et al. (2015) would have removed any potential difference in milk fat production between the CMA and CON treatments. In a previous experiment carried out by our research group (Neville et al., 2019), there were no differences in fat concentration or fat yield between the CMA treatment and the sodium bicarbonate treatment. Milk fat synthesis is commonly associated with ruminal fermentation (Allen, 1997) and whether biohydrogenation pathways for fatty acids in the rumen are complete or not (Fuentes et al., 2009). While ruminal pH or ruminal fermentation variables were not measured in the current study, there is consistent evidence from previous studies that CMA has a positive effect on ruminal pH and VFA production. Our previous research (Neville et al., 2019) is the most comparable research to the current experiment, in terms of diet composition, environment, and ingredients used. Results from this

work showed increased mean ruminal pH and reduced time spent below pH 5.5, alongside the increase in milk fat production. Therefore, improved rumen fermentation and more complete biohydrogenation pathways, related to increased ruminal pH, is the most likely mechanism behind the improvements in milk fat concentration and yield.

Protein concentration, protein yield and fat + protein yield were not affected by the inclusion of CMA during this experiment. Similar findings were reported by Wu et al. (2015), Cruywagen et al. (2015), and Bernard et al. (2014) for milk protein concentration. Results of this experiment disagree with previous work at our research group (Neville et al., 2019), and Cruywagen et al. (2015) where both studies noted improvements in protein yield and fat + protein yield with the CMA treatment compared with a control treatment. Our previous work (Neville et al., 2019) also reported an increase in protein concentration with cows fed CMA compared with cows fed sodium bicarbonate and an increase in protein concentration with CMA plus marine MgO compared with the control treatment. Wu et al. (2015) found that CMA supplemented during the prepartum period increased protein yield postpartum, which also differs with the results of the current experiment.

The CMA treatment had no effect on postpartum serum Ca, Mg, Na, K, or Cl concentrations compared with the CON treatment. This agrees with work carried out by Wu et al. (2015). Calcareous marine algae provides a source of Ca and Mg in a different physical and chemical form to typical Ca sources such as limestone and typical Mg sources such as MgO. In contrast to the other serum mineral concentrations measured during this study, serum P concentration was increased on the CMA treatment during the postpartum period. This finding is not consistent with previous work carried out by Wu et al. (2015). Work carried out by Leno et al. (2017), investigating Ca-Mg dolomite as an alternative to limestone and MgO, reported similar results to our study. They observed an increase in plasma P concentration and no effect on plasma Ca concentration during the postpartum period. Phosphorous is a major component to bone mineral, second only to Ca (Goff, 1999). Phosphorous concentrations are closely related to plasma PO_4 concentrations which are indirectly regulated by parathyroid hormone (PTH)/ calcium negative feedback loop (Lean et al., 2006). Calcareous marine algae provides minimal amounts (1g / cow per day) of supplemental P, which is not likely to affect serum P concentrations. Cruywagen et al. (2015) described CMA as sparingly soluble in water due to its chemistry, fine particle size, and unique physical form. This would indicate that CMA is solubilised in rumen conditions and its mineral contents (Ca and Mg) released into rumen fluid. The knock-on effects on the absorption of Ca and Mg, from the rumen may have affected Ca

metabolism and resulted in higher serum P concentrations in cows supplemented with CMA. Dairy cows secrete PTH during periods of low serum Ca, consequently stimulating renal conservation of Ca and the release of Ca from bone stores (Goff et al., 1986). Therefore, potential differences in dietary Ca absorption between CMA and CON could have been masked by PTH secretion and hence why there was no effect of CMA on serum Ca concentration to coincide with the increase in serum P concentration. In monogastrics, limestone can have antagonistic effects on dietary P absorption during the digestion process (Li et al., 2021). The difference in Ca sources used during this study, CMA instead of limestone, may have affected the availability of P for absorption in the lower digestive tract and hence differences in serum P concentration. These findings will need to be investigated further to determine mechanisms behind the effect of CMA on serum P concentration.

The results of this experiment partially agree with our hypothesis as plasma SAA concentration was reduced but plasma Hp concentration was unaffected by the CMA treatment compared with the CON treatment. No previous experiments have reported the effects of CMA on SAA and Hp in dairy cows. The positive effects of CMA on ruminal pH (Neville et al., 2019 and Cruywagen et al., 2015) and the findings of O Gorman et al. (2012), where CMA had an anti-inflammatory effect in rodents, contributed to the hypothesis of this study that CMA would reduce the concentration of inflammatory markers, SAA and Hp, in the early postpartum period. Ruminal fermentation has been cited as a common precursor of diet induced inflammation (Zebeli et al., 2012) and different types of SARA can determine the extent of dietary induced inflammation experienced by the dairy cow. The source of supplemental Ca offered to transition dairy cows may be very important as Ca status is pivotal to the functionality of the cow's immune system (Martinez et al., 2014). A recent study by Al-Qaisi et al. (2020) reported how an oral supplement containing soluble Ca and live yeast lessened the extent of LPS induced hypocalcemia, with authors citing the symbiotic effects of the live yeast's improvement in rumen conditions and the oral Ca effects on Ca metabolism as an explanation of these findings. The reduction in plasma SAA, reported in the current experiment, may have been caused by improvements in ruminal pH and fermentation with the CMA treatment, and potential increased supply of soluble Ca in the rumen provided by the CMA treatment will need to be investigated further due to the absence of treatment differences for serum Ca concentration.

3.6 Conclusion

Calcareous Marine Algae supplemented to transition dairy cows increased DMI both pre- and postpartum and some important milk production variables such as ECM yield and milk fat concentration compared with cows fed the CON diet, which contained limestone as the primary Ca source. Estimated energy balance postpartum tended to be higher with the CMA treatment, likely as a result of increased DMI. The concentration of plasma SAA tended to be reduced in the CMA treatment postpartum, indicating possible links between CMA, inflammation, and feed intake around parturition. This phenomenon warrants further investigation. Other blood variables such as P concentration postpartum was positively affected by the addition of CMA to the diet. These results will offer dairy producers and nutritionists' further knowledge on the implications of adding CMA to transition cow programmes and a possible tool to improve the health and production of dairy cows during this critical period.

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Table 3.1 Ingredient composition of the control, and calcareous marine algae diets during prepartum and postpartum periods.

	Dietary treatments[1]			
	Prepartum		Postpartum	
Item	CON	CMA	CON	CMA
Ingredients, % DM				
Maize silage	43.56	43.56	25.7	25.7
Straw (barley)	22.29	22.29	-	-
Grass silage	18.31	18.31	25.7	25.7
Soybean meal (48% CP)	7.43	7.43	10.72	10.72
Wheat grain (finely ground)	-	-	9.48	9.49
Barley grain (finely ground)	-	-	8.00	8.04
Parlour concentrate[2]	-	-	7.94	7.94
Soychlor[3]	6.76	6.76	-	-
Maize grain (finely ground)	-	-	4.54	4.54
Molasses (sugar cane)	-	-	3.28	3.28
Soya hulls	-	-	2.47	2.47
Dry cow premix[4]	1.22	1.22	-	-
Milking cow premix[5]	-	-	0.23	0.23
White salt (NaCl)	-	-	0.47	0.47
Mono-dicalcium phosphate	-	-	0.47	0.47
Vegetable oil	-	-	0.23	0.23
Limestone	0.34	0.00	0.61	0.14
Calcined Magnesite	0.04	0.00	0.14	0.09
Calcareous Marine Algae[6]	0.00	0.43	0.00	0.47

[1] Treatments: CON = control; CMA = calcareous marine algae (95% Ash, 30% Ca, 5.5% Mg, 1% P, and 0.7% K)

[2] Parlour concentrate ingredients (DM basis): 22.5% barley grain, 22.5% maize grain, 10% maize distillers dried grains with solubles, 9% sugar beet pulp, 21% soybean meal, 2.5% soya hulls, 1.5% palm oil, 4.5% sugar cane molasses, 1.6% mono-dicalcium phosphate, 1.6% limestone, 1.8% salt, 1.5% MgO.

[3] SoyChlor = anionic salt supplement (Landus Cooperative, IA, USA). Contains -2,980 mEq DCAD/ kg, 20.1% CP, 0.99 Mcal/ kg, 4.54% Ca, 0.53% P, and 2.84% Mg.

[4] Dry cow premix contained: 24% maize grain [carrier], 1% Ca, 10% P, 12% Mg, 4% Na, 6% Cl, 2,767 mg/kg of Mn, 5,000 mg/kg of Zn, 2,000 mg/kg of Cu, 33 mg/kg of Se, 333 mg/kg of I, 67 mg/kg of Co, 134 mg/kg of Biotin, 500 IU/kg of Vitamin A, 167 IU/kg of Vitamin D3, 8,000 IU/kg of Vitamin E.

[5] Milking cow premix contained: 30% Ca, 50 mg/kg of Co, 10,400 mg/kg of Cu, 390 mg/kg of I, 130 mg/kg of Se, 21,600 mg/kg of Mn, 32,500 mg/kg of Zn, 10,000 IU of Vitamin A, 3,000 IU of Vitamin D3, 13 IU of Vitamin E.

[6] Calcareous marine algae = Lithothamnion calcareum

Table 3.2 The analysed and predicted chemical and nutrient profile of the control and calcareous marine algae diets during the prepartum and postpartum periods.

	Dietary treatment[1]			
	Prepartum		Postpartum	
Item	CON	CMA	CON	CMA
Chemical composition, % DM				
DM	40.7	40.1	40.0	40.6
CP	13.0	13.1	16.4	15.9
PDIN[2]	8.11	8.11	10.85	10.85
PDIE[2]	7.39	7.39	10.42	10.42
NDF	44.2	46.3	29.2	28.0
f-NDF[3]	93.16	93.16	25.4	25.4
ADF	29.2	29.2	17.2	17.2
Ash	9.81	9.70	7.98	7.88
Starch	14.9	14.5	24.8	24.9
Sugar	4.3	4.0	4.08	4.36
Ca	0.87	0.87	0.83	0.84
P	0.35	0.35	0.45	0.46
Mg	0.46	0.46	0.32	0.33
K	1.80	1.80	1.75	1.76
Na	0.08	0.08	0.30	0.31
Cl	1.50	1.50	0.85	0.85
S	0.20	0.20	0.18	0.18
DCAD (mEq/kg)	-52.2	-52.2	226	233
Metabolizable energy[2] (MJ/kg)	9.91	9.91	12.0	12.0
Net energy[2] (UFL/kg)	0.76	0.76	0.99	0.99
Net energy[4] [NE_L] (Mcal/kg DM)	1.33	1.33	1.74	1.74

[1] Treatments: CON = control; CMA = calcareous marine algae

[2] As calculated by using INRAtion 4.07 feed formulations program, based on ingredient analyses: similar for all treatments. PDIN = protein truly digestible in the small intestine where nitrogen is limiting microbial protein synthesis, PDIE = protein digested in the small intestine where energy is limiting microbial protein synthesis, UFL = unit of energy for lactation.

[3] NDFforage = contribution of the forage component of the diet to NDF

[4] NE_L = Net energy for lactation, at production level, NRC (2001).

Table 3.3 The effect of control and calcareous marine algae on DMI, BCS, serum energy metabolites, and serum mineral concentration during the prepartum period.

	Dietary Treatments[1]			
Variable	CON	CMA	SEM	*P*[2]
DMI, kg/d	12.23	13.31	0.39	0.03
BCS	2.99	3.01	0.10	0.82
Serum energy metabolites (m*M*)				
NEFA[3]	0.22	0.11	0.05	0.10
BHB	0.52	0.56	0.04	0.46
Serum mineral concentration, m*M*				
Ca	2.28	2.27	0.02	0.72
Mg	0.86	0.86	0.02	0.85
P	2.18	2.20	0.08	0.85
Na	145.0	145.8	0.67	0.44
K	5.33	5.41	0.16	0.70
Cl	106.7	107.7	1.02	0.52

[1] Treatments: CON = control; CMA = calcareous marine algae
[2] *P*-value for treatment effect.
[3] NEFA = nonesterified fatty acid
No significant treatment × week interactions were detected for any of the above variables

Table 3.4 The effect of control and calcareous marine algae on DMI, energy balance, BCS, BW, and energy metabolites during the postpartum period.

	Treatments[1]			*P*-value[2]	
Variable	CON	CMA	SEM	Trt	Wk
DMI (kg/d)	19.14	20.08	0.39	0.05	<0.01
Energy balance					
Mean (UFL/d)[3]	0.02	0.55	0.44	0.40	<0.01
AUC (UFL x wk)[3]	0.69	3.51	1.06	0.06	-
BCS and BW					
Mean BCS	2.79	2.81	0.08	0.65	0.72
BCS change wk 1-3	-0.06	-0.05	0.05	0.85	-
BCS change wk 4-6	0.12	0.08	0.05	0.62	-
Mean BW, kg	598.0	620.5	16.32	0.33	<0.01
Serum and plasma energy metabolites (m*M*)					
BHB	0.60	0.63	0.02	0.31	<0.01
NEFA[4]	0.27	0.25	0.03	0.64	<0.01
Glucose	3.24	3.20	0.04	0.54	<0.01

[1] Treatments: CON = control; CMA = calcareous marine algae

[2] Trt = *P*-value for treatment effect. Wk = *P*-value for week effect

[3] UFL = unit of energy for lactation. AUC = Area under the curve

[4] NEFA = nonesterified fatty acid

No significant treatment × week interactions were detected for any of the above variables

Table 3.5 The effect of control and calcareous marine algae on milk production during the postpartum period.

	Treatments[1]			*P*-value[2]	
Variable	CON	CMA	SEM	Trt	Wk
Output, kg/d					
Milk yield	31.04	30.71	0.76	0.75	<0.01
FCM	30.09	31.65	0.52	0.03	<0.01
ECM	34.45	35.76	0.53	0.07	<0.01
Fat yield	1.18	1.29	0.04	0.03	0.15
Protein yield	1.16	1.17	0.02	0.96	<0.01
Fat + protein yield	2.33	2.44	0.06	0.16	<0.01
Composition %					
Fat	3.81	4.24	0.09	<0.01	<0.05
Protein	3.72	3.82	0.05	0.17	<0.01
Lactose	4.43	4.42	0.02	0.81	<0.01
Casein	2.87	2.95	0.05	0.21	<0.01
Urea (mg/dL)	14.00	14.33	0.77	0.74	0.71

[1] Treatments: CON = control; CMA = calcareous marine algae

[2] Trt = *P*-value for treatment effect. Wk = *P*-value for week effect

No significant treatment × week interactions were detected for any of the above variables

Table 3.6 The effect of control and calcareous marine algae on serum mineral concentration and plasma acute phase protein concentration during the postpartum period.

	Treatments[1]			*P*-value[2]	
Variable (m*M*)	CON	CMA	SEM	Trt	Wk
Ca	2.12	2.15	0.03	0.46	<0.01
Mg	0.92	0.92	0.02	0.88	<0.01
P	1.93	2.13	0.06	0.01	<0.01
Na	143.9	143.9	0.21	0.85	<0.01
K	5.12	5.07	0.12	0.79	<0.01
Cl	103.2	102.5	0.32	0.14	<0.01
Acute Phase proteins					
SAA (ug/ml)[3]	73.2	57.8	6.24	0.07	0.01
Hp (mg/ml)[4]	0.78	0.70	0.19	0.85	<0.01

[1] Treatments: CON = control; CMA = calcareous marine algae
[2] Trt = *P*-value for treatment effect. Wk = *P*-value for week effect
[3] SAA = Serum amyloid A
[4] Hp = Haptoglobin
No significant treatment × week interactions were detected for any of the above variables

Chapter 4.

The effect of calcareous marine algae, with or without marine magnesium oxide, and sodium bicarbonate on rumen pH, rumen fermentation and digestion in lactating dairy cows.

4.1 Abstract

Low rumen pH can negatively alter the rumen environment in dairy cows leading to impaired fibre digestion. The objective of this experiment was to investigate the effects of calcareous marine algae (CMA) [Acid Buf], with or without marine magnesium oxide (MM) [precipitated magnesia derived from seawater], and sodium bicarbonate (SB) on rumen pH parameters, volatile fatty acid (VFA) production, apparent total tract digestion, and the kinetics of digestion in lactating dairy cows fed a TMR based on grass silage, maize silage and ingredients typical of a confined northern European feeding system. Four multiparous cannulated Holstein cows were utilized in a 4 x 4 Latin square design. Dietary treatments consisted of the control (55% concentrate, 33% starch and sugar, and 20% NDF from forage) including no dietary buffer (CON); CON including CMA at 0.45% DM (CMA); CON including CMA at 0.45% DM and MM at 0.11% DM (CMA+MM); CON including SB at 0.9% DM (SB). The experiment contained 4 periods of 25 days (d), with each period consisting of 13 d acclimatization followed by 12 d of sampling and data collection. Both CMA treatments had the biggest impact on rumen pH. The CMA and CMA+MM increased mean, median and minimum rumen pH, and reduced time spent below pH 5.6 and 5.4 compared to CON. There was less variation in rumen pH across the d with CMA compared to SB as CMA reduced the range and CV of pH. The SB treatment tended to increase mean rumen pH compared to CON. Acetate: propionate ratio was reduced, and the molar proportion of propionate increased with CMA+MM compared to CON, CMA, and SB. The CMA+MM treatment increased NDF digestibility compared to CON. There were no differences in rumen fluid and particulate outflow rates or rumen retention time between treatments. *In-sacco* degradability constants of soya hulls in the rumen were not affected by treatment. These findings demonstrate how CMA, with or without MM, can successfully prevent rumen pH depressions and CMA products proved more effective in regulating rumen pH compared to the traditional buffer, SB. The combination of CMA and MM had a positive effect on NDF digestion and may provide itself as a useful tool in many ruminant diets.

4.2 Introduction

The formulation of high producing dairy cow diets that satisfy all of their nutrient requirements while maintaining normal rumen function is challenging (Khorrami et al., 2021). On one hand, there should be a focus on providing highly digestible diets containing large amounts of readily fermentable carbohydrates to provide cows with sufficient energy and nutrients to achieve their genetic potential for milk production (Plaizier et al., 2008). While on the other hand, the inclusion of physically effective fibre, which will lower the energy density of the diet, is essential to encourage rumination and the production of saliva to maintain rumen pH (Mertens, 1997). The highly fermentable fractions of the diet generate large amounts of VFA in the rumen (Humer et al., 2018). However, increased VFA concentration in the rumen can lead to a depression in rumen pH (Kolver and de Veth, 2002). Periods of persistently low rumen pH have many consequences, such as negatively altered microbial populations (Khafipour et al., 2009), reduced milk fat production (Allen, 1997) and health issues such as laminitis and rumen epithelial damage (Steele et al., 2011). Fibre digestion is also impaired during periods of rumen pH depression due to the sensitivity of cellulolytic bacteria to low rumen pH (Weimer, 1996). Furthermore, Mulligan et al. (2002) demonstrated that total tract digestibility of NDF and OM were positively correlated to rumen pH. Hours below threshold rumen pH values of 5.6 and 5.8 are commonly used to define SARA or risk of SARA (AlZahal et al., 2007). Previous studies, conducted in northern Europe, have shown that the prevalence of SARA can be substantial with 20% of TMR fed cows (Kleen et al., 2013) and 11% of pasture-fed cows (O'Grady et al., 2008) affected by the production disease.

Rumen buffers, such as sodium bicarbonate (SB), calcareous marine algae (CMA), and magnesium oxide (MgO), are routinely added to dairy cow diets to prevent low rumen pH, SARA, and the associated consequences (McGuffey, 2017). Sodium bicarbonate is the most commonly used rumen buffer (Hu and Murphy, 2005). The exact mechanism by which SB effects rumen pH and fermentation products has been disputed in the past with Russell and Chow (1993) proposing that additional Na consumed through SB increased rumen fluid osmolarity, leading to increased water consumption. Greater fluidity of the rumen fluid accelerated the passage of small feed particles associated with starch sources to post-ruminal sites of absorption, leaving a higher proportion of forage digestion in the rumen (McGuffey, 2017). Calcareous marine algae (Acid Buf; Celtic Sea Minerals) is a feed additive produced from *Lithothamnion*. Both human food and animal feed have been supplemented with CMA for many years now. Recent research found that CMA increased rumen pH and VFA

production (Cruywagen et al., 2015) increased milk fat production (Neville et al., 2019) and improved milk production efficiency (Bernard et al., 2014). Magnesium oxide has also been used to alleviate pH depressions in dairy cows. Evidence from the literature highlights the ability of MgO to increase rumen pH (Erdman, 1988) and improve total tract digestibility of DM in dairy cows (Erdman et al., 1982). Marine magnesium (MM) [precipitated magnesia derived from seawater] is a MgO, containing 56% magnesium with a very small particle size (< 20 µm). Previous research found that MM supplemented in combination with CMA improved rumen pH and increased milk fat concentration compared to a control, while reducing milk yield compared to CMA-only and SB and increasing milk protein concentration and improving milk production efficiency compared to SB (Neville et al., 2019). No previous work has been carried out investigating the effects of CMA, with or without MM, or SB on apparent total tract digestion or kinetics of digestion in dairy cows fed diets based on ingredients typical of a northern European feeding system. There is also a lack of research around the effects of CMA in combination with MM on VFA production and the impact of CMA on VFA production in cows fed diets with greater than 35% forage. The objective of this experiment was to investigate the effects of CMA, with or without MM, and SB on rumen pH parameters, VFA production, apparent total tract digestion, and the kinetics of digestion in lactating dairy cows fed a TMR based on grass silage, maize silage and ingredients typical of a confined northern European feeding system. We hypothesized that CMA, with and without MM, would improve rumen pH parameters compared to a control and SB, and increase total VFA concentration compared to the control. We also hypothesize that SB would increase rumen fluid passage rate and have a negative effect on apparent total tract digestion compared to the control, and CMA, with or without MM.

4.3 Materials and methods

All procedures described in this experiment were approved by the Animal Research Ethics Committee (AREC) at University College Dublin (UCD) and conducted under experimental license from the Health Products Regulatory Authority (HPRA) under the European directive 2010/63/EU and S.I. No. 543 of 2012. Each person who carried out procedures on experimental animals during the experiment was authorized to do so by means of individual authorization from the HPRA.

4.3.1 Experimental design

Four multiparous rumen cannulated Holstein cows were selected from the dairy herd at UCD Lyons Farm, Co. Kildare, IE. Cows were 202 ± 41 DIM and had an average BW of 618 ± 19 kg at the start of the experiment. Cows were assigned to one of four dietary treatments in a 4 x 4 Latin square design (n = 4). Total VFA concentration was considered to have one of the highest levels of variation among the variables that were measured in this experiment. A sample size calculation based on 80% power and a significance level of 0.05 based on total VFA concentration, was used to determine sample size for this experiment. A CV for total VFA concentration of 6.75% and an expected difference of 15% was estimated by averaging the results of previous studies (Al Ibrahim et al., 2012; Whelan et al., 2013) and it was determined that the sample size for this experiment was n = 4.

The four dietary treatments consisted of the control (**CON**); CON including CMA at 0.45% DM (CMA); the control including CMA at 0.45% DM and MM at 0.055% DM (CMA+MM); the control including SB at 0.9% DM (SB). Inclusion rates of dietary treatments were based on a predicted daily DMI of 18 kg / cow. Therefore, both CMA and CMA+MM treatments provided a daily CMA intake of 80 g / cow, and the SB treatment provided a daily SB intake of 160 g / cow. The dosage rates of CMA, MM, and SB were determined from previous experiments (Neville et al., 2019; Cruywagen et al., 2015). Limestone and MgO levels were adjusted in both CMA treatments to balance total diets for Ca and Mg concentration.

The total duration of the experiment was 100 d consisting of four periods of 25 d each. Each experimental period consisted of a 13-d dietary adjustment period whereby cows were acclimatized to their respective treatments, followed by a 12-d sampling and data collection period.

4.3.2 Animal care and housing

During the data collection period, animals were either housed individually in 5 × 7 m pens in a well-ventilated barn with a solid concrete floor or in metabolism stalls for 5 d / period to facilitate total faecal collection. Each pen contained a lying area with woodchip, a feeding trough, and a water trough. The metabolism stalls contained 1 cow / stall with a headlock to the front, a steel cubicle frame to either side and a rubber mat (Mayo Mats, Cow Comfort Ltd) underneath the cow. Cows had free access to feed and water throughout the day, and both water and feed troughs were emptied and cleaned daily before adding fresh feed and water. Cows received their diet as a TMR twice daily, after a.m. milking (0830) and after p.m. milking (1630). Each dietary treatment was mixed separately using an experimental diet feeder (American Callan), and each dietary treatment received the same total mixing time. Cows were milked twice daily in a 10-unit herringbone parlour (Dairymaster). Cows were also milked in the metabolism stalls, which were fitted with individual milking units (Dairymaster). The loose pens were cleaned, and new woodchip bedding was added twice daily. Metabolism stalls were cleaned every 4 h, and no woodchip or bedding material was used during the total faecal collection procedure.

4.3.3 Diets and feeding management

The cows feed allowance was restricted to 18 kg DM daily or approximately 3% of their BW. The concentrate portion of the diet was introduced gradually over three weeks before the start of the experiment to acclimatize the cows to the diets. Diets were formulated using the INRAtion 4.07 computer programme and designed to supply 100% of the energy requirements of a 600 kg lactating dairy cow producing 23 kg of milk per day containing 40 g/kg fat and 32 g/kg protein. The four dietary treatments were formulated to contain: 55% concentrate, 33% starch and sugar, and 20% NDF from forage, based on grass silage, maize silage, and concentrate. The forage: concentrate ratio was 45:55.

The grass silage used in this experiment consisted of primarily perennial ryegrass (*Lolium perenne*). The crop was felled using a mower-conditioner during the early booth stage of vegetation (growth stage 41) (Zadoks et al., 1974), wilted for 16 h and harvested with a fixed chamber round baling machine creating round, compact and airtight bales (mean particle length 800 mm) (Fusion baler, McHale Ltd). The crop was then ensiled by wrapping the bales in black polythene cover without the use of an additive. Maize silage (*Zea maiz*, variety Award) was

grown with the aid of plastic film (Samco Agricultural Manufacturing Ltd), as per Farrell and Gilliland, (2011). The crop was harvested at the dough stage (growth stage 85) (Lancashire et al., 1991) using a Claas Jaguar 860 (Claas GmbH & Co) precision chop forage harvester (mean particle length 100 mm). The harvester was equipped with a grain cracker to improve starch digestibility. The harvested maize was ensiled in a bunker under a black polythene cover without the use of an additive. Fresh samples of both grass and maize silage were sent to a forage analysis laboratory (AFBI- Hillsborough) for near infrared (NIR) analysis (FOSS NIR systems 5000, FOSS UK). The concentrate portion of the diet was fed in the form of a pellet to prevent unwanted dietary separation and aid mixing accuracy. The pellet was manufactured by Gain Feeds. Ingredients were added to the TMR in the following order: concentrate pellet, grass silage and maize silage.

4.3.4 Feed and milk samples

Days 14 – 25 of each period were used for the determination of milk yield and milk sampling. Daily milk yields were recorded for monitoring purposes. Cows were weighed on d 1 of each data collection period, after a.m. milking to monitor BW. Samples of concentrate, grass silage, and maize silage were collected daily and dried at 104°C for 16 h to establish the DM content of each ingredient daily to accurately determine the feed allocation of 18 kg DM per cow / d.

Subsamples of the composite TMR, grass silage, maize silage, and concentrate samples were dried at 55°C for 72 h. The dried samples were ground using a Norris hammer mill fitted with a 1 mm screen (Lab Mill, Christy Turner). Ash content was determined by incineration of a 5 g sample in a muffle furnace (Nabertherm GmbH) at 550°C for 5.5 h. The N content of the feed was determined by combustion using a Leco 528 instrument, (Leco Instruments UK). Crude protein was calculated using N × 6.25. Neutral detergent fibre and ADF were determined according to the method of Van Soest et al. (1991) using the Ankom 220 Fibre Analyzer (Ankom™ Technology). As part of the NDF procedure, both sodium sulphite (Na_2SO_3) (FSS, Ankom Technology) and heat-stable alpha-amylase (FAA, Ankom Technology) were used for the analysis of TMR and maize silage subsamples, while only sodium sulphite (Na_2SO_3) was used for grass silage subsamples. Starch content was determined using the Megazyme Total Starch Assay Procedure (product no: K-TSTA; Megazyme International Ireland Ltd). Water-Soluble Carbohydrates (WSC) were analysed according to the method used by Birch et al.

(1974). Ether Extract (EE) was measured using a Soxtec instrument (Tecator) according to the AOAC 107 (1970) method. The following formula was used to calculate OM:

OM % = 100 – Ash %

4.3.5 Rumen pH

Rumen pH measurements were collected over 3 d, starting on d 1 of each sampling period. Rumen pH was measured using internal pH probes linked to a data logger (Intech Instruments Ltd). The pH data loggers were connected to straps that were securely fastened around the cow's shoulder to prevent damage to the data logger while also avoiding irritation of the cow. The electrodes were housed in specially designed stainless-steel capsules and joined to the cannulas via water-tight hoses and fittings. This specially designed rumen cannula, holding the pH probe, allowed the pH probe to reside in the centre of the rumen. On d 1 of each data collection period, the pH loggers and probes were introduced at 0730 h. The device was removed from the rumen every 24 h to retrieve the pH measurements stored in the data logger. At the same time, the pH probes were cleaned, checked for accuracy and re-calibrated, with pH 4.0 and 7.0 standards. This process took approx. 15 min to complete. Continuous pH measurements from the indwelling probe were sent to the data logger every ten min. Measurements taken over the 3 d were averaged to create one 24 h diurnal pattern of rumen pH. Time spent below pH 5.4, 5.6, and pH 5.8 was calculated per cow and treatment as the total h / d that pH was below 5.4, 5.6, or 5.8.

4.3.6 Rumen fluid VFA

Rumen fluid samples were harvested twice per period, via the rumen cannula, on d 2 of the data collection period. Samples were collected before morning feeding (0830) and 12 h after morning feeding (2030). Rumen fluid was obtained using a collection tube (Bar Diamond Inc.) and a 60-mL disposable syringe. Four-millilitre subsamples were drawn off using an automatic pipette and acidified by mixing with 1 mL of trichloroacetic acid (50% wt/vol) before freezing at -20°C.

During analysis, samples were allowed to thaw in the refrigerator for 16 h at 4°C before centrifuging at 2,100 × g for 10 min at 4°C. Next, 250 µL of supernatant was mixed with 3.75 mL of distilled water (dH_2O) and 1 mL of internal standard solution (0.5 g of 3-methylvaleric acid in 1,000 mL of 0.15 *M* oxalic acid). The resulting solution was centrifuged at 1,600 × g and filtered through a syringe-tip filter (PTFE, 25-mm diameter, 0.45 µm) into 2-mL GC vials. Concentrations of VFAs were determined using Scion 456-GC (Scion Instruments, Scotland, UK) fitted with DB-FFAP capillary column (15m x 0.53mm; 1.00 µm, Agilent Technologies, USA).

4.3.7 Diet digestibility determination

Diet digestibility was determined by total faecal collection from d 1 to 5 of the data collection period. At 1000 h on each of the 5 d, faeces were collected and weighed. Daily faecal collections were thoroughly mixed before a representative subsample was taken (approx. 1 kg fresh) and stored at -20°C. Fresh feed allocations and feed refusals were carefully weighed each day to calculate actual DMI. Samples of TMR and refusals were also collected and stored at -20°C during each d of the total faecal collection procedure. Before chemical analysis, frozen samples of TMR, refusals, and faeces were thawed and dried at 55°C for 72 h. After drying, samples were mixed, and pooled by weight (DM-basis) for each cow and period. Pooled samples of TMR, refusals, and faeces were then ground using a Norris hammer mill fitted with a 1 mm screen (Lab Mill, Christy Turner). The TMR was analysed as per the routine feed analysis during the experiment, see "Feed and Milk Samples" above. The faecal and refusal's samples were analysed for DM, OM, NDF, and starch. Digestibility of DM, OM, NDF and starch were calculated using the following formula:

1- (faecal output in kg/d / diet intake in kg/d).

4.3.8 In-sacco degradability of soya hulls

In-sacco DM degradability of soya hulls was carried out on d 4 to 6 of each data collection period to determine the extent of ruminal digestion at time points. *In-situ* filter bags (5 x 10cm; 50µm pore size) (Ankom, Fairport) containing approximately 5 g of soya hulls were placed inside large mesh nylon fishnet bags and inserted into the rumen at 0800 h on d 4 of the data collection period and incubated in the rumen of each animal for 2, 4, 8, 16, 24, and 48 h. The soya hulls were previously ground using a Norris hammer mill fitted with a 2mm screen (Lab Mill, Christy Turner). After removal from the rumen, all bags were immediately submerged in ice-cold water, thoroughly washed, and stored at - 20 °C. On thawing, all bags were stomached

using a Lab Blender (Seward Medical) to remove any microbial debris, washed for 35 min in a washing machine and dried at 55 °C for 72 h so that the DM residue could be determined. The 0-h loss was determined by stomaching and washing 3 unincubated bags. Degradability constants *a*, *b*, and *c* for DM were determined according to the equation $P = a + b (1 - e^{-ct})$ (Orskov and McDonald, 1979) where 'a' is the rapidly degradable DM, 'b' is the potentially degradable DM not including *a*, and '*c*' is the fractional rate of degradation per hour of the '*b*' fraction with time '*t*'. Potential degradability was calculated as the sum of *a* and *b*. Effective degradability was calculated using the equation $P = a + bc/(c + k)$ (Orskov and McDonald, 1979), where k is the determined rumen particulate outflow rate (klp) [% per h] for each treatment.

Total DM degradability at 8, 16, 24, and 48 h was calculated with the following formula:

1 – (sample end weight – empty bag weight) / (sample start weight – empty bag weight)

4.3.9 Rumen particulate and fluid outflow rate

Chromium mordanted soya hulls were prepared as described by Uden et al. (1980). Cows were pulse dosed with 100g of Cr-mordanted soya hulls via the rumen fistula at 1600 h on d 9 of the data collection period. Faecal samples were taken at 16, 20, 24, 28, 32, 40, 46, 52, 64, 76, and 88 h post dosing. The fractional rumen outflow rate of Cr-mordanted soya hulls (klp) was calculated from the regression of the ln of faecal Cr concentration on time post dosing for samples occurring after the peak in faecal Cr concentration (Grovum and Williams, 1973). Final sample times were selected for inclusion in this regression based on maximum R^2 values.

The rumen fluid outflow rate was determined using Co-EDTA, prepared as described by Bosch et al. (1988). On d 7 of the data collection period, cows were pulse dosed with 400ml of Co-EDTA (690 mg of Co) at 0800 h. Ruminal fluid was sampled at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 36 h post dosing. The ruminal fluid samples were stored at -20°C and later centrifuged at 6000 × g for 20 min. Fractional ruminal fluid outflow rate (klf) was calculated from the regression of the ln of ruminal fluid Co concentration on time post dosing.

4.3.10 Data screening and statistical analyses

Residuals of data were examined for normality using the UNIVARIATE procedure of SAS (version 9.4, SAS Inst.). Following assessment of normality, outliers were removed (± 3 std from the mean). The results were analysed as a 4 × 4 Latin square, where four cows were randomly assigned to the rows of the squares, using the MIXED procedure of SAS (SAS, version 9.4). The model included fixed effects of treatment and period, and the random effect of cow within square. All data presented in the tables are expressed as least square means ± standard error of the mean (SEM). Statistical significance was declared at $P < 0.05$. *P* – values are presented as Tukey-Kramer adjusted values.

4.4 Results

4.4.1 Chemical analysis of TMR

The chemical analysis of the treatment diets is presented in Table 4.2. Each dietary treatment contained similar levels of DM, CP, protein digested in the small intestine where nitrogen is limiting microbial protein synthesis (PDIN), protein digested in the small intestine where energy is limiting microbial protein synthesis (PDIE), NDF, NDF from forage (f-NDF), ADF, ash, starch, water-soluble carbohydrates (WSC), Ca, Mg, Na, unit of energy for lactation according to INRA (UFL) and net energy for lactation according to NRC (2001) (NE_L). Values for PDIN, PDIE, Ca, Mg, Na, UFL, and NE_L are predicted based on the INRAtion 4.07 computer programme and NRC (2001), respectively.

4.4.2 Rumen pH

The effect of CMA, CMA+MM and SB on rumen pH mean, median, maximum, minimum, range, coefficient of variation (CV) and time spent below rumen pH 5.4, 5.6, and 5.8 is presented in Table 4.3. The CMA ($P = 0.03$) and CMA+MM ($P < 0.01$) treatments increased mean rumen pH compared to CON while SB ($P = 0.07$) tended to increase mean rumen pH compared to CON. The CMA ($P = 0.07$) and CMA+MM ($P = 0.03$) increased the median rumen pH compared to CON, with no other treatment differences detected for median pH. The 3 buffer treatments (CMA, CMA+MM, and SB) did not differ from each other. The maximum rumen pH was higher on CMA+MM ($P = 0.02$) and SB ($P = 0.02$) compared to CON and tended to be higher on SB ($P = 0.07$) compared to CMA. There were no differences between CMA and CON, or between CMA and CMA+MM. The CMA+MM was also similar to SB for maximum rumen pH. Minimum rumen pH was lower on CON compared to CMA ($P = 0.03$) and CMA+MM ($P = 0.04$). There were no other treatment differences detected for minimum rumen pH. The daily range in rumen pH was greater on SB (P = 0.02) compared to CMA and tended to be greater on CON (P = 0.09) compared to CMA. The CMA+MM treatment did not differ from CON, CMA, or SB. The CON and SB also had similar rumen pH ranges. The CV for daily rumen pH was lower on CMA compared to CON ($P = 0.01$) and SB ($P < 0.01$). The CMA+MM tended to have a lower rumen pH CV compared to SB ($P = 0.06$). The CON treatment did not differ from CMA+MM or SB for rumen pH CV. The CMA and CMA+MM were also similar for rumen pH CV. Time spent below pH 5.8 tended to be greater on CON compared to CMA ($P = 0.07$) with no differences detected between any of the other treatment comparisons. Time spent below pH 5.6 and 5.4 was greater for CON compared to CMA ($P =$

0.03) and CMA+MM (P = 0.04). There were no differences in time below pH 5.6 or 5.4 between CMA, CMA+MM, and SB. The CON and SB were also similar for time spent below pH 5.6 and 5.4.

Figure 4.1. displays the effect of CMA, CMA+MM, and SB on diurnal pH changes. The CMA treatment had a higher mean pH for 11 h, at 0900, 1000, 1100, 1200, and 1600 through to 2200 h (P < 0.05), compared to CON. The CMA treatment had a higher mean pH for 3 h, at 1400, 1700, and 1800 h (P < 0.05), compared to SB. The mean pH was higher on the CMA+MM treatment for 13 h, at 0300, 0400, 0600, 1000, 1100, 1200, and 1600 through to 2200 h (P < 0.05), compared to CON. The CMA and CMA+MM had different mean pH values for just 2 h, CMA+MM was higher at 0200, and 0400 h (P < 0.05). The CMA+MM treatment had higher mean pH for 4 h, at 1400, 1700, 1800, and 2100 h (P < 0.05) compared to SB. The SB treatment had higher mean pH for 8 h, at 0600, 0700, 0900, 1000, 1100, 1200, 1700, and 1900 h (P < 0.05), compared to CON. The mean pH was higher on the SB treatment for 2 h, at 0100 and 0200 h (P < 0.05), compared to CMA and higher than CMA+MM for 1 h, at 0900 (P < 0.05).

4.4.3 Rumen fluid VFA

The effect of CMA, CMA+MM, and SB on VFA concentrations and molar proportion of rumen fluid is outlined in Table 4.4. There were no differences between treatments for total VFA, propionate, butyrate, valerate or iso-valerate concentrations. Acetate tended to be higher on CMA compared to CMA+MM (P = 0.09) with no differences detected for any of the other treatment comparisons. Iso-butyrate concentration was higher on the CMA+MM treatment compared to CMA (P = 0.07). There were no differences between CON, CMA, and SB for iso-butyrate. There were also no differences between CON, CMA+MM, and SB for iso-butyrate concentration. The ratio of acetate: propionate was reduced with the CMA+MM treatment compared to CON (P = 0.04), CMA (P = 0.01), and SB (P = 0.01), and there were no differences detected among the other treatment comparisons for acetate: propionate ratio. The proportion of acetate in rumen fluid was higher on CMA compared to CMA+MM (P = 0.03), and CON tended to be higher than CMA+MM (P = 0.08). There were no differences between CON, CMA, and SB for acetate proportions. The CMA+MM had similar acetate proportions to SB. The proportion of propionate in rumen fluid was increased with CMA+MM compared to CON (P < 0.01), CMA (P < 0.01), and SB (P < 0.01). There were no other differences detected between treatment comparisons for propionate proportion. The proportion of butyrate, iso-butyrate, valerate, and iso-valerate in rumen fluid was similar for all treatments.

4.4.4 Diet digestibility determination

Table 4.5. illustrates the effect of CMA, CMA+MM and SB on DM, OM, NDF and starch total tract digestibility. There was no difference between any of the treatments for DM or OM digestibility. The digestibility of NDF was increased in CMA+MM compared to CON (P = 0.04), but there were no differences between CMA, CMA+MM and SB treatments. The CON was similar to CMA, and SB for NDF digestibility. Starch digestibility was reduced in the CMA treatment compared to CON ($P < 0.01$), CMA+MM ($P < 0.01$) and SB ($P = 0.01$). There was no difference between CON, CMA+MM and SB for starch digestibility. Starch digestibility was high for all treatments, being over 99%.

4.4.5 Rumen outflow rates and in-sacco degradability constants

The effect of CMA, CMA+MM, and SB on klp, klf, rumen retention time (RRT) and the rapidly degradable DM (*a* fraction), potentially degradable DM (*b* fraction), rate of DM digestion (*c* fraction), potential DM degradability, effective DM degradability and total DM degradability of soya hulls at 8, 16, 24, 48 h is outlined in Table 4.6. There were no differences detected between any of the treatments for any of the parameters outlined in Table 4.6., except for the rapidly degradable DM (*a*) of soya hulls. The CON had a higher *a* fraction compared to CMA+MM ($P = 0.07$). There were no other differences detected for *a* fraction.

4.5 Discussion

The objective of this experiment was to investigate the effects of CMA, with or without MM, and SB on rumen pH parameters, VFA production, apparent total tract digestion, and the kinetics of digestion in lactating dairy cows fed a TMR based on grass silage, maize silage and ingredients typical of a confined northern European feeding system. The basal diet for this experiment was designed to be very fermentable and cause a reduction in rumen pH. The rumen pH data for CON confirm that the basal diet challenged rumen pH and exceeded the threshold for SARA, 180 min/ d less than pH 5.6 (Gozho et al., 2005). Diurnal rumen pH data demonstrated that the nadir pH occurred between 1800 and 2000 h, 9 to 11 h post first feeding on all treatments. Compared to the CON, all the buffer treatments (CMA, CMA+MM, and SB) maintained a higher rumen pH during the late morning, from 1000 to 1200 h. The rumen acid load and steep decline in rumen pH were counteracted by the activity of the 3 buffer treatments during this time. A more significant differentiation of the rumen pH curves occurred from 1400 to 2200 h. For much of this time, CMA and CMA+MM maintained a higher rumen pH than both the CON and SB, demonstrating superior buffering ability. This agrees with Cruywagen et al. (2015) who illustrated higher rumen pH values with CMA on a diurnal pH curve compared to CON and SB. Cruywagen et al. (2015) also found greater differences between all treatments in the afternoon and evening time. This experiment shows that the three dietary buffer treatments positively impacted mean rumen pH but only CMA and CMA+MM affected time spent below pH 5.4 and 5.6, and CMA was the only treatment to affect time spent below pH 5.8 compared to CON. The work of AlZahal et al. (2007) suggests that a reduction in rumen pH for more than 150 or 282 min/d below pH 5.6 and 5.8, respectively, could be defined as SARA. In this experiment, CON and SB cows could be defined as having SARA at both thresholds, based on the suggested definitions of AlZahal et al. (2007). The effect of CMA on time spent below 5.4, 5.6, and 5.8 is similar to the reduction in time spent below pH 5.5 reported by Cruywagen et al. (2015). Cruywagen et al. (2015) found no difference in mean rumen pH between CMA, SB, and the control. Previous work had reported an increased rumen pH when SB was supplemented to the diet (Marden et al., 2008). However, Khorasani and Kennelly (2001) demonstrated that SB did not influence rumen pH in late lactation dairy cows. In a review conducted by Hu and Murphy (2005), SB increased rumen pH in maize silage-based diets but not in diets based on other forages such as alfalfa or grass silage. According to Cruywagen et al. (2015), CMA is sparingly soluble in water compared to SB which is highly soluble in water, and its effects are more short term than CMA. This may have also contributed

to the improved pH profile in the CMA and CMA+MM treatments compared to the SB treatment. According to Erdman (1988), MgO can increase mean rumen pH by 0.15 pH units, when numerous trials using low forage diets (< 30% DM) were examined. Erdman et al. (1982) reported that MgO supplemented to the diet of lactating dairy cows at 0.8% DM was the most effective treatment at increasing rumen pH compared to the control, while a later experiment (Erdman et al., 1982) reported that a combination of SB at 1% DM and MgO at 0.8% DM were most effective compared to the control and either buffer (SB, MgO) fed on its own. In this experiment, there were no differences between CMA and CMA+MM in mean pH, time below pH 5.4, 5.6, and 5.8, or any of the rumen pH parameters. The MM used in this trial was of a very small particle size which should have increased its ability to impact on rumen pH, according to data from Xin et al. (1989). The lack of difference between CMA and CMA+MM for rumen pH parameters may have been due to CMA providing enough buffering material to stabilize the rumen pH and additional buffer, added through MM, was not required or its effects over the CMA-only treatment were very slight and not detectable through rumen pH measurements.

There were few effects of treatment on VFA concentrations in the data from this experiment. The CMA treatment tended to increase acetate and *iso*-butyrate concentration compared to CMA+MM. The acetate: propionate ratio was reduced on the CMA+MM compared to all other treatments, despite CMA+MM maintaining the highest diurnal pH values and reduced time spent below pH 5.4 and 5.6 compared to CON. More significant changes were observed among the molar proportion of VFA between treatments. The CMA+MM had reduced proportions of acetate compared to CMA and CON, while propionate proportions were highest on CMA+MM compared to all other treatments. The changes in acetate proportion correspond to the reduced acetate concentration on the CMA+MM treatment compared to CMA. Using similar diets to those fed during this experiment, previous research reported a reduction in milk yield and total yield of fat + protein with CMA+MM compared to CMA (Neville et al., 2019). These differences in milk production by Neville et al. (2019) may be related to the differences in VFA outlined in this experiment. It is known that different VFA may influence the partitioning of energy into milk or body tissues (Morvay et al., 2011). For example, the primary function of acetate is to act as a precursor for long-chain fatty acid synthesis in the mammary glands, while propionate is a substrate for gluconeogenesis in the liver and the primary source of glucose in the animal (Bannink et al., 2006). Furthermore, Dijkstra et al. (2012) reported that isocaloric infusion of acetate resulted in more energy secreted as milk while propionate infusion favoured

more energy deposited in body tissue. Microbial species present in the rumen will also have a major influence on the type of VFA produced due to the many rumen microbes that are substrate-specific and have characteristic metabolic pathways (Bannink et al., 2008). Therefore, the difference in VFA profile between CMA and CMA+MM may be due to differences in rumen microbial populations and further investigation is warranted. The absence of any differences in VFA concentration or proportion between SB and CON disagrees with previous work by Marden et al. (2008) where the authors reported increased total VFA and increased acetate concentrations with a SB treatment compared to a CON. However, the total VFA concentrations were much lower, and the level of concentrate fed was reduced in this work by Marden et al. (2008) compared to the present experiment. Previous research comparing CMA and SB reported an increased acetate: propionate ratio and reduced propionate proportion on the CMA treatment compared to SB (Cruywagen et al., 2015). That same experiment found that CMA increased total VFA and acetate concentrations and reduced propionate concentrations compared to a control (Cruywagen et al., 2015). Therefore, the current experiment had different outcomes in VFA production for CMA versus CON than the previous work by Cruywagen et al. (2015). Discrepancies between experiments are likely due to diet formulation; Cruywagen et al. (2015) used a diet containing 35% forage, based on oat and alfalfa hay, whereas the current experiment used diets containing 45% forage, based on maize silage and perennial ryegrass silage. The power calculation for this experiment was based on total VFA concentration and a minimum replication required to detect a 15% difference between experimental groups was 3.18. Therefore, the design of this experiment was sufficient to detect any real treatment effects for VFA concentrations and the likelihood of a type-II error reduced.

Based on rumen pH data from this experiment, CMA and CMA+MM had the potential to positively alter fibre digestion in the rumen. The NDF digestibility was increased with the CMA+MM treatment compared to the control, while no difference was detected between any other treatments. Total tract NDF digestibility can be closely associated with rumen pH due to cellulose contributing to a large proportion of total NDF and the importance of cellulolysis in the rumen and its sensitivity to low rumen pH [< 6.0] (Weimer, 1996). Despite differences in rumen pH between the two treatments, no differences were detected between CMA and CON for DM, OM or NDF digestibility. There was no treatment lack of effect of CMA on NDF digestibility, even though it improved rumen pH, differs from most research describing the link between rumen pH and NDF digestion (Allen, 1997). Despite CMA and CMA+MM having

similar rumen pH profiles, the addition of MM to CMA appeared to enhance its effects on the digestive tract and NDF digestion. The alkalizing effect of MM (Erdman, 1988) in combination with CMA may have promoted a better rumen environment outside of the pH parameters measured during this experiment, compared to CMA on its own. The effect of rumen available magnesium supplied by MM may have had a beneficial impact on some rumen microbes. According to Hubbert et al. (1958), Mg was one of the nutrients most likely to be deficient for rumen bacteria in a prepared medium for an *in-vitro* experiment. The lack of SB effect on total tract digestibility parameters partially agrees with a previous meta-analysis by Erdman (1988) where the authors reported no effect of SB on DM or NDF digestibility in grass silage-based diets. However, the same meta-analysis found that SB had a positive effect on DM and NDF digestibility in maize silage-based diets. More recently, Marden et al., (2008) reported no effect of SB on DM, OM, or NDF total tract digestibility compared to a CON in cows fed a TMR based on maize silage (51.7% DM).

To the best of the authors' knowledge, the effect of CMA, CMA+MM, and SB on digestion kinetics such as k1p, k1f, RRT, soya hulls *in-sacco* degradation fractions (a, b, c) and effective degradability in lactating dairy cows have not been previously reported. There were no treatment effects detected for any of the digestion kinetics variables recorded during this experiment, apart from the impact of CMA+MM and SB on the rapidly degradable component of soya hulls (*a*-fraction). The CMA+MM treatment reduced the value of the *a*-fraction, and SB tended to reduce the *a*-fraction. The *a*-fraction is the rapidly degradable component of soya hulls. It is difficult to find any reason why a rumen buffer would reduce the rapidly degradable portion of soya hulls. Therefore, this finding may need to be investigated further. Russell and Chow (1993) questioned the mode-of-action behind SB-based buffers, suggesting that the increase in Na consumption increased rumen fluid osmolarity and subsequently increased the klf. A previous experiment by Perez Ruchel et al. (2014) found that a combination of SB and MgO, supplemented to wethers, increased rumen turnover rate and transit time. The findings of our experiment disagree with Perez Ruchel et al. (2014), albeit in a different species, and the theory proposed by Russell and Chow (1993) for SB. Our findings also prove that there is no difference in klf or klp between any of the buffer treatments. We can postulate that the effects of CMA, CMA+MM, and, to a lesser extent, SB on rumen pH was not caused by changes in k1f or k1p and the mode-of-action is more likely to be a direct effect of the buffer treatments on hydrogen ion consumption from rumen fluid, hence the increase in pH. The *in-sacco* technique was used to determine the degradability and rate of degradation of soya hulls

in potentially different rumen environments. It was hypothesized that increased time spent below rumen pH 5.4, 5.6, and 5.8 would correspond to reduced degradability of soya hulls using the *in-sacco* technique due to cellulolytic activity diminishing as the pH falls (Russell and Wilson, 1996). However, in this experiment, no differences were detected between treatments for *in-sacco* DM degradability or the rate of DM degradation of soya hulls in the rumen. Previous work by Mould et al. (1983) reported that DM degradation in the rumen, determined by the *in-sacco* technique, was influenced by rumen pH. No previous work has been carried out investigating the effects of CMA, with or without MM, and SB on the *in-sacco* degradability or rate of degradation of DM in the rumen. The present study used only DM degradation to investigate the effects of rumen pH on fibre digestibility in the rumen. However, NDF *in-sacco* degradability may have been more sensitive to changes in rumen pH due to the contribution of cellulose to total NDF. It must be noted that there exists some limitations and discrepancies associated with the *in-sacco* technique. The work of Nozière and Michalet-Doreau (1996) demonstrated that fibrolytic activity within the nylon bags used for the *in-sacco* degradability determination was much lower than the fibrolytic activity measured on the rumen contents outside the nylon bags. According to Nozière and Michalet-Doreau (1996), one of the explanations for this difference in fibrolytic activity is that feed particles within the nylon bags are not exposed to normal movement throughout the rumen and exchanges with rumen medium like feed particles outside the nylon bags. Another theory proposed by Nozière and Michalet-Doreau (1996) for this lack of fibrolytic activity, is that the pH of the contents within the nylon bags can be reduced compared to feed particles outside the bag, which may be due to an accumulation of VFA within the nylon bags used for *in-sacco* degradability determination. It is worth noting that there was a large numerical difference between the control and the three buffer treatments for *in-sacco* degradability at 48 h. Even though this difference was not significant, it could be seen as an indicator of improved rumen conditions for fibre digestibility and may warrant future investigations.

4.6 Conclusion

The supplementation of rumen buffering products can alter rumen fermentation in lactating dairy cows fed a diet containing 45% DM forage, using typical forages and feeds of the northern European region. Calcareous marine algae, with or without MM, and SB promoted a more positive pH profile with increased mean rumen pH. The SB failed to prevent prolonged pH depressions to the same extent as both CMA treatments. The CON used during this trial could be considered to have caused SARA when fed on its own and when supplemented with SB. The addition of MM to diets already containing CMA had a positive impact on total tract NDF digestibility compared to CON and altered the concentrations and proportion of VFA in rumen fluid compared to CMA-only. We can conclude from this experiment that CMA, CMA+MM, and SB had no effect on the kinetics of digestion and their effects on rumen pH were not related to changes in klf. The findings of this research support the use of CMA, with or without MM, in diets that contain high levels of fermentable carbohydrates as a tool to prevent low rumen pH and the negative production and health consequences of low rumen pH. Furthermore, the comparisons of different buffers (CMA, with or without MM, Vs SB) and their ability to affect rumen pH will be useful for dairy producers and nutritionists.

4.7 Literature cited

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Table 4.1 Ingredient composition of the control, calcareous marine algae, with or without marine magnesium oxide, and sodium bicarbonate diets.

	Dietary treatments[1]			
Item	CON	CMA	CMA + MM	SB
Ingredients, % DM				
Wheat grain (finely ground)		34.7		
Grass silage		22.5		
Maize silage		22.5		
Soybean meal (48% CP)		10.9		
Citrus pulp	2.80	2.80	2.80	2.50
Soy hulls	2.80	2.80	2.80	2.50
Sugar cane molasses	1.40	1.40	1.40	1.40
Limestone	0.78	0.45	0.45	0.78
White salt	0.45	0.45	0.45	0.18
Mono-dicalcium phosphate	0.36	0.36	0.36	0.36
PFAD (spray) blend[2]	0.34	0.34	0.34	0.34
Trace element & vitamin premix[3]	0.30	0.30	0.30	0.30
Magnesium oxide	0.16	0.10	0.00	0.16
Calcareous marine algae[4]	0.00	0.45	0.45	0.00
Sodium bicarbonate	0.00	0.00	0.00	0.90
Marine magnesium oxide[5]	0.00	0.00	0.11	0.00

[1] Treatments: CON = control; CMA = calcareous marine algae; CMA+MM = calcareous marine algae and marine magnesium oxide; SB = sodium bicarbonate

[2] PFAD = Palm fatty acid distillate

[3] Lactating cow trace element & vitamin premix supplied by Nutribio Ltd, Cork, IE. Formulated to contain (per kg of DM) 340g of Ca, 0.04g of Co, 7.4g of Cu, 0.3g of I, 0.1g of Se, 16.6g of Mn, 25g of Zn, 8,000 IU of Vitamin A, 2,000 IU of Vitamin D3 and 10 IU of Vitamin E.

[4] Calcareous marine algae = Lithothamnion calcareum

[5] Marine magnesium oxide = precipitated magnesia derived from seawater

Table 4.2 The analysed and predicted chemical and nutrient profile of the control, calcareous marine algae, with or without marine magnesium oxide, and sodium bicarbonate diets.

	Dietary treatment[1]			
Item	CON	CMA	CMA + MM	SB
Chemical composition, % DM				
DM	40.1	40.1	40.1	40.1
Crude protein	15.7	15.9	15.9	15.4
PDIN[2]	10.5	10.5	10.5	10.5
PDIE[2]	10.5	10.5	10.5	10.5
NDF	27.2	26.1	26.8	27.0
f-NDF[3]	19.9	19.9	19.9	19.9
ADF	12.9	12.9	12.9	12.9
Ash	6.80	6.80	6.80	7.00
Starch	27.5	27.9	28.0	27.1
Sugar	5.10	6.20	5.25	5.80
Ca [2]	0.79	0.79	0.79	0.79
Mg [2]	0.20	0.20	0.20	0.20
Na [2]	0.26	0.26	0.26	0.31
UFL[2]	0.96	0.96	0.96	0.96
NE_L (Mcal/kg DM)[4]	1.60	1.60	1.60	1.60

[1] Treatments: CON = control; CMA = calcareous marine algae; CMA+MM = calcareous marine algae and marine magnesium oxide; SB = sodium bicarbonate

[2] As calculated by using INRAtion 4.07 feed formulations program, based on ingredient analyses: similar for all treatments. PDIN = protein truly digestible in the small intestine where nitrogen is limiting microbial protein synthesis, PDIE = protein digested in the small intestine where energy is limiting microbial protein synthesis, UFL = unit of energy for lactation.

[3] NDFforage = contribution of the forage component of the diet to NDF

[4] NE_L = Net energy for lactation, at production level, NRC (2001).

Table 4.3 The effect of calcareous marine algae, with or without marine magnesium oxide, and sodium bicarbonate on daily rumen pH parameters.

	Dietary Treatments[1]					
Item	CON	CMA	CMA+MM	SB	SEM	*P*
Mean pH	5.92^{ax}	6.22^{b}	6.32^{b}	6.15^{y}	0.08	0.04
Median pH	5.88^{ax}	6.15^{y}	6.22^{b}	6.05	0.09	0.13
Maximum pH	7.00^{a}	7.18^{x}	7.45^{b}	7.50^{by}	0.11	0.05
Minimum pH	4.90^{a}	5.48^{b}	5.40^{b}	5.18^{ab}	0.14	0.10
pH range	2.10^{x}	1.70^{ay}	2.05	2.33^{b}	0.14	0.10
pH CV, %	9.45^{a}	6.93^{b}	8.23^{abx}	9.88^{ay}	0.50	0.02
Time below pH 5.8 (mins/d)	662.5^{x}	237.5^{y}	305^{xy}	527.5^{xy}	93.6	0.06
Time below pH 5.6 (mins/d)	497.5^{a}	127.5^{b}	150.0^{b}	342.5^{ab}	67.0	0.02
Time below pH 5.4 (mins/d)	337.5^{a}	42.5^{b}	37.5^{b}	175.0^{ab}	50.6	0.02

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

[x, y] Means within a row with different superscripts differ ($P<0.10$)

[1] Treatments: CON = control; CMA = calcareous marine algae; CMA+MM = calcareous marine algae + marine magnesium oxide; SB = sodium bicarbonate.

[2] SEM = standard error of the mean

Table 4.4 The effect of calcareous marine algae, with or without marine magnesium oxide, and sodium bicarbonate on rumen VFA concentrations and molar proportions in rumen fluid.

	Dietary Treatments					
Item	CON	CMA	CMA+MM	SB	SEM	*P*
Total VFA, m*M*	159.8	173.4	156.2	166.8	6.44	0.26
Acetate, m*M*	109.6	120.3^{x}	103.4^{y}	114.3	4.86	0.11
Propionate, m*M*	22.44	24.84	25.99	22.79	1.48	0.29
Butyrate, m*M*	15.23	15.59	14.33	16.53	0.71	0.20
Iso-butyrate, m*M*	1.26	1.32^{x}	1.11^{y}	1.24	0.06	0.09
Valerate, m*M*	5.78	5.75	6.57	6.30	0.37	0.34
Iso-valerate, m*M*	5.49	5.61	4.79	5.70	0.28	0.12
Acetate: Propionate	4.89^{a}	5.06^{a}	4.07^{b}	5.06^{a}	0.21	0.01
Protein derived VFA, m*M*	12.54	12.69	12.46	13.24	0.47	0.65
Molar proportions, mol/100 mol						
Acetate	68.8^{x}	69.3^{a}	66.1^{by}	68.4	0.76	0.03
Propionate	14.2^{a}	14.1^{a}	17.0^{b}	14.0^{a}	0.59	<0.01
Butyrate	9.51	9.35	9.23	9.96	0.39	0.58
Iso-Butyrate	0.95	0.92	0.88	0.90	0.05	0.80
Valerate	3.34	3.26	3.96	3.63	0.24	0.16
Iso-valerate	3.18	3.08	2.85	3.15	0.14	0.38

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

[x, y] Means within a row with different superscripts differ ($P<0.10$)

[1] Treatments: CON = control; CMA = calcareous marine algae; CMA+MM = calcareous marine algae + marine magnesium oxide; SB = sodium bicarbonate.

[2] SEM = standard error of the mean

[3] *P*-value for treatment effect

Table 4.5 The effect of calcareous marine algae, with or without marine magnesium oxide, and sodium bicarbonate on apparent total tract digestibility of the diet.

	Dietary Treatments[1]					
Apparent Digestibility (%)	CON	CMA	CMA+MM	SB	SEM[2]	*P*[3]
DM	70.85	72.51	72.51	70.02	0.90	0.16
OM	73.65	75.12	75.35	73.65	0.81	0.30
NDF	44.42[a]	48.13	51.16[b]	47.47	1.66	0.06
Starch	99.43[a]	99.14[b]	99.34[a]	99.34[a]	0.04	<0.01

[a, b] Means within a row with different superscripts differ ($P<0.05$)
[x, y] Means within a row with different superscripts differ ($P<0.10$)
[1] Treatments: CON = control; CMA = calcareous marine algae; CMA+MM = calcareous marine algae + marine magnesium oxide; SB = sodium bicarbonate.
[2] SEM = standard error of the mean
[3] *P*-value for treatment effect

Table 4.6 The effect of calcareous marine algae, with or without marine magnesium oxide, and sodium bicarbonate on rumen turnover rate, rumen retention time, and *in-sacco* degradability constants of soya hulls in the rumen.

	Dietary Treatments[1]					
Variable	CON	CMA	CMA+MM	SB	SEM[2]	*P*[3]
klp[4], % per h	2.55	4.28	3.95	3.58	0.56	0.24
klf[5], % per h	12.81	11.86	14.11	12.20	1.51	0.74
RRT[6], h	41.83	25.95	26.78	28.71	4.69	0.16
In-sacco degradability fractions, %						
a	16.59[ax]	13.95	12.53[b]	13.06[y]	0.89	0.07
b	67.16	83.88	81.34	75.87	4.94	0.18
c, % per h	3.32	3.00	3.55	4.31	0.50	0.38
Potential[7]	83.75	97.83	93.87	88.93	4.70	0.27
Effective[8]	54.96	49.40	50.81	52.69	4.17	0.80
Total *in-sacco* degradability, %						
8 h	29.92	33.48	30.48	33.58	1.75	0.38
16 h	46.17	44.03	47.32	53.18	2.53	0.17
24 h	49.96	52.28	52.81	55.03	3.01	0.71
48 h	70.47	81.05	81.77	79.25	3.85	0.24

[a, b] Means within a row with different superscripts differ ($P<0.05$)
[x, y] Means within a row with different superscripts differ ($P<0.10$)
[1] Treatments: CON = control; CMA = calcareous marine algae; CMA+MM = calcareous marine algae + marine magnesium oxide; SB = sodium bicarbonate.
[2] SEM = standard error of the mean
[3] *P*-value for treatment effect
[4] klp = rumen particulate turnover rate
[5] klf = rumen fluid turnover rate
[6] RRT = rumen retention time (1/klp)
[7] Potential degradability = a + b fractions
[8] Effective degradability, calculated using a, b, c constants and klp

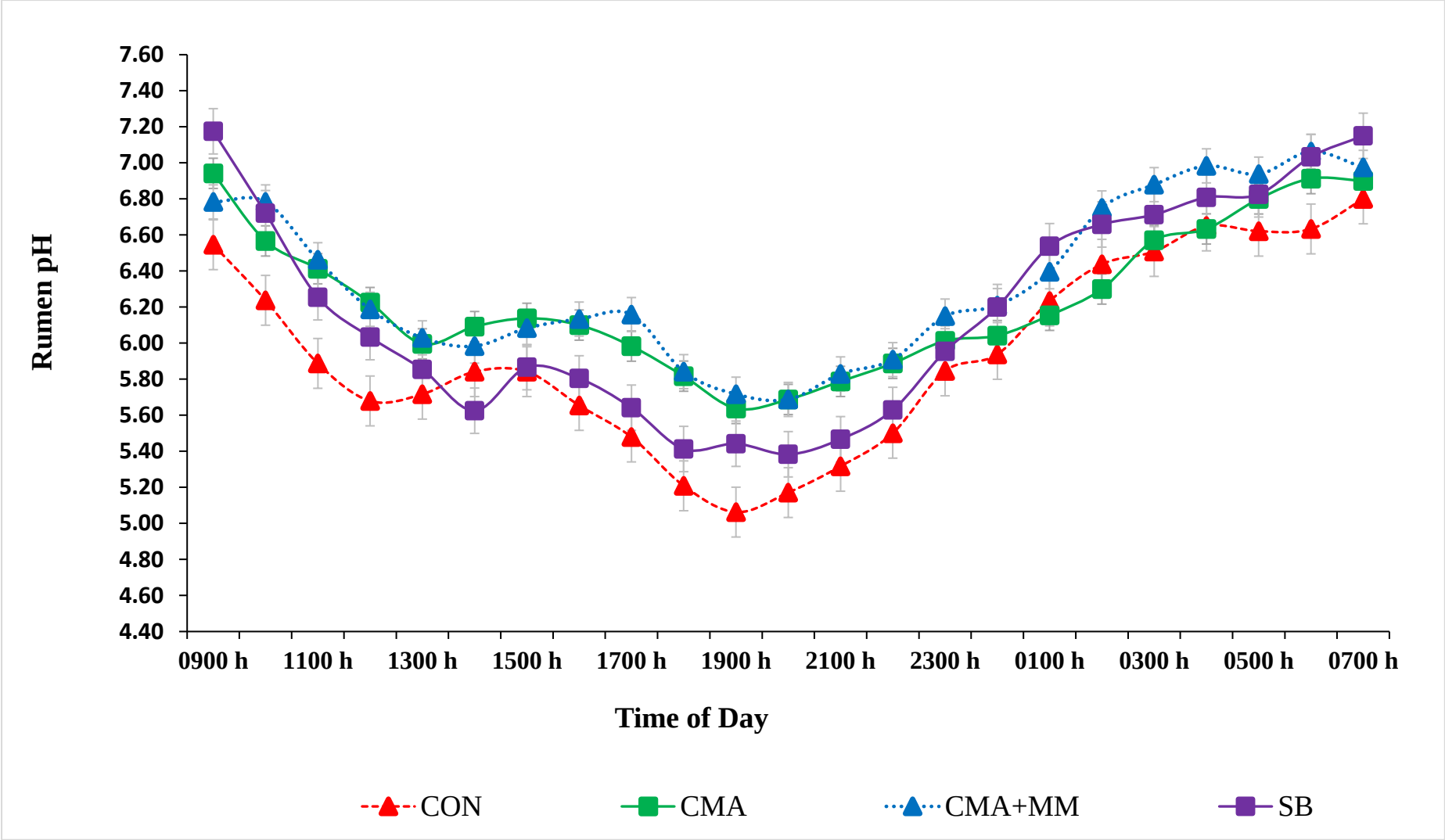

Figure 4.1 The effect of calcareous marine algae, with or without marine magnesium oxide, and sodium bicarbonate on diurnal rumen pH over a 24 h period.

Chapter 5.

Effects of calcareous marine algae on rumen pH and fermentation, and plasma inflammatory markers during a grain and ryegrass induced SARA challenge in dairy cows

5.1 Abstract

The primary objective of this experiment was to determine the effects of calcareous marine algae (CMA; Acid Buf, Celtic Sea Minerals) on rumen pH, rumen fermentation, and plasma inflammatory markers during a grain and ryegrass induced sub-acute ruminal acidosis (SARA) challenge. The secondary objective of this experiment was to compare the effects of a grain-induced (GR) SARA challenge and a ryegrass-induced (RY) SARA challenge. Eight ruminally cannulated Holstein dairy cows were assigned to 4 experimental treatments in a 2 × 2 split-plot crossover design. The main plot was diet during the SARA challenge, GR or RY, and the sub-plot was treatment, control (CON) or CMA. The experiment consisted of 3 phases: acclimatisation (ACC), d 1 – 20; challenge (CHL), d 21 – 30; and recovery (REC), d 31 – 38. The cows remained on their main plots, GR or RY, throughout the experiment and alternated from CON to CMA and vice-versa as they progressed from period 1 to period 2 of the experiment. For the GR CHL, each cow was offered a TMR consisting of 30% or 7.1 kg dry matter (DM) of ground wheat barley mix (50% wheat and 50% barley) along with grass silage, maize silage, soybean meal, and the experimental concentrate pellet. For the RY CHL, cows were offered fresh ryegrass, cut at the 3-leaf stage or equivalent to 1,000 - 1,200 kg DM/ ha of a pre-cutting sward mass, and 3.7 kg DM per cow of the experimental concentrate pellet fed in 2 equal portions during a.m. and p.m. milking each day. Both GR and RY diets successfully induced SARA. The CMA treatment reduced the minutes below rumen pH 5.4 (- 33.5 min/d), 5.6 (- 66.6 min/d), and 5.8 (- 76.6 min/d) during the ACC phase, and minutes below rumen pH 5.6 (- 76.5 min/d) and 5.8 (- 110.3 min/d) during the REC phase. Treatments or diets did not differ for rumen pH parameters during the CHL phase. Rumen lipopolysaccharide (LPS) concentration was increased during the CHL on both diets, but the RY diet had a lower concentration of rumen LPS (30,834 Vs 56,235 endotoxin units/ml) compared to the GR diet. Plasma serum amyloid A concentration was reduced on the RY-CMA treatment (- 37.39 µg/ml) compared to the RY-CON during the CHL phase. There was a greater reduction (- 138%) in SAA with the CMA treatment compared to the CON treatment from the ACC to the CHL phase. These findings demonstrate that a RY-induced SARA CHL can induce similar rumen pH depressions and levels of plasma inflammatory markers, but lesser concentrations of rumen LPS compared to a GR-induced SARA CHL. The use of CMA in such CHL diets can reduce the extent of inflammation and can have positive effects on rumen pH when feeding high forage (59%) diets.

5.2 Introduction

Dairy cows require highly fermentable diets to meet their nutritional demands during lactation. Consumption of diets containing highly fermentable carbohydrates and moderate levels of physically effective fibre stimulate microbial growth in the rumen and can lead to an accumulation of VFA and a reduction in rumen pH (Humer et al., 2018). Periods of persistently low rumen pH are defined as SARA. Thresholds such as 3 h/ d less than rumen pH 5.6 are commonly used to provide a more specific definition of SARA in dairy cows (Plaizier et al., 2008). The incidence of SARA is significant with some authors reporting 11% of cows in pasture-based systems (O'Grady et al., 2008) and 20% of cows in TMR feeding systems (Kleen et al., 2003) affected. Sub-acute ruminal acidosis and low rumen pH have many negative consequences such as reduced fibre digestion (Mulligan et al., 2002), negatively altered microbial populations (Khafipour et al., 2009a), reduced DMI (Penner et al., 2007), reduced milk fat production (Allen, 1997) and health issues such as laminitis and rumen epithelial damage (Steele et al., 2011). In more recent years, SARA as a predisposition to dietary induced inflammation has been investigated in more detail (Bradford et al., 2015). Khafipour et al. (2009a) reported that an experimentally induced grain-based SARA challenge triggered the translocation of lipopolysaccharide (LPS) from the rumen or digestive tract to the peripheral system. Lipopolysaccharides are derived from the cell wall of gram-negative bacteria and are released by lysing or shedding during periods of rapid growth by these gram-negative bacteria (Chiquette et al., 2015). The translocation of LPS into the blood circulation triggers an inflammatory response (Plaizier et al., 2014). During SARA or rumen fermentation disruptions, the rumen epithelium is damaged which greatly increases the chance of LPS translocation to the blood stream (Steele et al., 2011). Experimentally induced grain-based SARA challenges are effective in reducing mean rumen pH, increasing time below pH 5.6, increasing rumen LPS concentrations, and subsequently invoking an inflammatory response through increased concentrations of inflammatory markers detected in serum or plasma (Gozho et al., 2007; Emmanuel et al., 2008; Khafipour et al., 2009a). Rumen pH can be depressed, and SARA induced in diets containing low levels of starch through particle size reduction or a deficiency in physically effective fibre (Beauchemin et al., 2003). Khafipour et al. (2009b) experimentally induced an alfalfa-based SARA challenge, by replacing alfalfa hay with alfalfa pellets, and effectively reduced mean rumen pH, increased time below pH 5.6, and increased rumen LPS concentration but did not detect any increases in plasma or serum inflammatory markers. Therefore, the inflammatory response of cows fed highly fermentable or SARA

inducing diets is dependent on diet type. Over the last 20 years, the susceptibility of pasture-based dairy cows to SARA has been highlighted, despite consuming low to moderate levels of starch, and a forage that is not mechanically altered (e.g., pelleting) to reduce particle size (Bramley et al., 2008; O'Grady et al., 2008). To the best of the authors knowledge, the effects of a ryegrass-induced SARA on rumen pH parameters, rumen LPS concentrations, and plasma inflammatory markers have not been investigated previously.

The use of rumen buffers in dairy cows has been thoroughly investigated and proven to increase rumen pH and prevent SARA (Enemark, 2008). However, the role of rumen buffers in preventing SARA-induced inflammation has not received the same level of investigation. Calcareous marine algae (CMA) [Acid Buf; Celtic Sea Minerals] is an animal feed supplement produced from *Lithotamnion* sp. that has been used as a rumen buffer and source of Ca in dairy cow diets (Bernard et al., 2014). Previous research has demonstrated the ability of CMA to increase rumen pH, reduce the time spent below rumen pH 5.5, and increase total rumen VFA concentration (Cruywagen et al., 2015). Previous work by our research group (Neville et al., 2019) found that CMA increases rumen pH and prevents SARA, while also increasing milk fat yield and total milk fat and protein yield. *In-vitro* research using human macrophage cells, discovered CMA to have an anti-inflammatory role by inhibiting nuclear factor kappa B activation (O Gorman et al., 2012). Results from chapter 3 reported a tendency for reduced plasma inflammatory marker, serum amyloid A (SAA), concentration in cows fed CMA during the Transition period, a period where the risk of SARA is high (Penner et al., 2007). Most of the research carried out on CMA in dairy cows has been in TMR feeding systems. Rafferty et al. (2019) reported a reduction in time spent below reticulo-rumen pH 5.8 and an increase in milk yield when a CMA and marine MgO combination product was compared to a control treatment in pasture-fed dairy cows. The primary objective of this experiment was to determine the effects of CMA on rumen pH, rumen fermentation products, and plasma inflammatory markers during a grain and ryegrass induced SARA challenge. The secondary objective of this experiment was to compare the effects of a grain-induced SARA challenge and a ryegrass-induced SARA challenge. We hypothesise that the GR-CMA and ZG-CMA treatments will increase rumen pH, reduce time spent below rumen pH 5.6 and 5.8, and reduce the concentration of plasma inflammatory markers compared to the GR-CON and ZG-CON during both the grain-induced and ryegrass-induced SARA challenges. Our hypothesis was informed by a review of the previous experiments showing the effects of CMA on rumen pH and milk

production in dairy cows, and its anti-inflammatory effects in the *in-vitro* work carried out by O Gorman et al. (2012). Our secondary hypothesis was that both grain and ryegrass induced SARA challenges would reduce rumen pH and increase the time spent below pH 5.6 but only the grain-induced SARA challenge would invoke an inflammatory response.

5.3 Materials and methods

All procedures described in this experiment were approved by the Animal Research Ethics Committee (AREC) at University College Dublin (UCD) and conducted under experimental license from the Health Products Regulatory Authority (HPRA) under the European directive 2010/63/EU and S.I. No. 543 of 2012. Each person who carried out procedures on experimental animals, during this experiment, was authorized to do so by means of individual authorization from the HPRA.

5.3.1 *Experimental design and feeding management*

Total VFA concentration was considered to have one of the highest levels of variation among the variables that were measured in this experiment. A sample size calculation based on 80% power and a significance level of 0.05 based on total VFA concentration, was used to determine sample size for this experiment. A CV for total VFA concentration of 6.75% and an expected difference of 15% was estimated by averaging the results of previous studies (Al Ibrahim et al., 2012; Whelan et al., 2013) and it was determined that the sample size for this experiment was n = 4. Eight ruminally cannulated Holstein dairy cows averaging 685 ± 15 kg of BW, 72 ± 9 DIM, and 6,918 ± 242 kg of milk in their previous 305 d lactation were selected from the dairy herd at UCD Lyons Farm, Co. Kildare, IE and assigned to their experimental treatments in a 2 × 2 split-plot crossover design. Within the 2 × 2 split-plot design, the main plot was diet type during the SARA challenge, grain (GR) or ryegrass (RY), and the sub-plot was treatment, control (CON) or CMA (CMA) at 100 g DM/ cow per d. Therefore, the experiment design included 4 experimental treatments: GR-CON; GR-CMA; RY-CON; and RY-CMA. Two experimental periods consisted of 38 d divided into 3 phases: acclimatisation (ACC), d 1 – 20; challenge (CHL), d 21 – 30; and recovery (REC), d 31 – 38. The 20-d ACC phase was required as a rumen acclimatisation for the CMA additive. There was also an 8-d washout period between period 1 and 2. The cows remained on their main plots, GR or RY, throughout the experiment and alternated from CON to CMA and vice-versa as they progressed from period 1 to period 2 of the experiment. The ingredient compositions of the dietary treatments are outlined in Table 5.1. The CON and CMA treatments were fed through 2 different concentrate pellets. The only difference between the CON and CMA experimental concentrate pellets was the replacement of limestone (2.3%) with CMA (2.7%) and additional soya hulls (+ 0.4%) in the control. The experimental concentrate pellets, and hence the total diets, were balanced for

Ca and all other major minerals. The inclusion rate of the CMA additive was based on a predicted daily DMI of 22 kg DM during the ACC and REC, and 22.5 kg DMI during the CHL phase to ensure a daily intake of 100g of CMA per cow per day. The dosage rates of CMA were determined from previous experiments (Wu et al., 2015).

During the ACC and REC phases, cows received *ad-libitum* access to a low starch mid-lactation TMR containing a high level of NDF from forage (f-NDF). Two types of SARA challenge were induced during the CHL phase. The GR induced SARA consisted of *ad-libitum* access to a TMR consisting of 30% or 7.1 kg DM/ cow of ground wheat and barley mix (50% wheat and 50% barley) along with grass silage, maize silage, soybean meal, and the experimental concentrate pellet. The GR TMR was fed once per d at 0930 h and cows received 100% of their diet through the TMR. During the RY SARA challenge, cows were offered *ad-libitum* access to fresh ryegrass, cut at the 3-leaf stage or equivalent to 1,000 - 1,200 kg DM/ ha of a pre-cutting sward mass, and 3.7 kg DM per cow of the experimental concentrate pellet fed in 2 equal portions during a.m. and p.m. milking each day.

The ACC, CHL, and REC diets were formulated to supply 100 % of the energy requirements of a 650 kg lactating dairy cow yielding 32 kg of milk/ d containing 4.0 % of fat and 3.4 % of protein (INRA, 2018). The ACC and REC diets had a predicted DMI of 22 kg DM/ cow per d and a feed allocation of 23.1 kg DM/ cow per d, to allow for 5 % refusals. The CHL diets had a predicted DMI of 22.5 kg DM/ cow per d and a feed allocation of 23.6 kg DM/ cow per d. The ACC and REC diets were formulated to contain: 40% concentrate, 21% starch and sugar, and 29% NDF from forage, based on grass silage, maize silage, and concentrate. The GR CHL diet was formulated to contain: 58% concentrate, 39% starch and sugar, and 19.5% NDF from forage. The RY CHL diet was formulated to contain: 16% concentrate, 18% starch and sugar, and 33% NDF from forage.

Cows had ad-libitum access to TMR for 22 h every d through specific computerized feeding boxes (RIC System, Insentec B.V.) during the ACC and REC phases. The cows receiving the GR-CON and GR-CMA also had *ad-libitum* access to their TMR through the feeding boxes. The RY-CON and RY-CMA cows had *ad-libitum* access to zero-grazed ryegrass through the feeding boxes and received the experimental concentrate pellet in the milking parlour. The cows were trained to use the computerized feeding stations prior to the experiment. All cows were introduced to the feeding stations and fed an ACC CON diet for 7 d before the experiment as an acclimatization to the feeding system and the barn. Each TMR treatment was mixed

separately with a Keenan Feeder (Keenan Feeding Systems), receiving the same total mixing time, 10 min after all the ingredients were added to the mixer wagon. Water was available ad-libitum and water troughs were cleaned daily.

The fresh grass and grass silage used consisted predominantly of perennial ryegrass (*Lolium perenne*). The crop, for grass silage, was cut using a mower-conditioner during the early boot stage of vegetation (growth stage 41) (Zadoks et al., 1974), wilted for 16 h and harvested with a Krone Big X (Krone GmbH & Co) forage harvester (mean particle length 50 mm). The crop was then ensiled under a black polythene cover without the use of an additive. The maize used for maize silage (Zea maiz, variety Tekni) was grown with the aid of plastic film (Samco Agricultural Manufacturing Ltd). The crop was harvested at the dough stage (growth stage 85) (Lancashire et al., 1991) using a Krone Big X (Krone GmbH & Co.) precision chop forage harvester (mean particle length 25mm). The harvester was equipped with a kernel processor to improve starch digestibility. The harvested maize silage was ensiled under a black polythene cover without the use of an additive.

Prior to the experiment, fresh samples of both grass and maize silage were sent to a forage laboratory (Trouw Nutrition) for near infrared (NIR) analysis (FOSS NIR systems 5000) to aid with the accurate formulation of the basal diets for ACC, CHL, and REC. The experimental concentrate was fed in the form of a pellet and added to the TMR to aid in mixing accuracy. The experimental concentrate pellet was manufactured by McAuley Feeds Ltd.

5.3.2 Animal care and housing

The experiment was carried out between May and August 2019. Each cow was on the experiment for 84 d: 38 d in period 1, 8 d of a washout period, and 38 d in period 2. Cows were housed in a free stall barn with 3 stalls per cow and 1 computerized feeding box per cow available throughout the experiment. Cows were milked twice daily, at 0730 h and 1530 h, in a rotary milking parlour (Dairymaster) and stalls were also cleaned, with new sawdust bedding added, twice daily.

5.3.3 Data collection, sampling procedures, and sample analyses

Daily milk yields were automatically recorded using the Weighall milk meter system (Dairymaster). Milk samples were collected on the last two d during each phase. Each day consisted of an a.m. and p.m. sample, pooled in proportion to the specific a.m. and p.m. yields

to create one milk sample per day and two milk samples per cow for each phase. Samples were preserved (Broad Spectrum Microtabs II, D&F Control Systems Inc.) and stored at 4°C until analysed. Concentrations of total milk fat, protein, and lactose were determined in a commercial milk laboratory (Progressive Genetics) using infrared analysis (CombiFoss 5000, Foss Analytical). Values for ECM were calculated using the following formula:

ECM = (0.3273 × milk yield kg) + (7.65 × milk protein kg) + (12.97 × milk fat kg) (Tyrrell and Reid, 1965).

Rumen pH measurements were collected over the last 5 d of each phase. Rumen pH was measured every 15 min using intraruminal pH boluses (eCow Ltd, Devon, UK). The pH boluses were weighted so that they would sit in the ventral sac region of the rumen. Boluses were removed from the rumen, cleaned, re-calibrated with pH 4.0 and 7.0 standards, and pH data downloaded every day at 0800 h. Measurements recorded over the 5 d were averaged to create one 24 h diurnal pattern of rumen pH. Daily mean, median, maximum, minimum, range, CV, and time spent below pH 5.4, 5.6, and pH 5.8 were also calculated from this data.

Rumen fluid samples were harvested, from different parts of the rumen, on the final d of each phase (ACC d 20; CHL d 30; and REC d 38) via the rumen cannula. Samples were collected 3 times per d: before morning feeding (0900 h); 6 h after feeding (1530 h); and 12 h after feeding (2130 h). Rumen fluid was obtained using a collection tube (Bar Diamond Inc.) and a 60-mL disposable syringe. Once collected, samples were strained through 4 layers of cheesecloth, and a 4-mL aliquot drawn off using an automatic pipette. Samples were subsequently acidified by mixing with 1 mL of trichloroacetic acid (50% wt/vol) before freezing at -20°C. During analysis, samples were allowed to thaw in the refrigerator for 16 h at 4°C before centrifuging at 2,100 × g for 10 min at 4°C. One millilitre of supernatant was diluted 1 in 5 with distilled water (dH_2O) and then centrifuged at 1,600 × g for 15 min at 4°C. Next, 200 µL of supernatant was combined with 3 reagents and used to determine NH_3 concentrations using a spectrophotometer. Rumen fluid was prepared for VFA analysis by mixing 250 µL of rumen fluid with 3.75 mL of dH_2O and 1 mL of internal standard solution (0.5 g of 3-methylvaleric acid in 1,000 mL of 0.15 *M* oxalic acid). The resulting solution was centrifuged at 1,600 × g and filtered through a syringe-tip filter (PTFE, 25-mm diameter, 0.45 µm) into 2-mL GC vials. Concentrations of VFAs were determined using Scion 456-GC (Scion Instruments, Scotland, UK) fitted with DB-FFAP capillary column (15m x 0.53mm; 1.00 µm, Agilent Technologies, USA). Osmolality of rumen fluid samples was determined by vapour pressure osmometry using Wescor Vapro 5600 osmometer.

For rumen LPS analysis, a separate 40 mL portion of filtered rumen fluid was transferred into a 50-mL sterile tube and kept on ice until centrifugation (10,000 × g at 4°C for 45 min). The supernatant was filtered through a 0.22-µm LPS-free filter (Sarstedt). The filtrate was collected in a sterile, depyrogenated glass tube (previously heated at 180°C for 4 h) and then heated at 100°C for 30 min in a water bath. Samples were cooled at room temperature (19°C) for 10 min and stored in pyrogen-free glass tubes at −20°C for subsequent LPS measurement according to the method described in Gozho et al. (2005). Briefly, free rumen LPS content was determined by a chromogenic limulus amoebocyte lysate (LAL) end-point assay (Kinetic-QCL, Lonza Group Ltd). Pre-treated rumen samples were diluted until their LPS concentrations were in the range of 0.1 to 1 endotoxin units (EU)/mL relative to the reference endotoxin (Escherichia coli O111:B4) and assayed. The assay was performed using a 96-well microplate kit (Lonza Group Ltd) with absorbance read at 405 nm on a microplate reader.

Blood samples were harvested by jugular venepuncture on the last d of each phase (ACC d 20; CHL d 30; and REC d 38), at 0900 h and 1530 h to correspond with the 1st and 2nd rumen fluid sampling. Blood samples were collected into evacuated tubes (BD, Oxford, UK), which were coated with lithium heparin. Upon collection, tubes were placed on ice until processed accordingly. Samples were immediately centrifuged at 2,100 × g and 4 ^{o}C for 10 mins to obtain plasma for serum amyloid A (SAA), haptoglobin (Hp), and lipopolysaccharide binding protein (LBP) determination. Serum amyloid A and Hp were measured using ELISA kits (Tridelta Diagnostics Ltd.) as described by Gozho et al. (2007). Plasma concentrations of LBP were measured using a commercially available kit (HK503, Hycult Biotech).

Samples of TMR, fresh ryegrass, and refusals were collected daily and dried at 104°C in a forced air oven for 16 h to establish the DM content of the TMR, fresh ryegrass, and refusals. Individual daily feed intakes were recorded on the computerized feeding system and used, in combination with daily TMR, ryegrass, and refusals DM content to calculate DMI. Samples of TMR and fresh ryegrass were taken from their respective dietary treatments during feed-out 3 times per week, pooled into weekly samples by treatment, and stored at - 20°C until they were analysed. Concentrate pellets, soybean meal, unmolassed beet pulp, grass silage, maize silage and straw samples were collected weekly and stored at - 20°C prior to analysis. Subsamples of the composite TMR, ryegrass, maize silage, grass silage, barley straw, soybean meal, unmolassed beet pulp, concentrate pellets, and refusals were dried at 55°C for 72 h. The subsequent dried samples were ground using a Norris hammer mill fitted with a 1 mm screen (Lab Mill, Christy Turner). Ash content was determined by incineration of a 5 g sample in a muffle furnace (Nabertherm GmbH, Lilienthal, Germany) at 550°C for 5.5 h. The N content of

the feed was determined by combustion using a Leco 528 instrument (Leco Instruments UK). Crude protein was then calculated using N × 6.25. Neutral detergent fibre and acid detergent fibre (ADF) were determined according to the method of Van Soest et al. (1991) using the Ankom 220 Fiber Analyzer (Ankom™ Technology). As part of the NDF procedure, both sodium sulphite (Na_2SO_3) (Ankom Technology) and heat stable alpha-amylase (Ankom Technology) were used for the analysis of TMR, maize silage, barley straw, and concentrate pellet subsamples while only sodium sulphite (Na_2SO_3) was used for ryegrass, grass silage, and unmolassed beet pulp subsamples. Starch content was determined using the Megazyme Total Starch Assay Procedure (product no: K-TSTA; Megazyme International Ltd). Water Soluble Carbohydrate (WSC) was analysed according to the method used by Birch et al. (1974). Ether extract was measured using a Soxtec instrument (Tecator) according to the method of AOAC 107 (1970).

5.3.4 Data screening and statistical analyses

Data residuals were examined for normality using the UNIVARIATE procedure of SAS (version 9.4, SAS Inst.). Following assessment of normality, outliers were removed (± 3 STD from the mean). Milk production, rumen pH, rumen fermentation products, rumen LPS, and plasma inflammatory marker data were analysed as repeated measures using the MIXED procedure (SAS, version 9.4). The effect of whole-plot and sub-plot treatments were analysed within each phase of the experiment separately and the overall effect of phase during this experiment was also analysed. The model included fixed effects of whole-plot (diet), sub-plot (treatment), period, sequence within whole-plot, time, whole-plot by sub-plot interaction, and whole-plot by sub-plot by time interaction with cow nested within whole-plot considered as the random effect. The time variable for milk production and rumen pH variables was d while h within d was the time variable for rumen VFA, ammonia, osmolality, and LPS, and the plasma inflammatory markers. Day was modelled as the repeated measure for the milk production and rumen pH data and the variance-covariance structures were selected based on the lowest Bayesian Information Criterion (BIC). All the milk production variables, and DMI during ACC, CHL, REC, and the overall phase effect required a compound symmetry covariance structure. The time below rumen pH 5.4, 5.6, and 5.8 during ACC, CHL, REC, and the overall phase effect required a compound symmetry heterogenous covariance structure. All the other daily rumen pH variables during ACC, CHL, REC, and the overall phase effect were

analysed using a compound symmetry covariance structure. Hour within d was modelled as the repeated measure for rumen VFA, ammonia, osmolality and LPS, and the plasma inflammatory markers. The variance-covariance structures for these variables were also selected based on the lowest BIC. All the plasma inflammatory markers during ACC, CHL, REC, and the overall phase effect required a compound symmetry covariance structure. Rumen ammonia, total VFA, butyrate, valerate, and protein derived VFA during ACC, CHL, REC, and the overall phase effect required a compound symmetry covariance structure, while all the other rumen parameters, including rumen LPS, during ACC, CHL, REC, and the overall phase effect required a compound symmetry heterogenous covariance structure. All data presented in Tables 5.3. – 5.11. are expressed as LSM ± SEM. Statistical significance was declared at $P < 0.05$.

5.4 RESULTS

5.4.1 Chemical analysis of TMR

The nutrient composition of the dietary treatments during ACC, CHL, and REC are presented in Table 5.2. Values for PDIN, PDIE, UFL and NE_L are predicted based on the INRAtion 4.07 computer program and NRC (2001).

5.4.2 Overall phase effects

The effect of phase on DMI, milk production, rumen pH, rumen fermentation products, rumen LPS, and plasma inflammatory markers is outlined in Table 5.3. Dry matter intake ($P < 0.01$) and milk protein concentration ($P = 0.03$) were higher during the CHL phase compared to the ACC phase, while ECM yield ($P = 0.03$), fat + protein yield ($P = 0.03$), and fat concentration ($P < 0.01$) were reduced during the CHL phase compared to the ACC phase. There was no difference in milk yield or lactose concentration between the ACC and CHL phases. Apart from milk protein concentration ($P = 0.01$), which was higher during the REC phase, there was no difference in DMI or any of the milk production variables when the ACC phase was compared to the REC phase. Dry matter intake was reduced ($P < 0.01$), and milk fat concentration increased ($P = 0.03$) during the REC phase compared to the CHL phase. There was no other difference in milk production variables between the CHL and REC phases.

Mean pH ($P < 0.01$) and minimum pH ($P < 0.01$) were reduced, and mins below pH 5.6 ($P < 0.01$) and 5.8 ($P < 0.01$) increased during the CHL phase compared to the ACC and REC phases. Mean pH ($P < 0.01$) was greater and mins below pH 5.6 ($P = 0.02$) and 5.8 ($P < 0.01$) lesser during the ACC phase compared to the REC phase. There was a tendency for minimum pH ($P = 0.08$) to be greater in the ACC phase compared to the REC phase. Rumen osmolality ($P < 0.01$) and concentrations of propionate ($P < 0.01$) and LPS ($P < 0.01$) were increased during the CHL phase compared to the ACC and REC phases. There was no difference in the rumen concentrations of total VFA, acetate, and butyrate during the ACC and CHL phase. Rumen concentrations of total VFA ($P = 0.05$), butyrate ($P = 0.05$), and LPS ($P = 0.03$) were greater during the REC phase compared to the ACC phase. There were no differences in rumen osmolality or rumen concentrations of acetate and propionate between the ACC and REC phases. Rumen concentration of acetate ($P = 0.02$) was lower, while total VFA and butyrate were similar during the CHL phase and the REC phase. The concentration of SAA in plasma was lower during the REC phase compared to the ACC ($P < 0.01$) and CHL ($P = 0.01$) phase but there was no difference between the ACC and CHL phases. Plasma concentration of Hp did not differ between any of the phases. Concentrations of LBP in plasma was lower during the ACC compared to the CHL ($P < 0.01$) and REC ($P < 0.01$) phases but CHL and REC did not differ from each other.

5.4.3 Acclimatisation

The effect of diet was not included during the ACC phase as the different diet types were not imposed until the CHL phase. The effect of the CON and CMA treatments during the ACC phase is reported in Table 5.4. There was no difference in DMI or any of the milk production variables between cows fed CON and cows fed CMA during the ACC phase. The CV ($P < 0.01$) and range ($P = 0.05$) of daily rumen pH, and the minutes below rumen pH 5.4 ($P < 0.01$), 5.6 ($P < 0.01$), and 5.8 ($P < 0.01$) were reduced on the CMA treatment compared to the CON treatment. There were no treatment differences detected among the other rumen pH variables. The CON treatment increased rumen *iso*-butyrate concentration ($P = 0.04$) compared to the CMA treatment. Rumen osmolality, acetate: propionate ratio, and concentrations of ammonia, total VFA, acetate, propionate, butyrate, valerate, *iso*-valerate, and protein derived VFA were similar between the CON and CMA treatments. Rumen LPS concentration was not different between the CON and CMA treatments. Plasma concentrations of SAA ($P < 0.01$) and LBP (P

< 0.01) were higher on the CMA treatment compared to the CON treatment during the ACC phase. There was no difference in plasma Hp concentration between the CON and CMA treatments.

5.4.4 Challenge

The effects of the GR-CON, GR-CMA, RY-CON, and RY-CMA treatments, diet, and diet by treatment interaction on DMI and milk production during the CHL phase are outlined in Table 5.7. There was a significant diet by treatment interaction for DMI ($P = 0.02$), due to numerically higher DMI on the GR-CMA versus GR-CON but numerically lower DMI on RY-CMA versus RY-CON. The RY diet increased milk fat concentration ($P = 0.04$) and tended to increase milk fat yield ($P = 0.10$) compared to the GR diet during the CHL phase. There were no other treatment differences detected for DMI or any of the other milk production variables. The effects of the GR-CON, GR-CMA, RY-CON, and RY-CMA treatments, diet, and diet by treatment interaction on daily rumen pH, rumen osmolality, and rumen concentrations of ammonia and VFA during the CHL phase are displayed in Table 5.8. There was no effect of treatment on any of the rumen pH variables. The GR and RY diets were similar for mean rumen pH, time below pH 5.4, 5.6, and 5.8, and all the other rumen pH parameters. The RY diet increased rumen concentrations of ammonia ($P < 0.01$), acetate ($P = 0.03$), and butyrate ($P = 0.05$) while tending to reduce valerate ($P = 0.07$) compared to the GR diet during the CHL phase. There were no treatment differences detected among the other rumen fermentation products.

Table 5.10. reports the effects of GR-CON, GR-CMA, RY-CON, and RY-CMA treatments, diet, and diet by treatment interaction on rumen LPS concentration and plasma concentrations of SAA, Hp, and LBP. The RY diet had lower rumen LPS ($P < 0.01$) compared to the GR diet. There were no other treatment effects for rumen LPS concentration. There was an overall effect of treatment on plasma concentration of SAA, with CMA reducing concentrations of SAA compared to the CON treatment ($P = 0.05$). Plasma SAA tended to be reduced on the RY-CMA ($P = 0.08$) treatment compared to the RY-CON treatment. There were no treatment or diet effects detected for plasma Hp or LBP concentration during the CHL phase.

5.4.5 Recovery

The effects of the GR-CON, GR-CMA, RY-CON, and RY-CMA treatments, diet, and diet by treatment interaction on daily rumen pH, rumen osmolality, and rumen concentrations of ammonia and VFA during the REC phase are outlined in Table 5.9. The CV of daily rumen pH was reduced on the RY-CMA ($P < 0.01$) treatment compared to the RY-CON treatment. Maximum rumen pH was reduced on the GR-CMA ($P = 0.03$) treatment compared to the GR-CON treatment, and on the RY-CMA ($P < 0.01$) treatment compared to the RY-CON treatment. The RY-CMA treatment reduced the mins spent below pH 5.6 ($P = 0.05$) and tended to reduce the mins spent below pH 5.8 ($P = 0.10$) compared to the RY-CON treatment. There were no other treatment differences detected across the other daily rumen pH variables. Acetate to propionate ratio tended to be reduced ($P = 0.06$) on the RY diet compared to the GR diet during the REC phase. The CMA treatment increased *iso*-butyrate ($P = 0.01$) and valerate ($P = 0.02$) and tended to increase *iso*-valerate ($P = 0.06$) compared to the CON treatment in both the GR and RY diets. There were no other differences detected among the other treatment comparisons for rumen concentrations of ammonia, osmolality, and VFA. The effects of the GR-CON, GR-CMA, RY-CON, and RY-CMA treatments, diet, and diet by treatment interaction on rumen LPS concentration and plasma concentrations of SAA, Hp, and LBP during the REC phase are displayed in Table 5.10. Neither individual treatment nor diet influenced rumen LPS concentrations. The RY diet had lower concentrations of LBP compared to the GR diet ($P = 0.05$) during the REC phase. There were no other differences detected among the other treatment comparisons for plasma SAA, Hp, or LBP concentrations.

5.4.6 Inflammatory marker relative change

The effect of GR-CON, GR-CMA, RY-CON, and RY-CMA treatments, diet, and diet by treatment interaction on relative change (%) in plasma SAA, Hp, and LBP concentrations from ACC to CHL, ACC to REC, and CHL to REC is outlined in Table 5.11. There was a greater reduction in SAA with the CMA treatment compared to the CON treatment from the ACC to the CHL phase ($P = 0.02$). There was a tendency for a greater reduction in SAA with the CMA treatment compared to the CON during the REC phase compared to the ACC phase ($P = 0.02$). There were no other differences detected for any of the other treatment or diet comparisons for SAA relative change. The RY-CMA treatment had a greater reduction in plasma Hp concentration compared to the RY-CON treatment from the ACC to the REC phase ($P = 0.03$).

There were no other differences among any of the other treatments or diets for plasma Hp relative change. There was an overall tendency for a reduction in plasma LBP concentration with the CMA treatments compared to the CON treatments from the ACC phase to the REC phase ($P = 0.06$). There were no other treatment differences detected for LBP relative change.

5.5 Discussion

The primary objective of this experiment was to determine the effects of CMA on rumen pH, rumen fermentation products, and plasma inflammatory markers during a grain and ryegrass induced SARA challenge. The secondary objective of this experiment was to compare the effects of a grain-induced SARA challenge and a ryegrass-induced SARA challenge. Both GR and RY CHL diets effectively induced SARA temporarily. The CHL phase reduced mean, median, and minimum pH, and increased time spent below pH 5.6 and 5.8 compared to both the ACC and REC phases. These changes are similar to that of previous authors who have induced SARA in an experimental setting (Khafipour et al., 2009a). The rumen pH results from the CHL phase agree with the SARA threshold outlined by Gozho et al. (2007). Predicting rumen pH based on diet nutrient composition, using variables such as effective NDF (eNDF) or NDF from forage (f-NDF) and starch and sugar concentration, is somewhat reliable with TMR. The ACC and REC diets contained more than adequate levels of f-NDF (24.52 %) and moderate levels of starch and sugar (18.8 %) and therefore, were not expected to cause a reduction in rumen pH or disrupt rumen fermentation. The GR CHL diets contained a much lower concentration of f-NDF (18.65%) and higher concentration starch and sugar (37.25%). The differences in f-NDF and starch and sugar concentration between the ACC and REC diets compared to the GR CHL diets explain the sharp increase in time spent below rumen pH 5.6 on the GR CHL diets compared to all the treatments during the ACC and REC phases. Using dietary variables to predict rumen pH in ryegrass-based diets is much less accurate compared to TMR. Therefore, it is difficult to highlight major differences in dietary variables between the ACC and REC diets compared to the RY CHL diet. The measurement of eNDF or peNDF based on the particle size method is not applicable to ryegrass (Kolver and de Veth, 2002). However, Kolver et al. (1998) assigned an eNDF of between 40 to 50% for ryegrass pasture, depending on the ryegrass quality. Based on this methodology, the total diet eNDF of the RY CHL diet is between 16.13 and 20.15% and would help to explain the sharp increase in time spent below rumen pH 5.6 on the RY CHL treatments compared to all the treatments during ACC and REC, similar to the effects of the GR CHL.

The GR and RY diets had similar effects on the magnitude of rumen pH depression experienced by cows during the CHL phase and rumen pH returned to similar levels on both diets during the REC phase. O'Grady et al. (2008) previously measured rumen pH in pasture-based dairy cows using the rumenocentesis method. They diagnosed 11% of cows across 12 farms with SARA based on a threshold of rumen pH 5.5. This is a considerably lower prevalence compared

to studies using similar methodology in TMR-fed cows where 26% of cows were diagnosed with SARA (Garret et al., 1997). However, SARA was successfully induced in this study where high-quality ryegrass was fed by zero-grazing, as reported by Holohan et al 2021. The REC phase had increased time spent below pH 5.6 and 5.8 compared to the ACC phase, despite receiving identical diets in the ACC and REC phases. Therefore, there appeared to be a carryover effect of phase on rumen pH parameters.

The results for mean pH and time spent below pH 5.6 and 5.8 on the CON and CMA treatments during the ACC and REC phase were not indicative of a problematic rumen fermentation or SARA-like conditions in these phases. Some of the earlier research on the use of rumen buffers in dairy cow diets suggested that rumen buffers were only required in diets containing less than 30% forage (Erdman, 1988). A more recent review by Hu and Murphy (2002) concluded that sodium bicarbonate-based buffers have limited effects on rumen pH when maize silage is not the sole or main forage in the diet. The ACC and REC diet used in this experiment contained 59% total forage with baled ryegrass silage as the primary forage source (34.1% of total diet DM). The reduction in time spent below rumen pH 5.4, 5.6, and 5.8 on the CMA treatment during the ACC phase, and the reduction in time spent below rumen pH 5.6 with both GR-CMA and RY-CMA compared to GR-CON and RY-CON during the REC phase disagrees with some of this earlier work around the limitation of rumen buffers in diets containing higher levels of forage (Erdman, 1988) and diets based on alternative forages to maize silage (Hu and Murphy, 2002). However, these findings agree with previous work outlining the benefits of CMA on rumen pH, albeit using diets containing lower inclusions of total forage (Cruywagen et al., 2015; Neville et al., 2019). The CV and range of daily rumen pH were reduced in cows fed CMA compared to cows fed CON during the ACC phase, and cows fed the RY-CMA treatment compared to the RY-CON treatment during the REC phase. A reduction in rumen pH variation could lead to a more stable rumen fermentation and rumen microbial profiles, as the acid sensitive cellulolytic bacteria will continue to proliferate in the higher pH range (> pH 6.0) while the amylolytic bacteria also proliferate in the slightly lower pH range (pH 5.5 – 6.0) (Weimer et al., 1996).

The CMA treatment had no effect on any of the rumen pH variables measured during the CHL phase compared to the CON treatment. These findings disagree with our hypothesis and previous experiments (Cruywagen et al., 2015; Neville et al., 2019). This is the first experiment to test the effect of CMA during an experimentally induced SARA and further similar research will be required to investigate the mechanisms behind these effects. The dosage rate of CMA

for this experiment was based on the experiment conducted by Wu et al. 2015 and was 10% and 25% higher than the dosage rate usage for Cruywagen et al. (2015) and Neville et al. (2015), respectively. It is possible that the 100 g/ cow per d CMA dosage rate was not sufficient to counteract the fermentation acids generated by the highly fermentable GR and RY CHL diets. Cruywagen et al. (2015) previously described CMA as sparingly soluble in water and rich in rumen soluble Ca (30%) and Mg (5.5%). The power calculation for this experiment was based on a CV of 6.75% for total VFA concentration. The CV for rumen pH on all treatments was less than 6.75% which would indicate that there was sufficient replication to detect treatment effects. There may be underlying mechanisms with CMA that indirectly aid rumen fermentation and stabilise rumen pH through effects of Ca on the rumen epithelium and VFA absorption or supply of Ca and Mg to rumen microbes. The CHL diets were akin to less common scenario's where cows abruptly consume large amounts of very fermentable ingredients and less like a typical lactating diet. Such scenarios include formulation or feeding error, cows sorting their diets in favour of smaller grain particles, or the first week of lactation where cows are being introduced to their lactating diet. Therefore, the beneficial effects of CMA on rumen pH observed during the REC indicate that it is a worthwhile dietary additive following one of the aforementioned scenarios.

There was less differentiation between phases in rumen concentrations of fermentation products compared to rumen pH. The increase in rumen osmolality during the CHL phase compared to ACC and REC phases is consistent with previous work where SARA was induced (Khafipour et al., 2009a). Increased rumen osmolality is caused by the rapid increase in rumen VFA concentration that coincides with SARA (Kent-Dennis et al., 2020). The increase in rumen propionate concentration during the CHL phase compared to the ACC and REC phases is likely caused by the increased level of starch on the GR CHL and increased level of sugar on the RY CHL (Bannink et al., 2006). The rumen concentration of total VFA was higher during the REC compared to the ACC, despite cows consuming identical diets. This was likely due to changes in the microbial population during the CHL having a residual effect on VFA production in the REC phase. Higher concentration of rumen available protein, and digestible cellulose and hemi-cellulose, commonly associated with RY, may have led to increased rumen concentrations of ammonia, and acetate, with the RY diet compared to the GR diet. Valerate is produced from *Megasphaera elsdenii*, the main lactate utilizing bacteria in the rumen (Bramley et al., 2008). The reduction in valerate on the RY diet compared to the GR diet may be explained by the considerable reduction in dietary starch and likely subsequent reduction in

rumen lactate observed with the GR CHL diet. With the exception of reduced rumen iso-butyrate concentration on the CMA treatment during the ACC phase, there was no differences in rumen VFA, osmolality, or ammonia concentrations between the CON and CMA treatments across the ACC, CHL, and REC phases. This agrees with the results of chapter 4 but disagrees with the previous work of Cruywagen et al. (2015). The power calculation for this experiment was based on total VFA concentration which indicates that there was sufficient replication to detect treatment differences for rumen concentrations of VFA, osmolality, and ammonia.

As expected, rumen LPS concentration was increased during the CHL phase with both GR and RY diets in the CHL phase compared to the ACC and REC phases. This agrees with previous work where SARA was induced (Gozho et al., 2006). Rumen LPS was higher during the REC phase compared to the ACC phase, likely due to the lag effect of the increased LPS during the preceding CHL phase. There were no differences in absolute concentration of rumen LPS between the CON and CMA treatments during the ACC, CHL, or REC phases. The RY diet had lower concentration of rumen LPS compared to the GR diet during the CHL phase. Increased rumen LPS concentration has long been associated with high grain diets (Garcia et al., 2017). The results of the current experiment agree with previous authors who have reported increased rumen LPS concentrations during grain-induced SARA (Khafipour et al., 2009a; Emmanuel et al., 2008). Khafipour et al. (2009b) reported increased rumen LPS concentration when they induced SARA using alfalfa pellets, similar to levels of rumen LPS associated with GR-induced SARA. Despite feeding moderate levels of starch in the CHL diets used by Khafipour et al. (2009), they still contained 3-times more starch than the RY CHL diet used during this experiment, 22.4% versus 7.15%. Therefore, discrepancies in rumen LPS concentration during the CHL phase between the RY diet in this experiment and alfalfa-based CHL's in previous experiments could be partly explained by level of starch inclusion.

Plasma SAA concentrations were highest during the ACC and CHL phases compared to the REC phase. The similarity in plasma SAA concentration between the ACC and CHL phases were not expected and disagree with previous experiments that induced a SARA challenge (Gozho et al., 2006). However, Chiquette et al. (2015) reported SAA and LBP data for one of their direct-fed microbial treatments during the SARA challenge that did not differ greatly from the adaptation phase. Interestingly, Zebeli et al. (2012) reported that only 15 to 21% of the overall variation in the SAA concentrations were due to rumen pH in a review of 10 different SARA challenge experiments. Plasma concentrations of LBP was also highest during the CHL phase, but there appeared to be a carryover effect of LBP into the REC phase. Based on a

combination of plasma LBP and SAA increases during the CHL phases and numerically higher plasma Hp concentrations, we can assume that the CHL phase invoked an inflammatory response. Both GR and RY diets had similar levels of plasma SAA, Hp, and SAA during the CHL phase, despite differences in rumen LPS concentrations. However, the RY diet had reduced the plasma concentration of LBP compared to the GR diet during the REC phase. This suggests that the cows fed the RY diets returned to pre-CHL levels sooner than the cows fed the GR diets. The RY fed cows experienced much lower levels of rumen LPS during the CHL phase compared to the GR fed cows, and hence had a lesser burden and a reduced potential for rumen LPS translocation. Therefore, plasma LBP concentrations returned to pre-CHL levels much earlier. The CMA treatment had higher levels of plasma SAA and LBP during the ACC phase compared to the CON. Rumen LPS concentrations were similar between CMA and CON, and rumen pH parameters improved on the CMA treatment compared to the CON treatment during the ACC phase. Therefore, a mechanism for this increase in SAA and LBP is not likely due to changes in rumen pH or translocation of LPS from the digestive tract. However, plasma SAA was reduced by the CMA treatment after the SARA CHL's were induced. There was also a greater reduction in plasma SAA and Hp on the CMA treatments compared to the CON treatments as cows progressed from the ACC phase to the CHL phase. These changes in inflammatory markers took place in the absence of corresponding changes to rumen pH so there must be other mechanism influencing the effects of CMA on plasma SAA and Hp change. Previous research using human macrophage cells, discovered CMA to have an anti-inflammatory role by inhibiting nuclear factor kappa B activation (O Gorman et al., 2012). Results from chapter 3 support the results of this experiment. Chapter 3 reported a tendency for reduced plasma inflammatory marker, serum amyloid A (SAA), concentration in cows fed CMA during the Transition period. The absence of previous experiments within the literature using similar methodology and environments to the current study meant that the CV of these plasma inflammatory markers could have been underestimated and the current experiment under-powered and unable to detect all of the treatment's differences for these inflammatory markers.

Yields of ECM and fat + protein was reduced during the CHL phase compared to the ACC phase, caused by a reduction in fat concentration. Milk fat synthesis is reduced when rumen biohydrogenation pathways are not complete due to a reduction in mean rumen pH or increase in time spent below rumen pH 5.6 (Allen, 1997). Changes in rumen pH parameters between ACC and CHL provide a good explanation for the reduction in milk fat concentration. Despite

having similar rumen pH profiles, the RY fed cows had higher milk fat concentration compared to the GR fed cows. While the main constraints to milk fat synthesis are low rumen pH and high dietary concentrations of polyunsaturated fatty acids (Bauman and Griinari, 2003), the original building blocks for milk fat synthesis is rumen derived acetate. One of the primary functions of acetate is to act as a precursor for long-chain fatty acid synthesis in the mammary glands (Bannink et al., 2006). Rumen acetate concentration was higher in RY fed cows compared to GR fed cows during the CHL phase of this experiment and can further explain the differences in milk fat concentration between the RY fed and GR fed cows. This experiment was not sufficiently powered for milk production as milk production was not the sole focus of this experiment. Therefore, milk production results should be interpreted with this in mind.

5.6 Conclusion

Ryegrass-induced SARA CHL can induce similar rumen pH depressions and levels of plasma inflammatory markers, but lesser concentrations of rumen LPS and valerate compared to a GR-induced SARA CHL. RY fed cows also had reduced LPBP in the recovery phase. Milk fat concentration was reduced during the GR-induced CHL but not during the RY-induced CHL. Calcareous marine algae reduced the time spent below rumen pH 5.4, 5.6, and 5.8 compared to a CON treatment during the ACC phase when cows were fed a 59% forage diet. However, the CMA treatment failed to have any effect on rumen pH during the CHL phase, when cows were abruptly changed onto highly fermentable diets. The CMA treatment had minor effects on rumen VFA and no effect on rumen osmolality, ammonia, or LPS concentrations. The use of CMA in both GR and RY-induced SARA CHL diets can reduce the extent of inflammation buts its mechanisms are not well understood and may not be based on rumen pH changes, hence further investigation is needed. The findings of this experiment demonstrate the effects of a RY induced SARA alongside a GR induced SARA and will offer researchers a greater understanding of challenges relating to rumen pH depressions using different diet types. Additionally, results of this study demonstrate that CMA is a worthwhile dietary additive to reduce plasma inflammatory markers and it may have a beneficial effect on the overall health of cows during dietary induced challenges. The application of CMA to higher forage diets may also prove useful in preventing long periods of low rumen pH.

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Table 5.1. Ingredient composition of the control and calcareous marine algae treatments, and grain and ryegrass diets during the acclimatisation, challenge, and recovery phases.

	Experimental diets[1]			
% DM	ACC	GR-CHL	RY-CHL	REC
Ryegrass silage (baled)	34.1	20.4	0.0	34.1
Maize silage	22.7	20.0	0.0	22.7
Fresh ryegrass	0.0	0.0	83.6	0.0
Experimental Nut[2]	16.8	16.4	16.4	16.8
Wheat/ Barley	0.0	31.1	0.0	0.0
Beet Pulp	14.1	0.0	0.0	14.1
Soya	10.2	12.0	0.0	10.2
Straw	2.1	0.0	0.0	2.1

[1] Experimental diets: ACC = acclimatisation; GR-CHL = grain-induced SARA challenge; RY-CHL = ryegrass-induced SARA challenge; REC = recovery

[2] Control experimental concentrate ingredients (DM basis): 30.4% maize grain, 30% barley grain, 25.5% soya hulls, 4% sugarcane molasses, 3.2% salt, 1.35% magnesium oxide, 2.3% limestone, 2.7% mono-calcium phosphate, 0.55% vitamins and trace elements.

CMA experimental concentrate ingredients (DM basis): 30.4% maize grain, 30% barley grain, 25.1% soya hulls, 4% sugarcane molasses, 3.2% salt, 1.35% magnesium oxide, 2.7% calcareous marine algae, 2.7% mono-calcium phosphate, 0.55% vitamins and trace elements.

Table 5.2. The analysed and predicted chemical and nutrient profile of the experimental diets fed during the acclimatisation, challenge, and recovery phases.

	Experimental diets[1]			
Item, % DM	ACC	GR-CHL	RY-CHL	REC
DM	42.35	43.84	17.09	42.35
CP	15.57	16.64	18.83	15.57
PDIN[2]	10.23	10.90	12.83	10.23
PDIE[2]	10.39	11.00	8.89	10.39
NDF	37.35	30.84	44.13	37.35
f-NDF[3]	24.52	18.65	40.28	24.52
ADF	21.72	17.70	22.36	21.72
Ash	8.88	7.08	11.29	8.88
Starch	15.11	34.80	7.15	15.11
Sugar	3.68	2.45	8.91	3.68
Net energy (UFL/kg)[2]	1.00	1.06	1.04	1.00
Net energy [NE_L] (Mcal/kg)[4]	1.76	1.86	1.84	1.76

[1] Experimental diets: ACC = acclimatisation; GR-CHL = grain-induced SARA challenge; RY-CHL = ryegrass-induced SARA challenge; REC = recovery

[2] As calculated by using INRAtion 4.07 feed formulations program, based on ingredient analyses: similar for all treatments. PDIN = protein truly digestible in the small intestine where nitrogen is limiting microbial protein synthesis, PDIE = protein digested in the small intestine where energy is limiting microbial protein synthesis, UFL = unit of energy for lactation.

[3] NDFforage = contribution of the forage component of the diet to NDF

[4] NE_L = Net energy for lactation, at production level, NRC (2001).

Table 5.3. Effect of phase on dry matter intake, milk output and composition, rumen pH, rumen fermentation products, and plasma inflammatory markers.

	Phase[1]				*P*-value[2]		
Variable	ACC	CHL	REC	SEM[3]	A V C	A V R	C V R
DMI[4], kg	20.86	22.25	21.39	0.63	<0.01	0.16	<0.01
Milk output, kg/ d							
Milk yield	29.64	28.48	28.24	1.07	0.23	0.12	0.94
ECM	34.07	31.77	32.39	1.27	0.03	0.13	0.76
Fat + protein yield	2.31	2.16	2.21	0.09	0.03	0.20	0.67
Milk composition, %							
Fat	4.23	3.96	4.17	0.15	<0.01	0.78	0.03
Protein	3.59	3.67	3.68	0.07	0.03	0.01	0.95
Lactose	4.48	4.46	4.47	0.04	0.45	0.60	0.97
Rumen pH							
Mean pH	6.23	5.96	6.10	0.04	<0.01	<0.01	<0.01
Minimum pH	5.44	5.23	5.36	0.03	<0.01	0.08	<0.01
Minutes below pH 5.6	85.8	309.0	154.8	28.07	<0.01	0.02	<0.01
Minutes below pH 5.8	173.4	492.1	312.0	42.52	<0.01	<0.01	<0.01
Rumen fermentation products, m*M*							
Osmolality (mOsm/kg)	299.9	313.1	299.8	3.74	<0.01	0.99	<0.01
Total VFA	148.2	156.6	161.4	4.59	0.28	0.05	0.67
Acetate	92.67	90.51	99.36	2.71	0.77	0.10	0.02
Propionate	30.45	38.86	32.54	1.39	<0.01	0.46	<0.01
Butyrate	19.37	20.28	22.31	0.93	0.75	0.05	0.24
Rumen fluid LPS[5], EU/ml	8,323	43,868	16,632	2,568	<0.01	0.03	<0.01
Plasma inflammatory marker, ug/ml							
SAA[5]	72.64	65.65	37.36	9.18	0.74	<0.01	0.01
Hp[5]	426.2	419.4	422.7	22.51	0.97	0.99	0.99
LBP[5]	2.60	3.24	3.42	0.17	<0.01	<0.01	0.52

[1] Phases: ACC = acclimatisation; CHL = challenge; REC = recovery
[2] *P*-value for phase effect: A V C = acclimatisation versus challenge; A V R = acclimatisation versus recovery; C V R = challenge versus recovery
[3] SEM = standard error of the mean
[4] DMI = dry matter intake
[5] LPS = lipopolysaccharide; SAA = serum amyloid A; HP = Haptoglobin; LBP = lipopolysaccharide binding protein

Table 5.4. Effect of control and calcareous marine algae on dry matter intake and milk production during the acclimatisation phase.

	Treatments[1]			
Variable, kg	CON	CMA	SEM[2]	*P*-value[3]
DMI[4]	21.03	20.69	0.75	0.30
Milk yield	29.84	29.45	1.09	0.70
ECM	34.43	33.71	1.66	0.57
Fat yield	1.28	1.23	0.08	0.45
Protein yield	1.06	1.06	0.04	0.99
Fat + protein yield	2.33	2.29	0.12	0.62
Composition, %				
Fat	4.27	4.19	0.17	0.49
Protein	3.57	3.60	0.06	0.44
Lactose	4.48	4.49	0.04	0.75

[1] Treatments: CON = control; CMA = calcareous marine algae
[2] SEM = standard error of the mean
[3] *P*-value for treatment effect
[4] DMI = dry matter intake

Table 5.5. Effect of control and calcareous marine algae on rumen pH and rumen fermentation products during the acclimatisation phase.

	Treatments[1]			
Variable	CON	CMA	SEM[2]	*P*-value[3]
Mean pH	6.22	6.25	0.06	0.42
Median pH	6.27	6.28	0.06	0.82
pH CV[4], %	5.44	4.56	0.32	<0.01
Maximum pH	6.82	6.75	0.03	0.12
Minimum pH	5.40	5.48	0.05	0.14
pH range	1.42	1.28	0.05	0.05
Minutes below pH 5.4	44.6	11.1	10.4	<0.01
Minutes below pH 5.6	118.2	51.6	20.6	<0.01
Minutes below pH 5.8	209.3	132.7	39.6	<0.01
Rumen fermentation products, mM				
Ammonia	1.97	1.98	0.21	0.99
Osmolality (mOsm/kg)	298.4	301.3	4.10	0.49
Total VFA	147.8	149.9	4.25	0.61
Acetate	93.02	93.70	3.29	0.81
Propionate	30.13	30.82	0.91	0.49
Butyrate	18.93	19.89	0.72	0.28
Iso-butyrate	1.17	0.99	0.08	0.04
Valerate	2.72	2.49	0.27	0.47
Iso-valerate	2.18	2.00	0.22	0.22
Acetate: propionate	3.16	3.08	0.09	0.53
Protein derived VFA	6.09	5.47	0.47	0.11

[1] Treatments: CON = control; CMA = calcareous marine algae
[2] SEM = standard error of the mean
[3] *P*-value for treatment effect
[4] CV = coefficient of variation

Table 5.6. Effect of control and calcareous marine algae on rumen lipopolysaccharide concentration and plasma inflammatory markers during the acclimatisation phase.

	Treatments[1]			
Variable	CON	CMA	SEM[2]	*P*-value[3]
Rumen fluid LPS[4], EU/ml	8,145	8,500	1,453	0.86
Plasma inflammatory marker, ug/ml				
SAA[4]	43.6	103.7	6.78	<0.01
Hp[4]	407.2	462.5	27.4	0.13
LBP[4]	2.33	2.87	0.11	<0.01

[1] Treatments: CON = control; CMA = calcareous marine algae
[2] SEM = standard error of the mean
[3] *P*-value for treatment effect
[4] LPS = lipopolysaccharide; SAA = serum amyloid A; HP = Haptoglobin; LBP = lipopolysaccharide binding protein

Table 5.7. Effect of control and calcareous marine algae treatments, grain and ryegrass diets, and their interactions on dry matter intake and milk production during the challenge and recovery phases.

	Experimental treatments[1]					*P*-value[3]		
Challenge phase	GR-CON	GR-CMA	RY-CON	RY-CMA	SEM[2]	Diet	Trt	Diet × Trt
Output, kg/ d								
DMI[4]	21.67	22.37	23.01	21.95	0.82	0.69	0.63	0.02
Milk yield	29.28	28.70	28.04	27.90	1.60	0.63	0.74	0.84
ECM[4]	31.42	30.91	32.43	32.33	1.57	0.56	0.77	0.85
Fat yield	1.06	1.05	1.18	1.19	0.06	0.10	0.99	0.83
Protein yield	1.07	1.04	1.03	1.01	0.06	0.68	0.49	0.96
Fat + protein yield	2.12	2.09	2.22	2.20	0.11	0.49	0.72	0.88
Composition, %								
Fat	3.62	3.70	4.23	4.30	0.18	0.04	0.53	0.98
Protein	3.66	3.68	3.69	3.64	0.11	0.97	0.67	0.36
Lactose	4.41	4.47	4.49	4.48	0.05	0.52	0.20	0.08
Recovery phase								
Output, kg/ d								
DMI[4]	20.55[a]	21.68[b]	21.63	21.71	0.90	0.67	0.05	0.09
Milk yield	26.01	28.49	28.71	29.76	1.93	0.47	0.08	0.47
ECM[4]	29.16	32.34	33.54	34.52	1.99	0.24	0.12	0.40
Fat yield	1.04	1.18	1.22	1.26	0.09	0.25	0.15	0.40
Protein yield	0.94	1.01	1.08	1.10	0.06	0.20	0.14	0.42
Fat + protein yield	1.98	2.19	2.31	2.36	0.14	0.20	0.13	0.38
Composition, %								
Fat	4.01	4.14	4.29	4.25	0.26	0.60	0.67	0.42
Protein	3.63	3.58	3.77	3.73	0.11	0.39	0.18	0.84
Lactose	4.44	4.50	4.46	4.47	0.07	0.99	0.24	0.28

[1] GR-CON = grain-induced SARA control; GR-CMA = grain-induced SARA with calcareous marine algae; ryegrass-induced SARA control; ryegrass-induced SARA with calcareous marine algae

[2] SEM = standard error of the mean

[3] Diet = *P*-value for diet effect; Trt = *P*-value for treatment effect; Diet × Trt = *P*-value for diet by treatment interaction

[4] DMI = dry matter intake; ECM = energy corrected milk

Table 5.8. Effect of control and calcareous marine algae treatments, grain and ryegrass diets, and their interactions on rumen pH and rumen fermentation products during the challenge phase.

	Experimental treatments[1]					*P*-value[3]		
	GR-CON	GR-CMA	RY-CON	RY-CMA	SEM[2]	Diet	Trt	Diet × Trt
Mean pH	5.98	5.96	5.94	5.94	0.07	0.80	0.75	0.80
Median pH	5.98	5.97	5.96	5.95	0.09	0.87	0.85	0.96
pH CV[4], %	6.01	6.40	5.77	5.95	0.49	0.59	0.39	0.76
Maximum pH	6.72	6.75	6.59	6.67	0.06	0.19	0.22	0.59
Minimum pH	5.26	5.17	5.23	5.22	0.07	0.93	0.30	0.37
pH range	1.47	1.57	1.37	1.45	0.08	0.31	0.14	0.90
Time below pH 5.4 (min/d)	157.5	171.5	133.5	154.7	55.93	0.79	0.60	0.91
Time below pH 5.6 (min/d)	287.3	389.6	306.8	320.5	84.18	0.83	0.20	0.33
Time below pH 5.8 (min/d)	446.9	564.0	494.3	515.5	104.0	0.99	0.21	0.39
Rumen fermentation products, m*M*								
Ammonia	1.27	1.28	4.04	3.79	0.34	<0.01	0.74	0.70
Osmolality (mOsm/kg)	310.2	303.7	319.1	319.1	5.63	0.10	0.57	0.57
Total VFA[4]	151.7	155.4	161.3	158.1	5.85	0.34	0.97	0.57
Acetate	86.53	81.71	97.55	95.56	3.65	0.03	0.36	0.70
Propionate	39.14	45.04	34.48	35.70	3.90	0.19	0.20	0.40
Butyrate	18.66	19.00	22.37	20.85	1.06	0.05	0.56	0.36
Iso-butyrate	1.47	1.61	2.03	1.61	0.28	0.37	0.62	0.33
Valerate	3.36	3.58	2.42	2.13	0.41	0.07	0.91	0.39
Iso-valerate	2.57	3.34	2.41	2.23	0.87	0.62	0.23	0.06
Acetate: propionate	2.44	2.00	2.85	2.76	0.24	0.10	0.06	0.22
Protein derived VFA[4]	7.39	8.11	6.86	5.97	1.19	0.43	0.88	0.17

[1] GR-CON = grain-induced SARA control; GR-CMA = grain-induced SARA with calcareous marine algae; ryegrass-induced SARA control; ryegrass-induced SARA with calcareous marine algae
[2] SEM = standard error of the mean
[3] Diet = *P*-value for diet effect; Trt = *P*-value for treatment effect; Diet × Trt = *P*-value for diet by treatment interaction
[4] CV = coefficient of variation; VFA = volatile fatty acid

Table 5.9. Effect of control and calcareous marine algae treatments, grain and ryegrass diets, and their interactions on rumen pH and rumen fermentation products during the recovery phase.

	Experimental treatments[1]					*P*-value[3]		
	GR-CON	GR-CMA	RY-CON	RY-CMA	SEM[2]	Diet	Trt	Diet × Trt
Mean pH	6.12	6.08	6.07	6.08	0.04	0.62	0.64	0.33
Median pH	6.14	6.11	6.05	6.09	0.07	0.61	0.98	0.38
pH CV[4], %	5.61	5.63	5.78[a]	4.46[b]	0.59	0.57	<0.01	0.01
Maximum pH	6.76[a]	6.65[b]	6.74[a]	6.61[b]	0.04	0.61	<0.01	0.69
Minimum pH	5.37	5.27	5.36	5.38	0.10	0.72	0.38	0.24
pH range	1.39	1.40	1.38	1.23	0.09	0.48	0.15	0.13
Mins below pH 5.4	100.9	71.9	45.8	22.1	22.03	0.13	0.08	0.86
Mins below pH 5.6	172.1	116.1	179.3[a]	82.4[b]	67.34	0.90	<0.01	0.44
Mins below pH 5.8	349.4	265.3	360.8[x]	224.4[y]	59.47	0.85	0.01	0.54
Rumen fermentation products, m*M*								
Ammonia	0.85	1.14	1.84	1.63	0.35	0.16	0.88	0.31
Osmolality (mOsm/kg)	292.6	297.4	305.7	303.7	6.21	0.27	0.75	0.45
Total VFA[4]	159.4	154.5	162.9	171.1	10.36	0.43	0.85	0.45
Acetate	97.2	98.9	100.0	106.0	5.68	0.44	0.49	0.70
Propionate	30.01	30.24	34.50	35.80	2.94	0.24	0.71	0.79
Butyrate	21.96	22.25	22.67	22.51	1.56	0.78	0.96	0.88
Iso-butyrate	1.21	1.62	1.27	1.61	0.17	0.89	0.01	0.79
Valerate	2.45	2.94	2.41	2.88	0.29	0.89	0.02	0.96
Iso-valerate	2.03	2.56	2.07	2.32	0.29	0.79	0.06	0.49
Acetate: propionate	3.38	3.26	2.97	3.01	0.13	0.06	0.78	0.52
Protein derived VFA[4]	10.30	6.68	5.75	6.81	1.61	0.24	0.44	0.16

[1] GR-CON = grain-induced SARA control; GR-CMA = grain-induced SARA with calcareous marine algae; ryegrass-induced SARA control; ryegrass-induced SARA with calcareous marine algae
[2] SEM = standard error of the mean
[3] Diet = *P*-value for diet effect; Trt = *P*-value for treatment effect; Diet × Trt = *P*-value for diet by treatment interaction
[4] CV = coefficient of variation; VFA = volatile fatty acid

Table 5.10. Effect of control and calcareous marine algae treatments, grain and ryegrass diets, and their interactions on rumen lipopolysaccharide concentration and plasma inflammatory markers during the challenge and recovery phases.

	Experimental treatments[1]					*P*-value[3]		
	GR-CON	GR-CMA	RY-CON	RY-CMA	SEM[2]	Diet	Trt	Diet × Trt
Challenge								
Rumen fluid LPS[4], EU/ml	59,969	52,501	31,119	30,549	6,839	<0.01	0.57	0.62
Plasma inflammatory marker, ug/ml								
SAA[4]	89.20	60.27	74.93[x]	37.54[y]	18.20	0.45	0.05	0.67
Hp[4]	427.2	438.2	413.7	398.3	37.02	0.53	0.95	0.68
LBP[4]	3.12	3.64	2.97	3.25	0.39	0.57	0.22	0.70
Recovery								
Rumen fluid LPS[4], EU/ml	14,954	14,921	18,129	17,955	2,795	0.39	0.96	0.97
Plasma inflammatory marker, ug/ml								
SAA[4]	48.67	50.12	31.55	36.31	18.02	0.55	0.58	0.76
Hp[4]	451.5	454.4	432.1	405.5	54.83	0.64	0.72	0.65
LBP[4]	3.91	3.97	2.80	2.97	0.37	0.05	0.45	0.71

[1] GR-CON = grain-induced SARA control; GR-CMA = grain-induced SARA with calcareous marine algae; ryegrass-induced SARA control; ryegrass-induced SARA with calcareous marine algae

[2] SEM = standard error of the mean

[3] Diet = *P*-value for diet effect; Trt = *P*-value for treatment effect; Diet × Trt = *P*-value for diet by treatment interaction

[4] LPS = lipopolysaccharide; SAA = serum amyloid A; HP = Haptoglobin; LBP = lipopolysaccharide binding protein

Table 5.11. The effect of control and calcareous marine algae treatments, grain and ryegrass diets, and their interactions on relative change (%) in plasma serum amyloid A, Haptoglobin, and lipopolysaccharide concentrations (µg/ml) during the acclimatisation, challenge, and recovery phases.

	Experimental treatments[1]					*P*-value[3]		
	GR-CON	GR-CMA	RY-CON	RY-CMA	SEM[2]	Diet	Trt	Diet × Trt
Serum amyloid A								
ACC to CHL[4]	115.79	- 38.36	48.71	- 72.71	44.16	0.38	0.02	0.71
ACC to REC[5]	16.38	- 5.10	- 26.66	- 77.60	46.12	0.40	0.07	0.38
CHL to REC[6]	- 10.24	10.46	142.67	- 34.88	111.51	0.65	0.51	0.41
Haptoglobin								
ACC to CHL[4]	11.48	17.40	4.00	- 38.61	17.74	0.37	0.43	0.34
ACC to REC[5]	13.62	16.27	7.78[a]	- 37.02[b]	15.47	0.41	0.02	0.02
CHL to REC[6]	16.17	4.98	8.12	6.42	18.45	0.90	0.52	0.63
Lipopolysaccharide binding protein								
ACC to CHL[4]	30.38	24.14	31.57	17.22	9.08	0.81	0.15	0.53
ACC to REC[5]	61.77	36.84	25.08	9.27	15.19	0.17	0.06	0.60
CHL to REC[6]	24.94	9.43	- 3.91	- 8.30	8.02	0.03	0.27	0.52

[1] GR-CON = grain-induced SARA control; GR-CMA = grain-induced SARA with calcareous marine algae; ryegrass-induced SARA control; ryegrass-induced SARA with calcareous marine algae
[2] SEM = standard error of the mean
[3] Diet = *P*-value for diet effect; Trt = *P*-value for treatment effect; Diet × Trt = *P*-value for diet by treatment interaction
[4] Relative difference (%) between the acclimatisation and challenge phases
[5] Relative difference (%) between the acclimatisation and recovery phases
[6] Relative difference (%) between the challenge and recovery phases

Chapter 6

Effects of calcareous marine algae on feeding behaviour, milk fatty acid profiles, and rumen fermentation in early lactation dairy cows

6.1 Abstract

Dairy cows undergo significant behavioural change in the early postpartum period. The consumption of highly fermentable diets coupled with irregular feeding patterns can lead to periods of low rumen pH. The objective of this experiment was to build on the results of Chapter 3 & 4 by investigating the effects of calcareous marine algae (CMA; Acid Buf, Celtic Sea Minerals) on feeding behaviour, rumen fermentation products, milk fatty acid profiles, and total tract digestibility of dry matter and organic matter in early lactation dairy cows. Twenty-two multiparous and 10 primiparous cows were assigned to 2 treatments from 25 days (d) before expected parturition until 42 d postpartum. Cows were assigned to treatment according to a randomized complete block design with repeated measures based on parity, pre-experimental body condition score, previous 305-d milk yield and fat + protein yield for multiparous cows and predicted transmitting ability for milk yield and fat + protein yield for primiparous cows. Cows were fed a negative dietary cation-anion difference (DCAD) [-50 mEq/kg] total mixed ration (TMR) based on maize silage, grass silage, and straw during the prepartum and a 50:50 forage: concentrate TMR based on grass silage, maize silage and concentrate during the postpartum period. The two dietary treatments consisted of a control (CON), which contained limestone as the primary calcium source, and CMA at 0.42% and 0.47% DM for the pre- and postpartum periods, respectively. The CMA treatment reduced the proportion *trans*-9 18:1 (- 0.10 g/ 100g of total fatty acid) in milk, and the omega-6 to omega-3 fatty acid ratio (- 0.2:1) compared to CON. Daily TMR intake (+ 1.11 kg) and feeding rate (+ 0.018 kg/ min) were increased in cows on the CMA treatment compared to cows on the CON treatment. Daily feeding time (- 15.7 min/ d) tended to be less on the CMA treatment compared to the CON treatment. There was an increase in TMR intake within the 2nd (between 1500 h and 2100 h) and 3rd (between 2100 h and 0300 h) daily quartiles on the CMA treatment compared to the CON. The feeding rate of cows on the CMA treatment was greater (+ 0.03 kg/ min) in the 2nd daily quartile compared to cows on the CON treatment. These findings show that CMA can alter milk fatty acid profiles and positively effect feeding behaviour toward larger intakes of feed throughout the day during the early postpartum period.

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6.2 Introduction

The transition to early lactation is one of the most critical stages of a cow's lactation. Mineral balance, immune function, and energy status are severely challenged in early lactation as a result of this transition (Mulligan and Doherty, 2008). The greatest change relates to the diet consumed by the cow, changing from high levels of physically effective fibre and low levels of fermentable ingredients during the prepartum period to moderate peNDF intake and high levels of fermentable carbohydrates during the postpartum (Shi et al., 2019). It is therefore not surprising that the early lactation period is cited as the highest risk period for sub-acute ruminal acidosis (SARA) (Penner et al., 2007). Perhaps the most critical aspect of the early postpartum period is the inevitable reduction in dry matter intake (DMI) and the associated negative consequences (Grummer et al., 2004). Successful transition cow nutritional management can alleviate this depression in DMI during the early postpartum period (Cardoso et al., 2020). An understanding of both nutrition and feeding behaviour is necessary to achieve this increased DMI (Nielson, 1999). Feeding behaviour has a direct effect on the level of DMI achieved by a cow and the subsequent effects on rumen fermentation and digestion (DeVries, 2017). One of the major behavioural changes experienced by the dairy cow in the early postpartum period is the abrupt increase in time dedicated to milking and being outside of her pen, which can lead to less time available for resting and eating (DeVries, 2017).

Feed additives with proven effects on rumen fermentation can benefit feeding and rumination behaviour (DeVries, 2017). Researchers have demonstrated the ability of rumen modifying feed additives such as live yeast (DeVries and Chevaux, 2014), essential oils (Tager and Krause, 2011), sodium bicarbonate (Martins et al., 2021), and monensin (Mullins et al., 2012) to have a positive influence on feeding behaviour. Rumen modifying feed additives stabilise rumen pH and fermentation, but also affect feeding behaviour as a secondary effect (DeVries and Chevaux, 2014). One of the more novel rumen modifying feed additives of the last decade is calcareous marine algae (CMA; Acid Buf or Calmin, Celtic Sea Minerals) which is produced from *Lithothamnion*, harvested off the coast of Iceland. Calcareous marine algae has been used effectively to increase mean rumen pH and reduced hours below pH 5.5 in dairy cows across different feeding systems (Cruywagen et al., 2015; Neville et al., 2019; Rafferty et al., 2019). As a consequence of increased rumen pH, CMA has been shown to increase milk fat concentration compared to a control diet (Neville et al., 2019). The relationship between milk fat production and rumen pH is well established (Allen, 1997) and the altering of rumen biohydrogenation pathways by increased production of *trans*-10, *cis*-12 conjugated linoleic

acid (CLA) was cited as the main mechanism behind this negative relationship (Bauman and Griinari, 2003). Chapter 3 investigated the feeding of CMA to cows during the transition period and found a large increase in milk fat concentration, as well as increased DMI during the postpartum period. Milk fat production in dairy cows can be further examined by analysing the fatty acid (FA) profile of milk. The production of specific FA in milk can be altered through changes in biohydrogenation pathways in the rumen (Colman et al., 2010). Chapter 4 sought to establish whether CMA could alter rumen fermentation and total tract digestibility in a 45% forage diet based on grass silage and maize silage. The results from chapter 4 show improvements in rumen pH but no effect on volatile fatty acid (VFA) production with CMA inclusion. Total tract digestibility of neutral detergent fibre (NDF) was increased when CMA was supplemented with marine magnesium oxide but not when supplemented on its own. The objective of this experiment was to build on the results of chapter 3 and 4 by investigating the effects of CMA on feeding behaviour, rumen fermentation products, milk FA profiles, and total tract digestibility of dry matter (DM) and organic matter (OM) in early lactation dairy cows. We hypothesised that CMA would alter feeding behaviour toward increased feeding rate and would alter the fatty acid profile in milk by altering ruminal biohydrogenation.

6.3 Materials and methods

All procedures described in this experiment were approved by the Animal Research Ethics Committee (AREC) at University College Dublin (UCD) and conducted under experimental license from the Health Products Regulatory Authority (HPRA) under the European directive 2010/63/EU and S.I. No. 543 of 2012. Each person who carried out procedures on experimental animals, during this experiment, was authorized to do so by means of individual authorization from the HPRA.

6.3.1 *Experimental design*

Thirty-two (22 multiparous and 10 primiparous) Holstein cows were selected from the dairy herd at UCD Lyons Farm, Co. Kildare, IE. Cows were assigned to treatment according to a randomized complete block design with repeated measures based on parity, pre-experimental BCS (multiparous: 2.88 ± 0.33, primiparous: 3.3 ± 0.26), previous 305-day milk yield (7,009 ± 1,404) kg and fat & protein yield (556 ± 97) kg for multiparous cows and PTA for milk yield (255 ± 127) kg and fat & protein yield (22 ± 7) kg for primiparous cows.

The two dietary treatments consisted of a control (CON) and CMA (CMA), where limestone was replaced by CMA at 0.42% and 0.47% DM for the pre- and postpartum periods, respectively. The ingredient composition of the two dietary treatments in pre- and postpartum are outlined in Table 6.1. The inclusion rate of the CMA additive was based on a predicted daily DMI of 11.7 kg DM for the prepartum and 21.4 kg DM for the postpartum to ensure a daily intake of 50g and 100g of CMA per cow per day in the pre- and postpartum periods, respectively. The dosage rates of CMA were determined from previous experiments (Wu et al., 2015). Limestone and magnesium oxide (MgO) levels were adjusted in the CMA treatment to balance diets for calcium and magnesium.

6.3.2 Animal care and housing

The experiment was carried out between September and November 2019. Each cow was on the experiment for a minimum of 57 d (57 – 67d), 25 d before expected calving date to 42 d postpartum. Sampling and data collection were carried out from 1 to 42 d postpartum. Cows were housed in a free stall barn with 1.25 stalls per cow available during the pre- and postpartum period. When cows were observed to be within 24 hours (h) of calving, they were moved to a loose pen to facilitate calving while still receiving their respective diet. Cows had

ad-libitum access to TMR for 22 h every day through specific computerized feeding boxes (RIC System, Insentec B.V.). Cows received their complete prepartum diet and most of their postpartum diet through a TMR once daily at 0900 h. Each treatment was mixed separately with a Keenan Feeder (Keenan Feeding Systems) and each dietary treatment received the same total mixing time, ten min after all the ingredients were added to the mixer wagon. During milking, cows received a pellet (1 kg as fed at am and 1 kg as fed at pm milking) to incentivize them into the milking parlour. Parlour troughs were inspected before and after each cow entered the parlour and any refusals were recorded. Water was available ad-libitum and water troughs were cleaned daily. Cows were milked twice daily, at 0800 h and 1600 h, in a rotary milking parlour (Dairymaster) and stalls were also cleaned, with new sawdust bedding added, twice daily.

6.3.3 Diets and feeding management

The diets were formulated using recommendations from INRA (2018). Prepartum diets were designed to supply 100% of the energy requirements of a 650 kg non-lactating dairy cow in late gestation with a predicted DMI of 11.7 kg DM per cow per d. Both prepartum diets were formulated to contain; 15% starch & sugar, 0.9% Ca, 0.46% Mg, and -53 mEq/kg DCAD, based on maize silage, grass silage, barley straw, and soybean meal. Postpartum diets were designed to supply 95 % of the energy requirements of a 650 kg lactating dairy cow yielding 32 kg of milk/d containing 3.9 % of fat and 3.3 % of protein with a predicted DMI of 21.4 kg DM per cow per d and a feed allocation of 22.5 kg DM per cow per d, to allow for 5 % refusals. The two postpartum diets were formulated to contain: 49% concentrate, 29% starch and sugar, and 22% NDF from forage. Multiparous and primiparous cows were trained to use the computerized feeding stations prior to the experiment. All cows were introduced to the feeding stations and fed a control diet from 28 d before expected calving date as an acclimatization to the diet, feeding system and the barn. The experimental diets were introduced to the cows from 25 d prepartum.

The grass silage used consisted predominantly of perennial ryegrass (Lolium perenne). The crop was cut using a mower-conditioner during the early boot stage of vegetation (growth stage 41) (Zadoks et al., 1974), wilted for 16 h and harvested with a Krone Big X (Krone GmbH & Co) forage harvester (mean particle length 50 mm). The crop was then ensiled under a black polythene cover without the use of an additive. Maize silage (Zea maiz, variety Tekni) was grown with the aid of plastic film (Samco Agricultural Manufacturing Ltd). The crop was

harvested at the dough stage (growth stage 85) (Lancashire et al., 1991) using a Krone Big X (Krone GmbH & Co.) precision chop forage harvester (mean particle length 25mm). The harvester was equipped with a kernel processor to improve starch digestibility. The harvested maize silage was ensiled under a black polythene cover without the use of an additive.

Fresh samples of both grass and maize silage were sent to a forage laboratory (Trouw Nutrition) for near infrared (NIR) analysis (FOSS NIR systems 5000). The ingredient composition of pre- and postpartum diets is presented in Table 6.1. The concentrate portion of the postpartum diet was fed in the form of a pellet, and added to the TMR, to prevent against unwanted dietary separation and aid in mixing accuracy. The concentrate pellet used in the TMR was manufactured by Brett Brothers Ltd. The milking parlour pellet was manufactured by Gain Feeds.

6.3.4 Data collection, sampling procedures, and sample analyses

Samples of TMR were collected daily and dried at 104°C in a forced air oven for 16 h to establish the DM content of the TMR. Individual daily feed intakes were recorded on the computerized feeding system and used, in combination with daily TMR DM content to calculate DMI. Samples of TMR were taken from each treatment during feed-out 3 times per week, pooled into weekly samples by treatment, and stored at - 20°C until they were analysed. Concentrate, grass silage, maize silage and straw samples were collected weekly and stored at - 20°C prior to analysis.

Faecal samples were collected from each cow on d 7 and 14 at 5 h post first feed. Approximately 100g of fresh faeces was collected and stored at - 20°C prior to analysis. Subsamples of the composite TMR, maize silage, grass silage, barley straw, soybean meal, concentrate, faeces, and refusals were dried at 55°C for 72 h. The subsequent dried samples were ground using a Norris hammer mill fitted with a 1 mm screen (Lab Mill, Christy Turner). Ash content was determined by incineration of a 5 g sample in a muffle furnace (Nabertherm GmbH, Lilienthal, Germany) at 550°C for 5.5 h. The N content of the feed was determined by combustion using a Leco 528 instrument (Leco Instruments UK). Crude protein was then calculated using N × 6.25. Neutral detergent fibre and acid detergent fibre (ADF) were determined according to the method of Van Soest et al. (1991) using the Ankom 220 Fiber Analyzer (Ankom™ Technology). As part of the NDF procedure, both sodium sulphite (Na_2SO_3) (FSS, Ankom Technology) and heat stable alpha-amylase (FAA, Ankom

Technology) were used for the analysis of TMR, maize silage, barley straw, and concentrate subsamples while only sodium sulphite (Na_2SO_3) was used for grass silage subsamples. Starch content was determined using the Megazyme Total Starch Assay Procedure (product no: K-TSTA; Megazyme International Ltd). Water Soluble Carbohydrate (WSC) was analysed according to the method used by Birch et al. (1974). Ether extract (EE) was measured using a Soxtec instrument (Tecator) according to the method of AOAC 107 (1970). Acid-insoluble ash (AIA) was determined using the 2N HCl procedure as outlined by Keulen and Young, 1977. Feed and faecal OM content was determined by difference using the equation: OM% = 100 – ash %.

Milk samples were collected on two separate days each week during the postpartum period. Each day consisted of an a.m. and p.m. sample, pooled in proportion to the specific a.m. and p.m. yields to create one milk sample per day and two milk samples per cow for each week. Samples were further pooled on a bi-weekly basis for milk FA analysis. Samples were preserved (Broad Spectrum Microtabs II, D&F Control Systems Inc.) and stored at 4°C until analysed. Daily milk yields were automatically recorded using the Weighall milk meter system (Dairymaster). Concentrations of total milk fat, protein, and lactose were determined in a commercial milk laboratory (Progressive Genetics) using infrared analysis (CombiFoss 5000, Foss Analytical). Values for 3.5 % fat-corrected milk (FCM) were calculated using the following formulae:

3.5% FCM = (0.4324 × milk yield kg) + (16.216 × fat yield kg) (Erdman, 2011).

6.3.5 Milk fatty acid analysis

Milk fat was extracted using the method of Folch (1957) while methylation of FA was performed according to Christie (1982). Fatty acid methyl esters (FAME) were quantified using a gas chromatograph (GCFID system Agilent 7890; Agilent, Santa Clara, CA, US) equipped with flame ionization detector (FID) and a CP-sil 88 fused silica capillary column for FAME (100 m × 0.25 mm [i.d.] with 0.2-µm film thickness), using helium as the carrier gas at a rate of 1 ml / minute. The initial temperature of 50°C was held for 4 min after which it was increased to 110°C at a rate of 8°C per minute. Temperature was then increased at a rate of 5°C per minute until it reached 170°C, at which it was held for 10 min and then increased at a rate of 2°C per minute until it reached 225°C, which was then held for 30 min. This resulted in a total analysis time of 64 min. FAME Methyltricosanoate (C23:0-Methyl ester) was used as an

internal standard. A reference standard (Supelco 37 Component FAME Mix) was used to determine recoveries and correction factors for individual FA as well as acting as a reference sample for routine quality control. Both reference and internal standards were purchased from Sigma-Aldrich (Arklow, Co. Wicklow, Ireland).

6.3.6 Milk mineral analysis

Milk samples from wk 1 – 3 for each cow were pooled for the determination of Ca, P, Mg, and K. Samples were digested in high purity nitric acid at 110°C, using a microwave. The digested sample was then used to determine the mineral concentration using an inductively coupled plasma emission spectrophotometer linked to a mass spectrometer.

6.3.7 Rumen fermentation parameters

Rumen fluid samples were collected on d 7, 14, 21, 28, 35, and 42 postpartum for each cow. Samples were harvested after p.m. milking at 1600 h (7 h post first feed) using the Flora Rumen scoop oral oesophageal sampler (Prof-Products). Once collected, samples were strained through 4 layers of cheesecloth, and a 4-mL aliquot drawn off using an automatic pipette and acidified by mixing with 1 mL of trichloroacetic acid (50% wt/vol) before freezing at -20°C.

During analysis, samples were allowed to thaw in the refrigerator for 16 h at 4°C before centrifuging at 2,100 × g for 10 min at 4°C. One millilitre of supernatant was diluted 1 in 5 with distilled water (dH_2O) and then centrifuged at 1,600 × g for 15 min at 4°C. Next, 200 µL of supernatant was combined with 3 reagents and used to determine NH_3 concentrations using a spectrophotometer. Rumen fluid was prepared for VFA analysis by mixing 250 µL of rumen fluid with 3.75 mL of dH_2O and 1 mL of internal standard solution (0.5 g of 3-methylvaleric acid in 1,000 mL of 0.15 *M* oxalic acid). The resulting solution was centrifuged at 1,600 × g and filtered through a syringe-tip filter (PTFE, 25-mm diameter, 0.45 µm) into 2-mL GC vials. Concentrations of VFAs were determined using Scion 456-GC (Scion Instruments, Scotland, UK) fitted with DB-FFAP capillary column (15m x 0.53mm; 1.00 µm, Agilent Technologies, USA). Osmolality of rumen fluid samples was determined by vapour pressure osmometry using Wescor Vapro 5600 osmometer.

6.3.8 Feeding behaviour

Feeding behaviour was evaluated using attendance data from the computerized feeding boxes (RIC System, Insentec B.V.). Total bouts were calculated as the number of times that a cow visited the feeding box. Feeding bouts was defined as visits to the feeding boxes where the cow consumed a minimum of 100g of TMR, whereas non-feed bouts was the number of visits without feed consumption or where the cow consumed less than 100g of TMR. Daily TMR intake was the sum of TMR (kg DM) consumed over the day. Bout size was the mean size (kg DM) of each individual feeding event. The CV of bout size was also calculated and presented as CV bout size (%). Daily feeding time was calculated as the total time that a cow spent eating each day (min/d). Bout duration was the mean time spent eating at each feeding event (min/d) and CV bout duration was the CV (%) of bout duration across the d. Feeding rate was calculated by dividing each individual bout size by bout duration (kg/min). The CV of feeding rate was also calculated and presented as CV feeding rate (%).

6.3.9 Data screening and statistical analyses

Data residuals were examined for normality using the UNIVARIATE procedure of SAS (version 9.4, SAS Inst.). Following assessment of normality, outliers were removed (± 3 STD from the mean). Feeding behaviour, milk output, total tract digestibility, rumen fermentation products, and milk FA data were analysed as repeated measures using the MIXED procedure (SAS, version 9.4). Milk mineral data was analysed using the MIXED procedure but not as a repeated measure. The model included fixed effects of treatment, week and parity as well as treatment by week interaction with cow considered as the random effect. Repeated measures were modelled for week and the variance-covariance structures were selected based on the lowest Bayesian Information Criterion. All data presented in Tables 6.3. – 6.7. and figures 6.1. – 6.3. are expressed as least square means ± standard error of the mean (SEM). Statistical significance was declared at $P < 0.05$ while a tendency was assumed at $P > 0.05$ and < 0.10.

6.4 Results

6.4.1 Chemical analysis of TMR

The nutrient composition of the CON and CMA treatments in the prepartum and postpartum periods are presented in Table 6.2. Values for PDIN, PDIE, UFL and NE_L are predicted based on the INRAtion 4.07 computer program and NRC (2001).

6.4.2 Feeding behaviour

The effect of CON and CMA on feeding behaviour parameters is presented in Table 6.3. Daily TMR intake (P = 0.02) and feeding rate (P = 0.05) were increased in cows on the CMA treatment compared to cows on the CON treatment. Daily feeding time (P = 0.07) tended to be less on the CMA treatment compared to the CON treatment. Total no. of bouts, no. of feeding bouts, no. of non-feed bouts, bout size, CV of bout size, bout duration, CV of bout duration, and CV of feeding rate were not different between experimental treatments.

Figures 6.1., 6.2.,6.3., and 6.4. shows the effect of CON and CMA on the no. of feeding bouts, TMR intake, feeding time, and feeding rate, respectively, across the four 6-h quartiles within the day. There were no differences in feeding bouts between treatments across the 4 quartiles of the day. There was an increase in TMR intake within the 2nd (P < 0.01) and 3rd (P = 0.02) daily quartiles on the CMA treatment compared to the CON. There were no treatment differences in TMR intake in the 1st and 4th daily quartiles. Feeding time was reduced in the 1st (P = 0.02) and 2nd (P = 0.02) daily quartiles on the CMA compared to CON. Both treatments had similar feeding time in the 3rd and 4th daily quartiles. Feeding rate tended to be greater (P = 0.08) in the 1st daily quartile on CMA versus CON. The feeding rate of cows on the CMA treatment was greater (P < 0.01) in the 2nd daily quartile compared to cows on the CON treatment. There were no treatment differences detected for feeding rate in the 3rd and 4th daily quartiles.

6.4.3 Milk production and total tract digestibility

Table 6.4. outlines the effects of CON and CMA on milk production, milk mineral concentration and the apparent total tract digestibility of DM and OM. Fat yield was higher (P = 0.03) on the CMA treatment compared to the CON. There were no treatment effects detected for FCM, protein yield, or lactose yield. There were no differences between treatments for milk

Ca, P, Mg, or K. The CMA treatment tended to increase the apparent total tract digestibility of DM ($P = 0.08$) and OM ($P = 0.09$).

6.4.4 Rumen fermentation parameters

Table 6.5. shows the effect of CON and CMA on osmolality, ammonia, and VFA concentrations in rumen fluid. The CMA treatment tended to reduce the osmolality of rumen fluid compared to CON ($P < 0.10$). There was no difference in VFA concentrations or molar proportions of VFA between CON and CMA. The acetate: propionate ration and ammonia concentrations were similar between the CON and CMA treatments.

6.4.5 Milk fatty acid analysis

Table 6.6. displays the effect of CON and CMA on the individual FA concentration of milk. The concentration of 4:0 ($P = 0.05$); *cis*-9, *cis*-12, *cis*-15 18:3 ($P = 0.03$); *cis*-7, *cis*-10, *cis*-13 *cis*-16, *cis*-19 22:5 ($P = 0.05$); and total omega-3 ($P = 0.02$) were increased on CMA compared to CON. The omega-3 to omega-6 ratio was reduced ($P = 0.04$) on the CMA treatment compared to CON. The CMA treatment tended to increase *cis*-9 18:1 ($P = 0.08$); *cis*-11 18:1 ($P = 0.07$), total saturated FA ($P = 0.10$), total mono-unsaturated FA (MUFA) ($P = 0.06$), and total omega-6 ($P = 0.06$) compared to CON. There were no other treatment differences detected among the concentrations of individual FA.

The effect of CON and CMA on individual FA as a proportion of total FA is shown in Table 6.7. The CMA treatment reduced the proportion *trans*-9 18:1 ($P = 0.05$), and omega-6 to omega-3 ratio ($P = 0.03$) compared to CON. Tendencies were detected for lower proportions of 14:0 ($P = 0.07$), and *cis*-5, *cis*-8, *cis*-11, *cis*-14 20:4 ($P = 0.07$) compared to the CON treatment.

6.5 Discussion

Increasing feed intake while maintaining optimum rumen function can be a challenge during early lactation. The abrupt increase in fermentation acids from the change in diet leads to an increased risk of SARA during the early lactation period (Penner et al., 2007). An important consequence of low rumen pH is the reduction in milk fat concentration (Allen, 1997) and change in milk fatty acid proportions (Bauman and Griinari, 2003). The use of CMA has been proven to increase milk fat concentration (Cruywagen et al., 2015; Neville et al., 2019). These findings were consistent with result of chapter 3 using transition dairy cows. The mechanism behind these effects is likely explained by rumen pH improvements with CMA (Neville et al., 2019). The biohydrogenation of FA in the rumen is influenced by the rumen pH and the presence of biohydrogenation intermediates such as *trans*-10, *cis*-12 CLA or *trans*-10 18:1 in milk can confirm the extent of rumen biohydrogenation (Colman et al., 2010). The effect of CMA on rumen pH has been previously established (Cruywagen et al., 2015: Neville et al., 2019) but its effects on milk FA profiles or specific biohydrogenation intermediates has not been investigated previously. Feeding behaviour will affect DMI in early lactation and subsequently effect rumen fermentation and digestion (DeVries, 2017). Results from chapter 3 show increased DMI with CMA in the early postpartum period and further research is required to establish if CMA can influence feeding behaviour. The objective of this experiment was to build on the results of chapter 3 and 4 by investigating the effects of CMA on feeding behaviour, rumen fermentation products, milk fatty acid profiles, and total tract digestibility of DM and OM in early lactation dairy cows.

Cows supplemented with CMA had higher daily TMR intakes, increased feeding rate, and a tendency for lower daily feeding time. Feeding behaviour across the daily quartiles followed a similar trend with greater TMR intake in the 2nd and 3rd quartiles and reduced feeding time in the 1st and 2nd quartile with the CMA treatment. These results are evidence that CMA allowed cows to consume more feed in shorter periods, subsequently leading to greater daily DMI. Early lactation cows have limited time available for feeding and resting due to increased time spent outside the pen for milking and other management procedures (DeVries, 2017). Evening milking occurred in the 2nd quartile of the day so it is likely that the increased TMR intake, increased feeding rate, and reduced feeding time experienced by CMA cows in the 2nd quartile had a knock-on effect on daily DMI as feeding time would have been limited and greater feeding rate required during this quartile of the day due to milking and related procedures. Greater feed intakes and reduced feeding times will likely lead to a greater challenge to rumen

pH due to larger amounts of fermentable carbohydrates entering the rumen and less time for papillae to absorb the fermentation acids (Steele et al., 2015). The ability of CMA cows to consume larger volumes of fermentable ingredients over shorter feeding periods suggests that CMA allowed the cows to cope better with increased rumen acid load compared to the CON cows. Previous research found that sodium bicarbonate reduced rumination time per kg of DMI, suggesting that sodium bicarbonate compensated for the reduction in natural buffering through saliva (Martins et al., 2021). Furthermore, it has been estimated that 170 g/d of sodium bicarbonate is equivalent to 23 L/d of saliva (Dijkstra et al., 2012). From a buffering perspective, 170 g of sodium bicarbonate is equivalent to 85 g of CMA (Cruywagen et al., 2015). Perhaps the CMA diet in our current study, 100 g/ cow per d, equates to a saving of 27 L in saliva and reduced the requirement for natural buffering from rumination. The current experiment did not measure rumen pH, but previous researchers have proven the ability of CMA to increase mean rumen pH and reduce rumen pH depressions below sub-optimal rumen fermentation thresholds (Cruywagen et al., 2015; Neville et a., 2019). Rumen modifying feed additives that can stabilise rumen pH and alter fermentation will also impact feeding behaviour, albeit as a secondary effect (DeVries and Chevaux, 2014). Feed additives with proven rumen modifying mechanisms, such as live yeast (DeVries and Chevaux, 2014), essential oils (Tager and Krause, 2011), sodium bicarbonate (Martins et al., 2021), and monensin (Mullins et al., 2012) have been proven to positively impact feeding behaviour.

Rumen VFA, ammonia, and osmolality concentrations were measured in this experiment but there were no treatment effects across all parameters except for osmolality which tended to be lower with CMA. The measurement of rumen fermentation products, like the aforementioned, does not always change in response to a rumen buffer. Chapter 4 investigates the effects of CMA in rumen cannulated cows, with increased rumen fluid sampling frequency compared to the current experiment, and also found no effect of CMA on rumen fluid VFA concentrations.

The tendency for increased DM and OM digestibility on the CMA treatment is at odds with the results of chapter 4 where we found no effect of CMA on apparent total tract digestibility of DM and OM. However, the CMA and marine magnesium oxide combination increased total tract digestibility of NDF in chapter 4. Previous research has demonstrated a positive relationship between rumen pH and the digestibility of NDF and OM (Mulligan et al., 2002). The challenge to rumen pH is highest in early lactation cows (Penner et al., 2007) so the difference in stage of lactation (early lactation in the current experiment and late lactation in

chapter 4) may have influenced the reduction in rumen pH and subsequent effects on digestibility.

The addition of CMA to diets during this experiment altered milk FA profiles. The increase in FA's 4:0; *cis*-9, *cis*-12, *cis*-15 18:3; *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19 22:5; and total omega-3 in total concentration of milk, as well as the tendency for increased *cis*-9 18:1, *cis*-11 18:1, total saturated, total MUFA, and total omega-9 FA's on the CMA treatment follow the same trend as total milk fat yield. The CMA treatment reduced *trans*-9 18:1 FA and the omega 6: omega 3 ratio as a proportion of total FA while also tending to reduce 14:0 FA and *cis*-5, *cis*-8, *cis*-11, *cis*-14 20:4 FA as a proportion of total FA. A more complete biohydrogenation pathway results in a reduction in biohydrogenation intermediates and an increase in the end-product, stearic acid (18:0) (Cabrita et al., 2009). Despite the increase in total fat yield, we did not detect any reduction in *trans*-10, *cis*-12 CLA or *trans*-10 18:1 FA during this study nor did we find any changes in the proportion or total concentration of stearic acid (18:0). However, absorbed 18:0 is extensively desaturated by the stearoyl-CoA desaturase to *cis*-9 18:1 in milk (Cabrita et al., 2009). The tendencies for increased *cis*-9 18:1 and the increased fat yield with the CMA treatment, during the current experiment, could be an indication of more complete rumen biohydrogenation pathways and provide explanation for the increase. Previous work investigating the effects of sodium bicarbonate-based buffers in high concentrate diets reported a reduction in *trans* 18:1 FA when the buffer was included and also confirmed the causative relationship of *trans* 18:1 FA on reduced total milk fat concentration (Kalscheur, 1997; Khorasani and Kennelly, 2001). While not normally associated with incomplete rumen biohydrogenation, we did detect a reduction in trans-9 18:1 with the CMA treatment. As most of the early research in this area reports total *trans* C18:1 as intermediates produced from incomplete rumen biohydrogenation, there may be a connection between the specific *trans*-9 18:1 FA, incomplete rumen biohydrogenation, and total milk fat yield. This finding and further literature around the *trans*-9 18:1 FA will need to be investigated. This is the first experiment to investigate the effects of CMA on milk FA profiles so it was difficult to hypothesize what effects the CMA might have, outside of the FA associated with rumen biohydrogenation and improved rumen conditions. An unexpected result of this experiment was the decrease in omega 6: omega 3 ratio, in milk and as a proportion of total FA, in favour of the CMA treatment. The concentration of total omega-3 FA was also increased in milk with the CMA treatment. These effects will need to be investigated further to determine a mode-of-action. Nevertheless, a reduced omega 6: omega3 ratio is viewed as beneficial for human health (Lock

and Bauman, 2004) and CMA may provide benefits as a tool for improving the nutritive value of milk.

6.6 Conclusion

Feeding cows CMA during the transition period influenced their feeding behaviour in early lactation. The CMA cows were able to consume more TMR over a shorter period compared to the CON. These effects were more pronounced during the first 12 h of the day. The increased DMI reported in chapter 3 can be explained by the feeding behaviour results of this experiment. The findings of this experiment offer dairy producers and nutritionists a tool to positively influence feeding behaviour in the early postpartum period. The proportions and total FA concentrations of milk were altered by CMA. The CMA treatment reduced the proportion of *trans*-9 18:1 FA in milk compared to the CON and reduced the ratio of omega-6 to omega-3 FA. These changes in FA profile could be due to changes in rumen biohydrogenation and warrants further work. A reduction in omega-6: omega-3 ratio can have benefits to human health and CMA could be a useful tool in improving the nutritive value of milk.

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Table 6.1. Ingredient composition of the control, and calcareous marine algae diets during prepartum and postpartum periods.

	Dietary treatments[1]			
	Prepartum		Postpartum	
Item	CON	CMA	CON	CMA
Ingredients, % DM				
Maize silage	43.56	43.56	25.70	25.70
Straw (barley)	22.29	22.29	-	-
Grass silage	18.31	18.31	25.70	25.70
Soybean meal (48% CP)	7.43	7.43	10.72	10.72
Wheat grain (finely ground)	-	-	9.48	9.49
Barley (finely ground)	-	-	8.00	8.04
Parlour concentrate[2]	-	-	7.94	7.94
Soychlor[3]	6.76	6.76	-	-
Maize grain (finely ground)	-	-	4.54	4.54
Molasses	-	-	3.28	3.28
Soya hulls	-	-	2.47	2.47
Dry cow premix[4]	1.22	1.22	-	-
Milking cow premix[5]	-	-	0.23	0.23
White salt	-	-	0.47	0.47
Mono-dicalcium phosphate	-	-	0.47	0.47
Vegetable oil blend	-	-	0.23	0.23
Limestone	0.34	0.00	0.61	0.14
Calcined Magnesite	0.04	0.00	0.14	0.09
Calcareous Marine Algae[6]	0.00	0.43	0.00	0.47

[1] Treatments: CON = control; CMA = calcareous marine algae

[2] Parlour concentrate ingredients (DM basis): 22.5% barley grain, 22.5% maize grain, 10% maize distillers dried grains with solubles, 9% sugar beet pulp, 21% soybean meal, 2.5% soya hulls, 1.5% palm oil, 4.5% sugar cane molasses, 1.6% mono-dicalcium phosphate, 1.6% limestone, 1.8% salt, 1.5% magnesium oxide.

[3] SoyChlor = anionic salt supplement (Landus Cooperative, IA, USA). Contains -2,980 mEq DCAD/ kg, 20.1% CP, 0.99 Mcal/ kg, 4.54% Ca, 0.53% P, and 2.84% Mg

[4] Dry cow premix contained: 24% maize grain [carrier], 1% Ca, 10% P, 12% Mg, 4% Na, 6% Cl, 2,767 mg/kg of Mn, 5,000 mg/kg of Zn, 2,000 mg/kg of Cu, 33 mg/kg of Se, 333 mg/kg of I, 67 mg/kg of Co, 134 mg/kg of Biotin, 500 IU/kg of Vitamin A, 167 IU/kg of Vitamin D3, 8,000 IU/kg of Vitamin E.

[5] Milking cow premix contained: 30% Ca, 50 mg/kg of Co, 10,400 mg/kg of Cu, 390 mg/kg of I, 130 mg/kg of Se, 21,600 mg/kg of Mn, 32,500 mg/kg of Zn, 10,000 IU of Vitamin A, 3,000 IU of Vitamin D3, 13 IU of Vitamin E.

[6] Calcareous marine algae = Lithothamnion calcareum (95% Ash, 30% Ca, 5.5% Mg, 1% P, and 0.7% K)

Table 6.2. The analysed and predicted chemical and nutrient profile of the control and calcareous marine algae diets during the prepartum and postpartum periods.

	Dietary treatment[1]			
	Prepartum		Postpartum	
Item	CON	CMA	CON	CMA
Chemical composition, % DM				
DM	40.7	40.1	40.0	40.6
OM	90.2	90.3	92.0	92.1
CP	13.0	13.1	16.4	15.9
PDIN[2]	8.11	8.11	10.85	10.85
PDIE[2]	7.39	7.39	10.42	10.42
NDF	44.2	46.3	29.2	28.0
f-NDF[3]	93.16	93.16	25.4	25.4
ADF	29.2	29.2	17.2	17.2
Ash	9.81	9.70	7.98	7.88
AIA	-	-	0.90	0.81
Starch	14.9	14.5	24.8	24.9
Sugar	4.30	4.00	4.08	4.36
Ca	0.87	0.87	0.83	0.84
P	0.35	0.35	0.45	0.46
Mg	0.46	0.46	0.32	0.33
K	1.80	1.80	1.75	1.76
Na	0.08	0.08	0.30	0.31
Cl	1.50	1.50	0.85	0.85
S	0.20	0.20	0.18	0.18
DCAD (mEq/kg)	-52.2	-52.2	226	233
Metabolizable energy[2] (MJ/kg)	9.91	9.91	12.0	12.0
Net energy[2] (UFL/kg)	0.76	0.76	0.99	0.99
Net energy[4] [NE_L] (Mcal/kg DM)	1.33	1.33	1.74	1.74

[1] Treatments: CON = control; CMA = calcareous marine algae

[2] As calculated by using INRAtion 4.07 feed formulations program, based on ingredient analyses: similar for all treatments. PDIN = protein truly digestible in the small intestine where nitrogen is limiting microbial protein synthesis, PDIE = protein digested in the small intestine where energy is limiting microbial protein synthesis, UFL = unit of energy for lactation.

[3] NDFforage = contribution of the forage component of the diet to NDF

[4] NE_L = Net energy for lactation, at production level, NRC (2001).

Table 6.3. The effect of control and calcareous marine algae on daily feeding behaviour.

	Treatment[1]			
Parameter	CON	CMA	SEM	*P*[2]
Total bouts	29.26	31.47	1.79	0.38
Feeding bouts	26.50	28.21	1.57	0.44
Non-feed bouts	2.65	3.15	0.30	0.23
Daily DMI[3], kg	19.14	20.08	0.39	<0.01
Daily TMR intake, kg	17.96	19.07	0.37	0.02
Bout size, kg	0.737	0.718	0.048	0.77
CV bout size, %	81.33	81.79	2.04	0.84
Daily feeding time, min	155.1	139.4	7.78	0.07
Bout duration, min	6.95	6.06	0.51	0.11
CV bout duration, %	87.27	86.00	2.96	0.73
Feeding rate, kg/min	0.152	0.170	0.008	0.05
CV feeding rate, %	61.90	62.54	2.75	0.86

[1] Treatments: CON = control; CMA = calcareous marine algae
[2] *P*-value for treatment effect.
[3] Includes intake of parlour concentrate.
All feeding behaviour parameters exclude intake of parlour concentrate
No significant treatment × interactions were detected for any of the above parameters

Table 6.4. The effect of control and calcareous marine algae on milk production, milk mineral concentration and apparent total tract digestibility.

	Treatments[1]			
Variable	CON	CMA	SEM	*P*[2]
Milk Output, kg				
3.5% FCM	30.90	32.50	1.11	0.27
Fat yield	1.18	1.29	0.04	0.03
Protein yield	1.16	1.17	0.02	0.96
Lactose yield	1.31	1.31	0.05	0.96
Milk mineral concentration, mg/kg				
Ca	1,264	1,298	20.8	0.26
P	1,213	1,184	25.9	0.43
Mg	106.4	104.6	1.60	0.43
K	1,552	1,537	18.1	0.56
Apparent total tract digestibility, %				
DMD	71.13	73.60	1.18	0.08
OMD	72.61	74.97	1.16	0.09

[1] Treatments: CON = control; CMA = calcareous marine algae
[2] *P*-value for treatment effect.
No significant treatment × interactions were detected for any of the above parameters

Table 6.5. The effect of control and calcareous marine algae on osmolality, ammonia, and VFA concentration of rumen fluid.

	Treatments[1]			
Parameter	CON	CMA	SEM	*P*[2]
Osmolality, mM	298.3	293.3	2.13	0.10
Ammonia, mM	1.34	1.46	0.10	0.36
VFA concentrations, mM				
Total VFA	115.7	124.9	5.05	0.17
Acetate	67.70	73.32	3.38	0.21
Propionate	29.05	31.06	1.54	0.32
Iso-butyrate	0.87	1.03	0.07	0.11
Butyrate	14.25	15.48	0.62	0.13
Iso-valerate	1.73	1.83	0.17	0.65
Valerate	2.54	2.42	0.20	0.65
Acetate: Propionate	2.37	2.39	0.08	0.82
Protein derived VFA	5.09	5.23	0.33	0.75
Molar proportions of VFA, %				
Acetate	57.1	57.6	0.80	0.59
Propionate	25.0	24.9	0.54	0.81
Iso-butyrate	0.81	0.89	0.08	0.43
Butyrate	13.0	12.9	0.71	0.92
Iso-valerate	1.70	1.60	0.17	0.73
Valerate	2.39	2.05	0.17	0.14

[1] Treatments: CON = control; CMA = calcareous marine algae
[2] *P*-value for treatment effect.
No significant treatment × interactions were detected for any of the above parameters

Table 6.6. The effect of control and calcareous marine algae on the individual fatty acid concentration of milk.

	Treatments[1]			
Fatty Acid, mg/ml	CON	CMA	SEM	P^2
C4:0	1.06	1.20	0.050	0.05
C6:0	0.76	0.83	0.035	0.16
C8:0	0.52	0.55	0.023	0.28
C10:0	1.32	1.38	0.060	0.51
C11:0	0.07	0.07	0.005	0.88
C12:0	1.62	1.70	0.080	0.50
C13:0	0.12	0.13	0.009	0.85
C14:0	4.32	4.56	0.200	0.39
C14:1 c9*	0.34	0.38	0.035	0.40
C15:0	0.62	0.65	0.049	0.76
C16:0	12.59	14.17	0.850	0.19
C16:1 c9*	0.66	0.69	0.062	0.72
C17:0	0.29	0.32	0.018	0.21
C18:0	2.16	2.43	0.200	0.33
C18:1 t9*	0.17	0.16	0.011	0.29
C18:1 t10*	0.36	0.31	0.062	0.61
C18:1 t11*	0.38	0.37	0.042	0.77
C18:1 c9*	4.42	5.11	0.270	0.08
C18:1 c11*	0.20	0.22	0.011	0.07
C18:2 c9,12*	0.58	0.62	0.025	0.21
C18:3 c9,12,15*	0.15	0.17	0.007	0.03
C18:2 (CLA)[3] c9, t11*	0.17	0.19	0.012	0.27
C20:0	0.03	0.03	0.003	0.53
C20:1 c11*	0.02	0.02	0.001	0.51
C20:3 c8, 11, 14*	0.02	0.02	0.001	0.32
C20:4 c5, 8, 11, 14*	0.05	0.05	0.002	0.67
C20:5 c5, 8, 11, 14, 17*	0.02	0.02	0.001	0.46
C21:0	0.02	0.02	0.001	0.72
C22:0	0.01	0.01	0.001	0.83
C22:1 c13*	0.01	0.01	0.001	0.76
C22:2 c13, 16*	0.01	0.01	0.001	0.91
C22:5 c7,10,13,16,19*	0.046	0.050	0.0018	0.046
Total saturated	23.94	26.97	1.41	0.099
Total MUFA[3]	5.65	6.45	0.29	0.06
Total PUFA[3]	0.91	0.99	0.04	0.12
Total omega-3	0.23	0.26	0.01	0.02
Total omega-6	0.67	0.72	0.03	0.22
Total omega-7	0.86	0.92	0.07	0.52
Total omega-9	4.61	5.29	0.27	0.06
Omega 6:3	3.1: 1	2.9: 1	0.06	0.04

[1] Treatments: CON = control; CMA = calcareous marine algae

[2] *P*-value for treatment effect.

[3] CLA = conjugated linoleic acid; MUFA = mono-unsaturated fatty acid; PUFA = poly-unsaturated fatty acid

* c = *cis*; t = *trans*

No significant treatment × interactions were detected for any of the above parameters

Table 6.7. The effect of control and calcareous marine algae on fatty acids as a proportion of total fatty acids.

Fatty acid, g/100g of FA[3]	Treatments[1]		SEM	*P*[2]
	CON	CMA		
C4:0	2.31	2.36	0.06	0.55
C6:0	1.94	1.91	0.04	0.61
C8:0	1.40	1.35	0.04	0.35
C10:0	3.78	3.58	0.10	0.14
C11:0	0.20	0.18	0.01	0.29
C12:0	4.81	4.59	0.12	0.22
C13:0	0.37	0.34	0.02	0.33
C14:0	13.06	12.54	0.22	0.07
C14:1 c9*	0.98	1.00	0.07	0.85
C15:0	1.89	1.80	0.10	0.52
C16:0	38.23	39.07	1.16	0.61
C16:1 c9*	1.91	1.85	0.12	0.72
C17:0	0.90	0.91	0.03	0.76
C18:0	6.89	7.02	0.51	0.85
C18:1 t9*	0.55	0.45	0.03	0.045
C18:1 t10*	1.19	0.96	0.21	0.45
C18:1 t11*	1.19	1.04	0.13	0.41
C18:1 c9*	13.95	14.67	0.63	0.43
C18:1 c11*	0.62	0.65	0.03	0.42
C18:2 c9,12*	1.86	1.80	0.06	0.50
C18:3 c9,12,15*	0.46	0.47	0.02	0.79
C18:2 (CLA)[4] c9, t11*	0.56	0.56	0.04	0.99
C20:0	0.10	0.10	0.01	0.80
C20:1 c11*	0.10	0.10	0.003	0.58
C20:3 c8,11,14*	0.07	0.06	0.003	0.82
C20:4 c5,8,11,14*	0.14	0.13	0.005	0.07
C20:5 c5,8,11,14,17*	0.07	0.06	0.005	0.69
C21:0	0.05	0.05	0.004	0.19
C22:5 c7,10,13,16,19*	0.13	0.13	0.005	0.97
Total saturated	76.09	75.95	0.80	0.90
Total MUFA[4]	18.48	19.37	0.52	0.18
Total PUFA[4]	2.81	2.75	0.10	0.64
Total omega-3	0.67	0.68	0.03	0.85
Total omega-6	2.14	2.07	0.06	0.43
Total omega-7	2.53	2.51	0.13	0.88
Total omega-9	15.25	16.16	0.56	0.19
Omega 6:3	3.2: 1	3.0: 1	0.06	0.03

[1] Treatments: CON = control; CMA = calcareous marine algae

[2] *P*-value for treatment effect.

[3] FA = fatty acid

[4] CLA = conjugated linoleic acid; MUFA = mono-unsaturated fatty acid; PUFA = poly-unsaturated fatty acid

* c = *cis*; t = *trans*

No significant treatment × interactions were detected for any of the above parameters

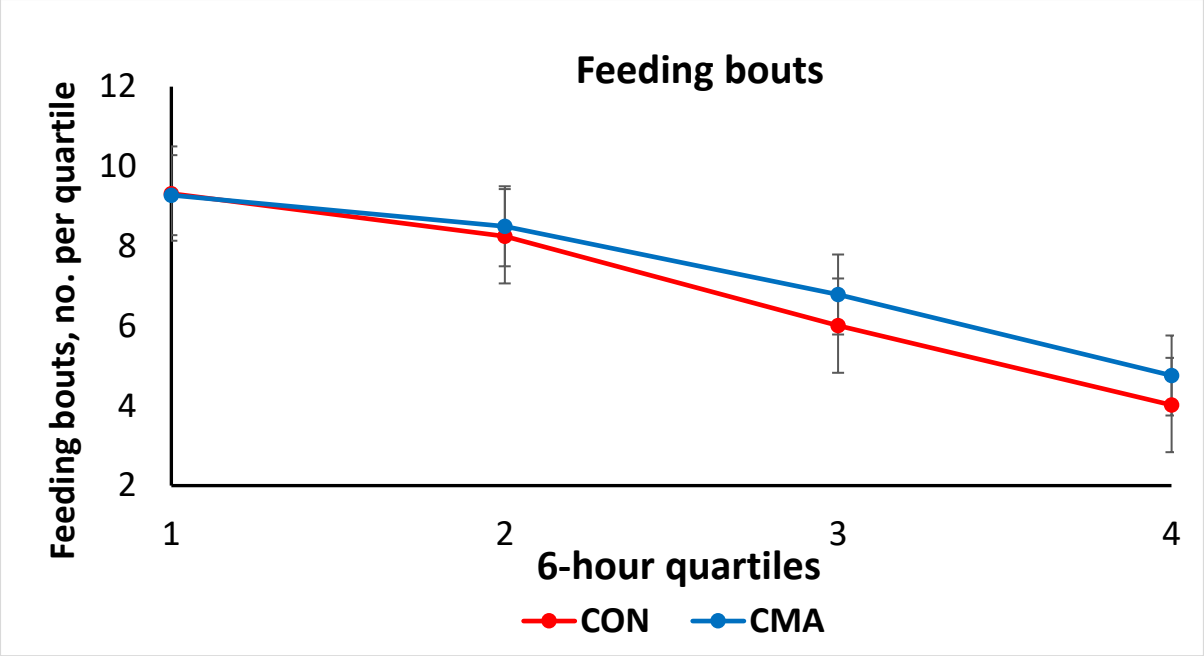

Figure 6.1. The effect of control and calcareous marine algae on feeding bouts during the 6-hour quartiles of the day.

Quartiles; 1 = 0900 to 1500 h; 2 = 1500 to 2100 h; 3 = 2100 to 0300 h; 4 = 0300 to 0900 h

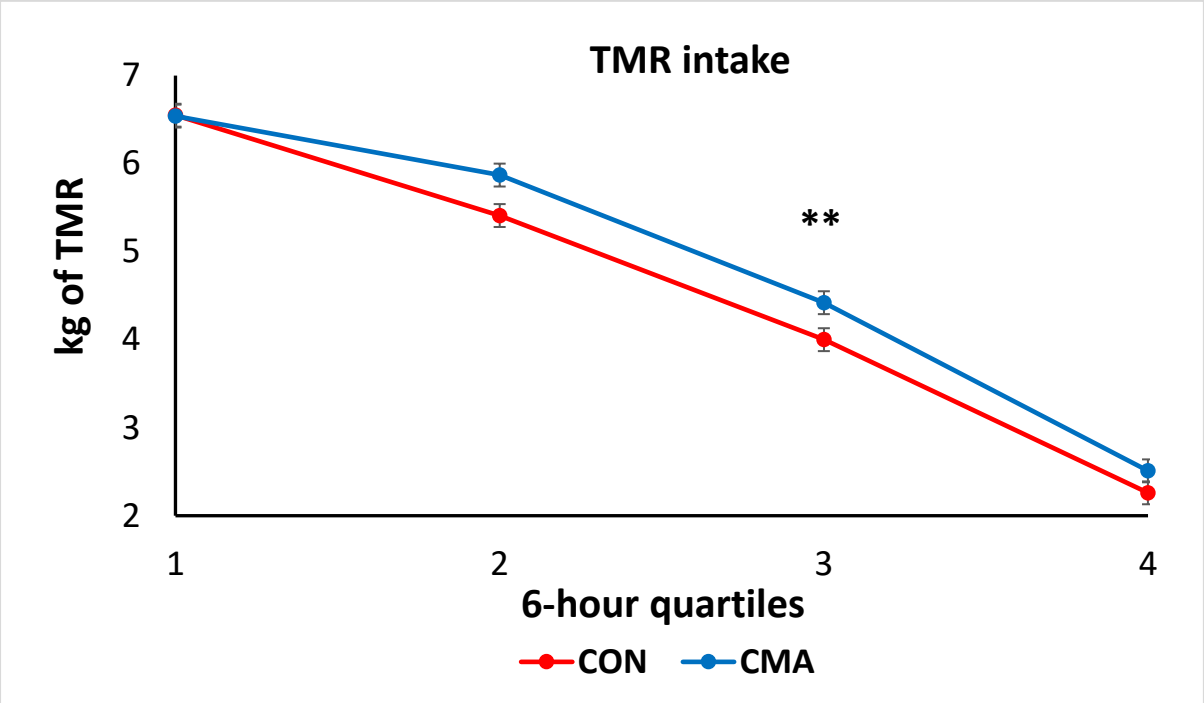

Figure 6.2. The effect of control and calcareous marine algae on TMR intake during the 6-hour quartiles of the day.

Quartiles; 1 = 0900 to 1500 h; 2 = 1500 to 2100 h; 3 = 2100 to 0300 h; 4 = 0300 to 0900 h

P-value for treatment effect: * = $P<0.10$; ** = $P<0.05$; *** = $P<0.01$.

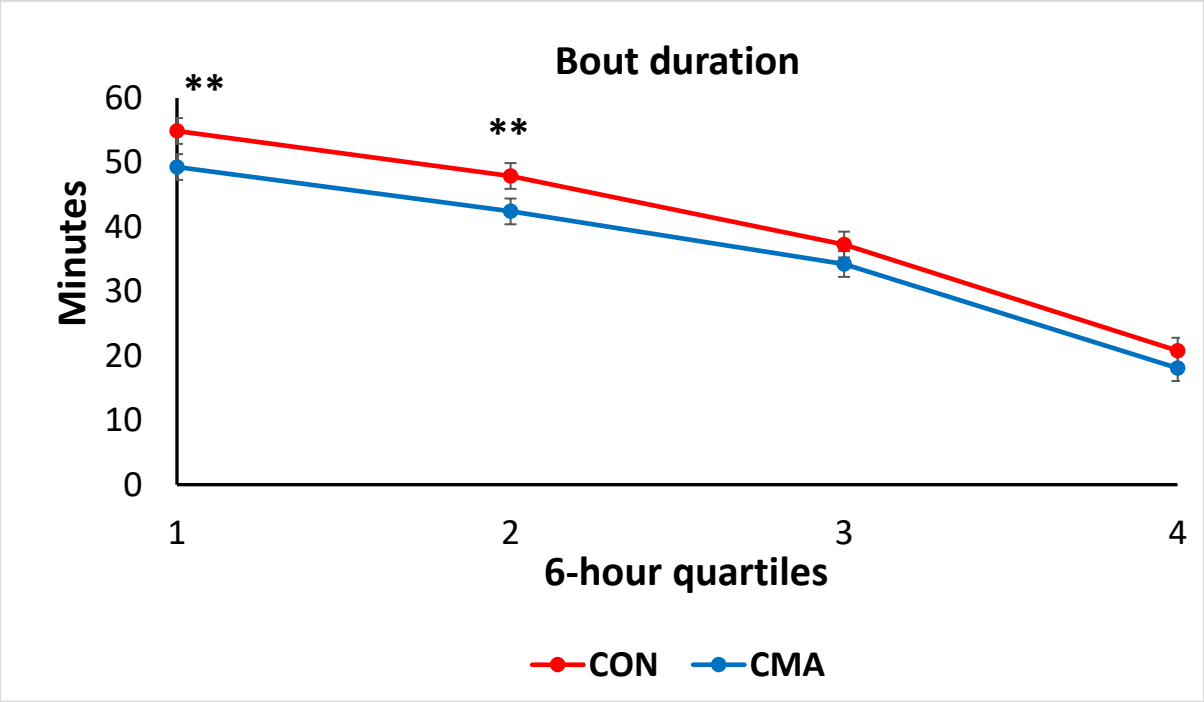

Figure 6.3. The effect of control and calcareous marine algae on bout duration during the 6-hour quartiles of the day.

Quartiles; 1 = 0900 to 1500 h; 2 = 1500 to 2100 h; 3 = 2100 to 0300 h; 4 = 0300 to 0900 h

P-value for treatment effect: ***** = *P*<0.10; ****** = *P* <0.05; ******* = *P* <0.01

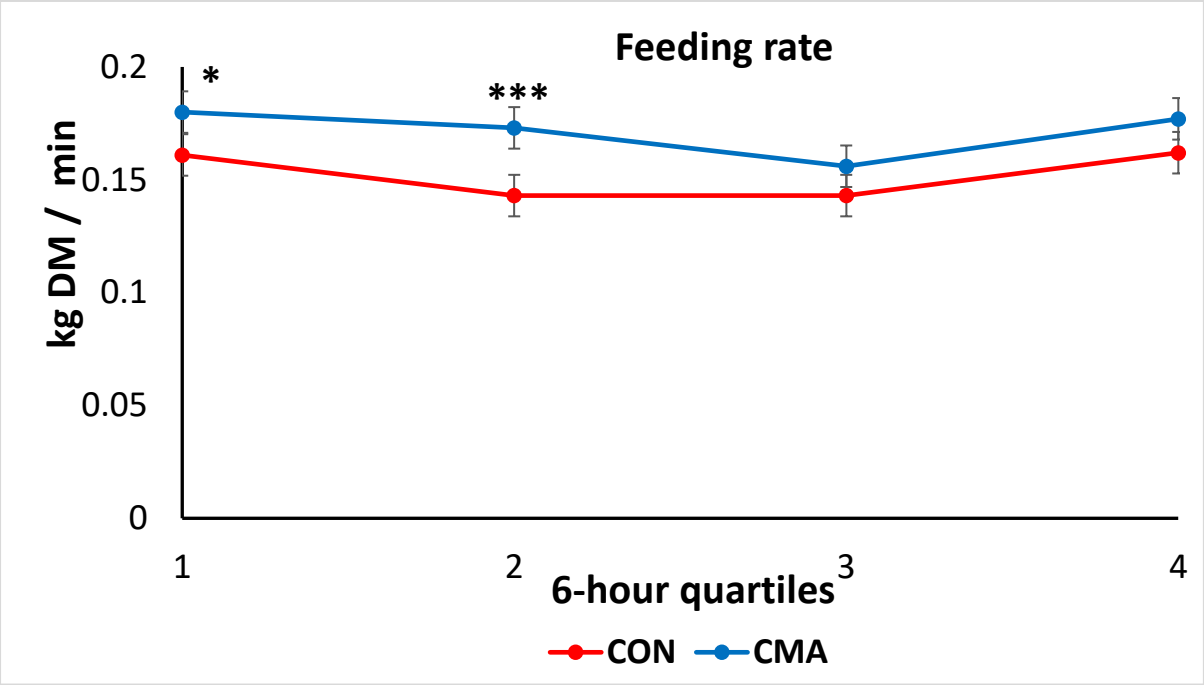

Figure 6.4. The effect of control and calcareous marine algae on feeding rate during the 6-hour quartiles of the day.

Quartiles; 1 = 0900 to 1500 h; 2 = 1500 to 2100 h; 3 = 2100 to 0300 h; 4 = 0300 to 0900 h

P-value for treatment effect: * = $P<0.10$; ** = $P<0.05$; *** = $P<0.01$

Chapter 7.

Thesis summary and conclusion

7.1 Summary

Dairy cows require large amounts of readily fermentable carbohydrates to provide them with sufficient energy and nutrients to achieve their genetic potential for milk production. Volatile fatty acids (VFA) generated from rumen fermentation contribute 75% of the cow's total energy requirement (Penner, 2009). Providing effective fibre that promotes saliva production and rumen function in dairy cow diets is also essential to maintain rumen pH within a normal physiological range. However, it can be difficult to include adequate effective fibre in the lactating cow diet whilst also providing sufficient energy due to the antagonistic relationship between both nutrients. A rapid breakdown of carbohydrates in the rumen provides substrates for rumen microbes and the subsequent production of VFA will lower rumen pH if allowed to accumulate (Humer et al., 2018). Dairy cows can experience abrupt dietary changes during lactation. Some of these changes are an inevitable part of the cow's production cycle, such as the transition period where cows change from dry cow to lactating cow diets within a short period. These dietary changes cause serious disruption to rumen fermentation due to fermentation acid generation exceeding rumen epithelial VFA absorptive capacity (Steele et al., 2015). Hence, VFA accumulate leading to prolonged periods of low rumen pH. Studies carried out on northern European dairy farms suggest that up to 20% of TMR-fed cows (Kleen et al., 2013) and 11% of pasture-fed cows (O'Grady et al., 2008) are suffering from prolonged periods of rumen pH depressions. Extended periods of low rumen pH have many negative consequences, from a production, economic and animal health point of view. Therefore, avoiding rumen pH depression is important to dairy farmers, nutritionists, and researchers.

Experiments have already illustrated these undesirable consequences, such as negatively altered milk composition (Krause and Oetzel, 2006), altered rumen microbial populations (Hook et al., 2011) and health issues such as laminitis and inflammation (Plaizier et al., 2014). Increasing milk production efficiency can be achieved by improving the utilisation of feedstuffs and a desirable rumen pH is important for optimal digestibility of feedstuffs in the rumen. The work of Mulligan et al. (2002) demonstrated that the total tract digestibility of dry matter (DM) and organic matter (OM) were positively correlated to rumen pH.

Chapter 2 gives a comprehensive overview of different rumen modifying feed additives and their effects on rumen pH and fermentation. Sodium bicarbonate (SB) is the most commonly used rumen buffer in dairy cow diets (Rauch et al., 2012). Magnesium Oxide (MgO) is also used to correct rumen pH problems in dairy cows (Bach et al., 2018) but is usually supplemented in combination with other buffers like SB. Calcareous marine algae (CMA) has been used successfully to improve rumen pH and fermentation, milk production, and milk production efficiency (Bernard et al., 2014; Cruywagen et al., 2015; Neville et al., 2019). Further research has demonstrated the effects of CMA, in combination with marine MgO (MM), on rumen pH parameters and milk production in TMR and pasture-fed cows (Neville et al., 2019; Rafferty et al., 2019). The database of research documenting the use of SB and MgO in dairy cows is extensive and much older than that of CMA research. There are several research gaps around the use of CMA in dairy cows, such as the effects of CMA on digestion, inflammation, milk fatty acid profiles, and on rumen pH parameters during different dietary challenges. There is limited work around the effects of CMA in transition dairy cows. The purpose of this thesis was to deepen our understanding of the mechanisms underpinning these effects of CMA in dairy cows. The thesis objective was to investigate the effects of CMA on rumen pH and fermentation, digestion, feed intake, milk production, and inflammation in dairy cows during a dietary challenge. Different types of dietary challenges were used to achieve our objective. The dietary challenges included: the transition period; a high starch total mixed ration (TMR), a grain-induced sub-acute ruminal acidosis (SARA); and a ryegrass induced SARA.

Chapter 3 compared the effects of CMA to a limestone-based control on feed intake, milk production, energy balance, serum mineral metabolites and inflammatory markers in transition dairy cows. Twenty-two multiparous and 10 primiparous cows were assigned to 2 treatments in a randomized complete block design from 25 days (d) before expected parturition until 42 d postpartum. Cows were fed a negative dietary cation-anion difference (DCAD) [-50 mEq/kg] TMR based on maize silage, grass silage, and straw during the prepartum and a 50:50 forage: concentrate TMR based on grass silage, maize silage and concentrate during the postpartum period. The 2 dietary treatments consisted of a control (CON), which contained limestone as the primary calcium source, and CMA where limestone was replaced by CMA at 0.42% and 0.47% DM for the pre and postpartum periods, respectively.

Chapter 4 investigated the effects of CMA with or without marine magnesium oxide (MM), and SB on rumen pH parameters, VFA production, apparent total tract digestion, and the kinetics of digestion in lactating dairy cows fed a high starch TMR based on grass silage, maize silage and ingredients typical of a confined northern European feeding system. Four multiparous cannulated Holstein cows were utilized in a 4 x 4 Latin square design. Dietary treatments consisted of the control (55% concentrate, 33% starch and sugar, and 20% neutral detergent fibre [NDF] from forage) including no dietary buffer (CON); CON including CMA at 0.45% DM (CMA); CON including CMA at 0.45% DM and MM at 0.11% DM (CMA+MM); CON including SB at 0.9% DM (SB). The experiment contained 4 periods of 25 d, with each period consisting of 13 d acclimatization followed by 12 d of sampling and data collection. Both CMA treatments had the biggest impact on rumen pH.

Chapter 5 focused on determining the effects of CMA on rumen pH, rumen fermentation, and plasma inflammatory markers during a grain (GR) and ryegrass (RY) induced SARA challenge. Eight ruminally cannulated Holstein dairy cows were assigned to 4 experimental treatments in a 2 × 2 split-plot crossover design. The main plot was diet during the SARA challenge, GR or RY, and the sub-plot was treatment, control (CON) or CMA. The experiment consisted of 3 phases: acclimatisation (ACC), d 1 – 20; challenge (CHL), d 21 – 30; and recovery (REC), d 31 – 38. The cows remained on their main plots, GR or RY, throughout the experiment and alternated from CON to CMA and vice-versa as they progressed from period 1 to period 2 of the experiment. For the GR CHL, each cow was offered a TMR consisting of 30% or 7.1 kg DM of ground wheat barley mix (50% wheat and 50% barley) along with grass silage, maize silage, soybean meal, and the experimental concentrate pellet. For the RY CHL, cows were offered 19.7 kg DM of fresh ryegrass, cut at the 3-leaf stage or equivalent to 1,000 - 1,200 kg DM/ ha of a pre-cutting sward mass, and 3.7 kg DM per cow of the experimental concentrate pellet fed in 2 equal portions during a.m. and p.m. milking each day.

Chapter 6 investigated the effects of CMA on feeding behaviour, rumen fermentation products, milk fatty acid profiles, and total tract digestibility of DM and OM in early lactation dairy cows. Twenty-two multiparous and 10 primiparous cows were assigned to 2 treatments from 25 d before expected parturition until 42 d postpartum. Cows were assigned to 2 treatments according to a randomized complete block design. Cows were fed a negative DCAD [-50

mEq/kg] TMR based on maize silage, grass silage, and straw during the prepartum and a 50:50 forage: concentrate TMR based on grass silage, maize silage and concentrate during the postpartum period. The two dietary treatments consisted of a control (CON), which contained limestone as the primary calcium source, and CMA at 0.42% and 0.47% DM for the pre and postpartum periods, respectively.

7.1.1 Rumen pH and fermentation

An important consideration when testing rumen additives is to have a negative control (un-supplemented diet) and a positive control (common industry standard product). Therefore, chapter 4 investigates the effects of CMA compared to a control (CON) and a SB treatment. Marine MgO was also supplemented in combination with CMA, as a fourth treatment, to examine potential synergies between CMA and MM. The CMA and CMA and MM treatments increased mean, median and minimum rumen pH, and reduced time spent below rumen pH 5.6 and 5.4 compared to the CON treatments. While there was no difference between the CMA treatments and SB for the rumen pH parameters, the SB treatment failed to reduce the time spent below rumen pH 5.6 and 5.8 compared to the CON treatment. Results from chapter 5 agree with chapter 4 for the effects of CMA on rumen pH parameters, but only during the ACC and REC phases. Surprisingly, the CMA treatment had no effect on rumen pH parameters during the GR and RY induced SARA challenges. This differs from results of chapter 4 and most previous experiments examining CMA in dairy cows (Cruywagen et al., 2015; Neville et al., 2019). The 100 g per cow/ d dosage rate of CMA may not have been sufficient to buffer the excess fermentation acids created during the SARA challenge. This will need to be investigated further. The range and coefficient of variation (CV) for daily rumen pH was reduced on the CMA treatment compared to the SB treatment in chapter 4 and compared to the CON during the ACC phase and compared to the CON within the ryegrass diets during the REC phase of chapter 5. A reduction in rumen pH variation could lead to a more stable rumen fermentation and rumen microbial profiles, as the acid sensitive cellulolytic bacteria will continue to proliferate in the higher pH range (> pH 6.0) while still maintaining an environment for the amylolytic bacteria to proliferate in the slightly lower pH range (pH 5.5 – 6.0) (Weimer et al., 1996).

Other than the effect of CMA treatment reducing *iso*-butyrate compared to the CON treatment during the ACC phase of chapter 5 and the CMA treatment reducing rumen osmolality

compared to the CON treatment, there were no other effects of CMA on rumen VFA, osmolality, and ammonia concentrations during chapter 4, 5, and 6. Increased rumen osmolality has been associated with a reduction in feed intake (Allen, 2000). The increased dry matter intake (DMI) and altered feeding behaviour with the CMA treatment compared to the CON treatment observed in chapter 3 and 6 may have been influenced by rumen osmolality. When CMA was supplemented with marine MgO in chapter 4, there was a reduction in rumen acetate and an increase in rumen propionate as a proportion of total VFA on the CMA+MM treatment compared to the CON and CMA-only treatment. Therefore, the addition of MM to diets already containing CMA caused this response in rumen VFA proportions.

7.1.2 Digestion

There has been no work carried out, previous to this thesis, describing the effects of CMA on total tract digestion, rumen degradability, or digestion kinetics in dairy cows. Chapter 4 found that CMA had no effect on the apparent total tract digestibility of DM and NDF compared to the CON and SB treatments. When MM was supplemented with CMA, there was an increase in apparent total tract digestibility of NDF compared to the CON treatment. The literature reported in chapter 2 shows strong links between rumen pH and NDF digestion (Allen, 1997). Despite the CMA and CMA+MM having similar rumen pH profiles, their effects on NDF digestion differed. Therefore, the addition of MM to diets already containing CMA appeared to enhance these effects. Chapter 6 used acid insoluble ash (AIA) as a marker of apparent total tract digestibility in dairy cows at 7 and 14 days in milk. There was an increase in both DM and OM digestibility with the CMA fed cows compared to the CON fed cows. These results differ slightly from chapter 4. The positive effects of CMA on DM and OM digestibility during early lactation could be explained by the positive effects of CMA on rumen pH parameters in chapter 4 and 5. The increased digestibility of DM and OM may explain the improvements in DMI during the same period, reported in chapter 3. Increased digestibility will allow cows to consume more feed due to increased rate of digested particles leaving the rumen. Chapter 4 results also suggest that the increased DMI observed in CMA fed cows during chapter 3 was not caused by changes in rumen fluid and particulate outflow rates. These results on digestion kinetics have deepened our understanding around the mechanisms behind CMA and is contrary to the theory of Russell and Chow (1993) that the buffering ability of SB was based on changes in rumen fluid outflow rates.

7.1.3 Feed intake and milk production

Chapter 2 outlines many factors that may cause a depression in feed intake during the early postpartum stage of the transition period. Low rumen pH may be a contributing cause in reduced feed intake experienced during the early postpartum period (Penner et al., 2007). The benefits of CMA on reducing rumen pH depressions, outlined in chapter 4 and 5 may provide an explanation for the DMI response during the postpartum period of chapter 3. Results from chapter 6 showed that cows fed CMA had greater feeding rates (kg DM/min) during the 1st and 2nd daily quartiles, from 0900 h to 2100 h each day. This increased feeding rate in the first 12 h after feed delivery is responsible for the greater DMI observed on the CMA treatment compared to the CON treatment. Diurnal rumen pH measurements outlined in chapter 4 show that CMA maintains a higher rumen pH in the late evening, from 1600 h to 2200 h. This provides further evidence that increased DMI with CMA in chapter 3 may have been due to improvements in rumen pH. The CMA treatment in chapter 5 increased DMI and reduced the time spent below rumen pH 5.6 and 5.8 during the REC phase. This provides further evidence to support rumen pH improvements as a mechanism for increased DMI.

Another factor involved in feed intake regulation in early lactation, outlined in the literature review; chapter 2, is the influence of Ca status. Cows that are deficient in Ca have reduced smooth muscle contractions and rumen motility, which leads to lower DMI (Goff, 2008). Cruywagen et al. (2015) described CMA as sparingly soluble in water, due to its chemistry and unique physical structure derived from its marine origin, and therefore effective as rumen buffer while also providing a soluble source of Ca in the rumen. While there is potential for a Ca derived CMA effect on increased DMI, we failed to find consistent evidence to support this theory. Chapter 3 also reported serum Ca concentrations, but no effect of CMA compared to the CON treatment was observed. The increased DMI pre and postpartum with the CMA treatment in chapter 3 resulted in lower levels of serum NEFA concentration prepartum and greater energy balance postpartum on the CMA treatment.

Chapter 2 describes previous experiments where CMA increased milk fat concentration in dairy cows (Cruywagen et al., 2015; Neville et al., 2019). This is consistent with the milk production results of chapter 3. The CMA fed cows had a sizeable increase (+ 0.4%) in milk fat concentration over the CON fed cows. Milk production was not measured in chapter 4 and chapter 5 reported no differences between the CMA and CON treatments for milk fat concentration across the ACC, CHL, and REC phases. However, the sample size used in

chapter 5 (n=4) was not sufficient to detect any statistical differences for milk production parameters as milk production was not the main focus of this chapter. Milk fat production in dairy cows is highly correlated with rumen pH (Allen, 1997). The biohydrogenation of fatty acids in the rumen is altered when rumen pH is low, leading to the production of biohydrogenation intermediates which may reduce fat production in the mammary gland (Bauman and Griinari, 2003). The presence of these biohydrogenation intermediates can be detected through milk fatty acid analysis (Colman et al., 2010). Chapter 6 was the first experiment to measure the fatty acid profile of milk from cows treated with CMA versus a CON. Results from chapter 6 showed tendencies for increased concentration of saturated fat and also increased *cis*-9 18:1 which can sometimes be indicative of improved rumen biohydrogenation of fatty acids (Cabrita et al., 2009). However, there was no changes detected for the biohydrogenation intermediates commonly associated with milk fat depression, *trans*-10, *cis*-12 CLA or *trans*-10 18:1, between the CMA and CON treatments. Therefore, we cannot conclusively say that CMA's effects on increased milk fat are due to improvements in rumen fatty acid biohydrogenation pathways. However, the links between rumen pH and milk fat are well proven and the effects of CMA on milk fat are most likely linked to rumen pH effects in some capacity.

7.1.4 Inflammation

Inflammation is an emerging aspect of dairy cow biology and may be an underlying cause of many negative health and production consequences experienced by dairy cows. Problems derived from inflammation are particularly relevant during the transition period when most health problems occur (Bradford et al., 2015). Chapter 2 cited some previous work that showed anti-inflammatory effects of CMA when used on human macrophage cells *in-vitro* (O'Gorman et al., 2012) but there has been no work undertaken to measure the effects of CMA on inflammation in dairy cows. Results from chapter 3 suggest that CMA may play a role in reducing the extent of inflammation experienced by dairy cows after they undergo a dietary change during the transition period. The CMA treatment tended to reduce plasma concentration of the inflammatory marker, serum amyloid A (SAA), compared to the CON treatment during the postpartum period. One of the main causes of systemic inflammation in dairy cows is the disruption to rumen fermentation and rumen pH depressions, usually as a result of an abrupt dietary change. Several researchers have proven that a GR-induced (SARA) leads to a

translocation of lipopolysaccharide (LPS) from the digestive tract to the peripheral system, subsequently invoking an inflammatory response (Gozho et al., 2007; Khafipour et al., 2009a; Li et al., 2012). Damaged rumen epithelium provides an entry point for LPS to translocate into the peripheral system (Steele et al., 2011). It has also been reported that alfalfa-induced SARA does not cause inflammation despite having a similar rumen pH profile to GR induced SARA (Khafipour et al., 2009b). Furthermore, Li et al. (2012) suggested that inflammation was caused by LPS translocation via intestinal epithelial damage in the hindgut and not in the rumen. This could explain why inflammation was caused by GR induced SARA but not by alfalfa induced SARA due to the characteristic passage of starch from the rumen to the hindgut experienced by cows during a GR induced SARA. The use of RY to induce a SARA challenge has not been investigated previously. In chapter 5, we discovered that the GR and RY-induced SARA caused similar depressions in rumen pH, and increase in rumen LPS and plasma lipopolysaccharide binding protein (LBP) compared to the ACC phase. However, the rumen LPS concentration was much higher on the GR induced SARA compared to the RY induced SARA. The CMA treatment reduced SAA concentration compared to the CON treatment within the RY diet during the CHL phase and had a greater reduction in plasma SAA concentration from the ACC to CHL phase on both GR and RY diets. The effects of CMA on plasma SAA concentration during a SARA challenge in chapter 5 supports the similar reduction in plasma SAA in transition cows on the CMA treatment in chapter 3. However, we have yet to determine the mechanism behind the effects of CMA on reducing inflammatory markers in dairy cows. The absence of rumen pH effects with the CMA treatments during the SARA challenges in chapter 5 suggest that this reduction in inflammatory markers is not related to pH changes in the rumen during a challenge episode. Zebeli et al. (2012) reported that only 15 to 21% of the overall variation in the SAA concentrations were due to rumen pH in a review of 10 different SARA challenge experiments.

7.2 Conclusion, implications, and future work

As the dairy industry strives to be more sustainable in the coming years, topics like increasing the efficiency of dairy production and reducing the environmental footprint of dairy products while also maintaining optimum health and welfare standards among the dairy livestock will be of utmost importance. Research from this thesis offers both knowledge and potential tools to increase the efficiency of dairy production and improve the health of dairy cows. The

positive effects of CMA on rumen pH parameters demonstrates its superiority as a rumen buffer and will help dairy farmers and nutritionists to choose the most effective rumen buffer for their diets. These improvements in rumen performance can translate to improvements in diet digestibility with CMA and CMA supplemented with MM allowing cows to extract more nutrients from their feed, and subsequently lead to increased milk production efficiency. Improvements in rumen pH translated to increased milk fat production and altered milk fatty acid composition with CMA. Increased milk fat production will allow dairy farmers, in many regions of the world where milk price is influenced by milk constituents, to achieve a better milk price. Increased feed intake and better energy balance during the transition period will be a real positive for dairy farmers during one of the most critical periods of the cow's production cycle and could translate into better health, fertility and production in the following lactation. Finally, the indications of reduced inflammation in cows fed CMA outlined in this thesis may become valuable to dairy farmers, nutritionists, and researchers as increased focus on disease prevention and reduced antibiotic usage will become more important.

One of the main implications of this thesis is that the addition of CMA can help maintain rumen pH in a desirable range for lactating dairy cows. Calcareous marine algae can be viewed as being more effective than the traditional rumen buffer sodium bicarbonate for regulating rumen pH. The knock-on effects of stabilising rumen pH were observed more so within the milk fat component than in milk yield. Previous literature has questioned the use of rumen buffers in medium to high forage diets (> 30% DM) (Erdman, 1988, Hu and Murphy, 2005). The research in this thesis has shown that lactating dairy cows consuming a variety of diet types containing different levels of forage, from 40 to 59%, can suffer from prolonged periods of rumen pH depression and the addition CMA can help regulate rumen pH, increase diet digestibility, and increase milk fat production. The increased DMI in cows fed CMA during the transition period is another important implication of this thesis. One of the primary goals of a good transition period programme is to increase DMI (Grummer et al., 2004), and CMA offers a tool to achieve this. Understanding how inflammation affects cows during the transition period and during dietary challenges will also be an important implication of this thesis. The effects of CMA on reducing inflammatory markers in transition cows and cows undergoing a SARA challenge is an important finding of this thesis. The information within this thesis on the RY-induced SARA challenge and its consequences on production and inflammation will answer some of the

unknowns that exist in this area of research. There is very little published literature if any, on the consequences of a RY induced SARA challenge.

Future work investigating the use of CMA in dairy cow diets needs to take a closer look at the rumen microbiome populations and the functionality of the microbiome. Lipopolysaccharide has received the most attention as a toxin that causes systemic inflammation. The fermentation of starch can lead to the production of gram-negative bacteria, from which LPS are derived (Chiquette et al., 2015). We also need to determine if the fermentation of ryegrass diets yields gram-negative bacteria that shed LPS or other toxins capable of inducing an inflammatory response. Initial work reported in this thesis would indicate that rumen LPS production in RY-based SARA episodes is reduced compared to GR-based SARA episodes. Future work is needed to investigate the interaction of CMA with different diet types and different microbes produced by these different diet types to assess LPS production. The mechanisms behind the effects of CMA on inflammatory markers in dairy cows is not yet fully understood. Future work is needed to carry out more detailed work on these inflammatory markers to determine if they are related to dietary changes. Measuring rumen epithelial morphology and gene expression would be useful in furthering our understanding around the effects of CMA in the rumen. In tandem with this, future research should look at the effects of CMA in the hindgut, such as its effects on caecal LPS concentrations and intestinal epithelial morphology and gene expression to assess barrier function. While this thesis was useful in strengthening the literature on the effects of CMA on milk fat production and identifying fatty acids that are altered, further work is required to investigate the effects of CMA on the biohydrogenation of fatty acids in the rumen and subsequent milk fat synthesis at the mammary gland.

7.2 Literature cited

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Chapter 8.

Publication List

Conferences, Presentations and Publications to Date

- Oral presentation at BSAS in April 2018:
  "**Neville, E. W.** 2018. The effect of calcareous marine algae, with or without marine magnesium oxide, and sodium bicarbonate on rumen pH and milk production in mid-lactation dairy cows."

- Oral Presentation at the 5th Herd Health Symposium, Antalya, Turkey, October 2018:
  "**Neville, E. W.** 2018. The effect of calcareous marine algae, with or without marine magnesium oxide, and sodium bicarbonate on rumen pH and milk production in mid-lactation dairy cows."

- Publication in Journal of Dairy Science 2019:
  "**Neville, E. W**., A. G. Fahey, V. P. Gath, B. P. Molloy, S. J. Taylor, and F. J. Mulligan. 2019. The effect of calcareous marine algae, with or without marine magnesium oxide, and sodium bicarbonate on rumen pH and milk production in mid-lactation dairy cows. J. Dairy Sci. 102:8027-8039. https:///dx.doi.org/10.3168/jds.2019-16244"

- Publication in Animal and Veterinary Sciences journal 2019:
  "Lawlor, J., A. Fahey, **E. Neville,** A. Stack, and F. Mulligan. 2019. On-farm safety and efficacy trial of cow start calcium bolus."

- All About Feed webinar in November 2020:
  "**Neville, E. W.** 2020. How plant derived Marine Minerals promote feed efficiency and sustainability in dairy production."

- Publication in Animal and Veterinary Sciences journal 2019:
  "Lawlor, J., A. Fahey, **E. Neville,** A. Stack, and F. Mulligan. 2020. Efficacy of Cow Start calcium bolus on metabolic status and milk production in early lactation."

- Oral presentation at ADSA annual conference in July 2021:
  "Neville, E. W., A. G. Fahey, and F. J. Mulligan. 2021. The effect of calcareous marine algae on feed intake, milk production, mineral status, energy balance, and inflammatory markers in transition dairy cows."