# Effect of feed additive supplementation on rumen bacterial amino acid profile and fermentation dynamics in dairy cows

**By**

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**Submitted in partial fulfilment of the requirements for the degree**

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# Declaration

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

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# Summary

## Effect of feed additive supplementation on rumen bacterial amino acid profile and fermentation dynamics in dairy cows

by

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Four late-lactation Holstein-Friesian cows were utilised in a 4 x 4 Latin square design experiment to evaluate the effect of a calcareous marine algae buffer, Acid Buf 10 (Celtic Sea Minerals), a direct-fed microbial product, Achieve$^{FE}$ (MicroBasics Inc.) and sodium monensin (Rumensin, Elanco Animal Health) on ruminal fermentation patterns, microbial crude protein (MCP) production, and the amino acid (AA) profile of the ruminal bacteria associated with the liquid and solid phases. The basal diet fed was a total mixed ration (TMR) comprised of 400 g/kg high quality Lucerne hay and 600 g/kg concentrates, of which 399 g/ kg of concentrate was ground corn (dry matter (DM) basis). Treatments were: (1) control diet (C), (2) control diet plus 3.75 g/kg DM of Acid Buf 10 (AB10), to achieve an intake of 90 g/cow/day, (3) control diet plus 10 g/day of Achieve$^{FE}$ (DFM) inserted directly into the rumen, and (4) control diet plus 10.84 mg/kg DM of monensin (MON), to achieve an intake of 260 mg/cow/day. The four experimental periods consisted of a 14 day "wash-out" period and an 11 day collection period. Cows were fed their respective TMR *ad libitum* twice daily at 08:00 and 14:00, with the DFM product being placed directly into the rumen daily prior to the 08:00 feeding. Cows were milked three times daily at 06:00, 12:00 and 19:00.

Dietary treatments had a minimal effect on performance parameters in this study, with no response being observed for DM intake, body composition and lactational performance ($P < 0.05$). The only exception being milk protein content which was reduced ($P = 0.04$) by supplementation with MON. Ruminal pH and fermentation parameters were monitored at intervals over a 24 hour period. Treatment had no significant effect on ruminal acidity, however, both C and DFM tended ($P = 0.07$) to present with lower mean ruminal pH values than AB10. The AB10 treatment tended to reduce the time pH remained below 5.5, the threshold for sub-acute ruminal acidosis, as compared to C (AB10 = 6.1 hours *versus* C = 10.1 hours, $P = 0.09$) and was numerically less than DFM (9.3 hours) and MON (7.1 hours). Treatment had a minimal effect on the ruminal volatile fatty acids (VFA's) and ammonia-nitrogen concentration. Treatment had no impact on microbial protein synthesis but MON tended ($P = 0.07$) to have a reduced MCP yield compared to AB10. Bacteria isolated from both the fluid and particulate phases of the rumen were found to differ in chemical and AA composition. Supplementation was shown to have an effect on the bacterial AA profile, with 9 and 10 of the 16 AA's being either altered ($P < 0.05$) or tending to be altered ($P \leq 0.10$), for the fluid-associated and particle-associated bacterial fractions, respectively. Of which the most limiting AA's for dairy cows, lysine, methionine and histidine were affected by treatment ($P < 0.05$). This study demonstrates the potential of feed additives to alter the composition of the MCP following to the duodenum, however, no clear pattern of alteration in the AA profile was identified. Owing to the significant contribution of this protein source to meeting the AA requirement of ruminants' further research on this topic is warranted.

# List of Abbreviations

| | |
|---|---|
| AA | amino acid |
| AACC | American Association of Cereal Chemists |
| ADF | acid detergent fibre |
| ADL | acid detergent lignin |
| ADY | active dry yeasts |
| AEC | Animal ethics committee |
| AL | allantoin |
| aNDF | NDF assayed with heat stable amylase |
| $aNDF_{OM}$ | aNDF free of residual ash |
| AOAC | Association of Official Analytical Chemists |
| ATP | Adenosine triphosphate |
| BCS | body condition score |
| BCVFA | Branched-chain volatile fatty acid |
| BHBA | β-hydroxybutyrate |
| BUN | blood urea nitrogen |
| BV | biological value |
| BW | body weight |
| Ca | calcium |
| $CaCO_3$ | limestone |
| CFU | colony forming units |
| $CH_4$ | methane |
| $Cl^-$ | chloride ion |
| CLA | conjugated linoleic acid |
| CMA | calcareous marine algae |
| CNCPS | Cornell Net Carbohydrate and Protein system |
| $CO_2$ | carbon dioxide |
| CP | crude protein |
| CR | creatinine |
| CV | coefficient of variation |
| d | day |
| DAPA | diaminopimelic acid |
| DE | digestible energy |
| DFM | direct-fed microbial |
| DIM | days in milk |
| DM | dry matter |
| DMD | dry matter digestibility |
| DMI | dry matter intake |
| DNA | deoxyribonucleic acid |
| EAA | essential amino acid |
| ECM | energy corrected milk |
| EE | ether extract |
| EU | European Union |
| FAO | Food and Agriculture Organisation |
| FAB | fluid-associated bacteria |
| FCM | fat corrected milk |
| g | gram |
| GE | gross energy |
| GRAS | generally recognised as safe |
| $H^+$ | hydrogen ion |
| $H_2$ | hydrogen gas |
| HAP | hyper-ammonia producers |
| $HCO^{3-}$ | bicarbonate |
| HPLC | high-performance liquid chromatography |
| iDM | initial dry matter |
| IFCN | International Farm Comparison Network |
| IVOMD | in vitro organic matter digestibility |
| K | potassium |
| $K^+$ | potassium ion |
| kg | kilogram |
| L | litre |
| LAB | lactic acid producing bacteria |
| LUB | lactic acid utilising bacteria |

| | |
|---|---|
| MCP | microbial crude protein |
| ME | metabolizable energy |
| MFD | milk fat depression |
| MFES | microbial free extraction solution |
| mg | milligram |
| Mg | magnesium |
| MgO | magnesium oxide |
| min | minutes |
| MP | metabolizable protein |
| MPO | Milk Producers Organisation |
| MPS | microbial protein synthesis |
| MUN | milk urea nitrogen |
| N | nitrogen |
| $N_2$ | nitrogen gas |
| $Na^+$ | sodium ion |
| $NaHCO_3$ | sodium bicarbonate |
| NAN | non-ammonia nitrogen |
| NDF | neutral detergent fibre |
| NE | net energy |
| NEAA | non-essential amino acid |
| NFC | non-fibrous carbohydrate |
| NGS | next generation sequencing |
| $NH_3$ | ammonia |
| $NH_3$-N | ammonia-nitrogen |
| NPN | non-protein nitrogen |
| NRC | National Research Council |
| NSC | non-structural carbohydrates |
| O | oxygen |
| OECD | Organisation for Economic Co-operation and Development |
| OM | organic matter |
| OMD | organic matter digestibility |
| P | phosphorous |
| PAB | particle-associated bacteria |
| PD | purine derivatives |
| PDC | purine derivative to creatinine index |
| PDE | total purine derivative excretion |
| pKa | acid-dissociation constant |
| $PO^{3-}_4$ | phosphate ion |
| RDP | rumen degradable protein |
| RNA | ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| RUP | rumen undegradable protein |
| SARA | sub-acute ruminal acidosis |
| SCC | somatic cell count |
| SD | standard deviation |
| SEM | standard error means |
| SG | specific gravity |
| SNF | solids-not-fat |
| TMR | total mixed ration |
| TTAD | total tract apparent digestibility |
| TTOMD | total tract organic matter digestibility |
| UN | United Nations |
| VFA | volatile fatty acid |
| WRC | whole rumen contents |

List of AA:

| | | | |
|---|---|---|---|
| Ala | alanine | Arg | arginine |
| Asp | aspartic acid | Cys | cysteine |
| Glu | glutamic acid | Gly | glycine |
| His | histidine | Ile | isoleucine |
| Leu | leucine | Lys | lysine |
| Met | methionine | Phe | phenylalanine |
| Pro | proline | Ser | serine |
| Thr | threonine | Tyr | tyrosine |
| Val | valine | | |

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# PREFACE

This dissertation consists of several chapters dealing with the topic of the effect of feed additive supplementation on the rumen bacterial amino acid profile and fermentation dynamics in dairy cattle, detailing research methodology, and evaluating research outcomes.

Protein nutrition is critical to the high production efficiency of lactating dairy cows, of late much focus has been on the environmental impact of dairy farming, with regards to nitrogen pollution, leading ruminant nutritionists to focus on the optimisation of milk production with minimal inclusion of dietary crude protein. This topic is briefly addressed as a general discussion in Chapter one, along with the project objectives and hypotheses.

Ruminants have the evolutionary advantage of utilising fibrous plant matter as their primary food source, owning to their capacious forestomach and symbiotic relationship with the inherent microbiome, this differentiates them from monogastric animals in terms of nitrogen an energy metabolism. Chapter two details this unique aspect of ruminant nutrition along with a review of the various feed additives in use to modify and improve ruminal fermentation with a resultant enhancement in the productive performance of lactating dairy cattle.

The method for the isolation and fractionation of the ruminal microbes is yet to be standardised, to date various techniques have been utilised to obtain bacterial and protozoal isolates, all of which give various recoveries and have limitations. In chapter three published research studies are examined in an attempt to detail the methodology behind the isolation of ruminal microbes, and come to a conclusion about an “ideal” procedure.

Experimental design, sample collection and analytical techniques employed are detailed in Chapter four.

The results from the research trial along with an in-depth discussion of the observed outcomes are presented in chapters five and six. Chapter five focuses on productive performance, ruminal pH and fermentation, whilst Chapter six details the results related to microbial protein production and the amino acid profile of the ruminal bacteria.

Following this general conclusions are drawn in Chapter seven, thereafter recommendations for future research and a critical evaluation of the work are presented in Chapter eight

# CHAPTER 1

# GENERAL INTRODUCTION

Animal agriculture currently faces the immense challenge of feeding the ever-growing global population, with the United Nations (UN) forecasting that the global population will reach 9.8 billion by the year 2050, with the majority of this growth attributable to African countries (UN, 2017). This global population growth along with increasing urbanisation and gross income are the principal drivers of the global average per capita consumption of dairy products, which according to the OECD-FAO Agricultural Outlook (2015) will increase by 13.7 % from 2013 to 2023, with much of this growth concentrated in developing countries (MPO, 2016). A recent analysis by the International Farm Comparison Network (IFCN, 2018) determined that the global dairy demand increased by 20 million tonnes in 2018, of which 6 million tonnes was as a result of population growth and 14 million tonnes a result of increased per capita dairy consumption (MPO, 2018). This IFCN analysis also forecasted a total increase in milk production and demand of 35 % by 2030. In order to meet this growing demand dairy producers face the challenge of enhancing productive performance in a cost-effective, environmentally friendly and sustainable manner. With limited availability of arable land the primary means by which global demand will be met is through improvements in production efficiency.

Protein nutrition is critical to the high production efficiency of lactating dairy cows, with a decrease in production observed when rations are not properly balanced for protein (Lanzas *et al.*, 2007a). Ultimately dairy producers aim to optimise ruminal efficiency and productive performance with rations lower in crude protein (CP) so as to reduce the cost of production as well as minimise any negative environmental impacts. Purchased feeds, particularly protein supplements are a major expense to the dairy producer (Klausner *et al.*, 1998), with total feed cost constituting approximately 60 % of the total cost of milk production (Beyers, 2001), countries such as South Africa have recently experienced higher production costs than others primarily as a result of increased feed costs (MPO, 2016) in response to drought. The dairy industry is subject to fluctuations in global milk prices, which can negatively affect profitability of dairy enterprises, for this reason producers need to be more mindful of production costs. Under typical conditions the dairy cow utilises dietary CP with a relatively low efficiency of only 25 % to 35 % (Sinclair *et al.*, 2014), this limited efficiency of utilisation translates to substantial losses of high cost nitrogen (N) with approximately 70 % of the remaining N being excreted in the urine and faeces (Reynal & Broderick, 2005). Through volatilisation, denitrification, runoff, and leaching, this N enters the environment (Tamminga, 1992; Hutson e*t al.*, 1998), contributing to ammonia emissions and nitrate contamination of surface and groundwater. For this reason, livestock enterprises have been identified as significant contributors to nonpoint sources of environmental pollution (NRC, 1993, 2003).

For decades, dairy nutritionists have fed CP in excess in an attempt to meet the amino acid (AA) requirements for desired milk yield, this has incurred unnecessary feed expenses, with reduced N efficiency, and no apparent benefit to animal wellbeing or performance. Moreover, feeding excessive CP increases the energy cost to synthesise and eliminate excess urea, approximately 12 Kcal of digestible energy (DE) per gram of urea synthesised (Van Soest, 1994), at the expense of milk production with the energy required to excrete excess N equating to approximately 2 kg of milk (Andrieu & Collins, 2013). Additionally, overfeeding protein results in excessive urinary N, the most environmentally labile form of excreted N (Varel *et al.*, 1999).

Animal agriculture is subject to scrutiny and criticism, and under the aforementioned economic and environmental constraints, improvement in the efficiency of N utilisation and the associated reduction in N excretion are paramount to ensure the improved production of marketable product (Reynal & Broderick, 2005) and the sustainability of dairy farms (Lanzas e*t al.*, 2007a). These days nutrition models serve as a valuable farm management tool for achieving these goals (Dinn e*t al.*, 1998; Van Amburgh e*t al.*, 2012). The last two decades have seen drastic improvements with regards to feed formulation, particularly with the development of the evolutionary Cornell Net Carbohydrate and Protein System (CNCPS), which since its release in 1992 (Fox e*t al.*, 1992; Russell *et al.*, 1992; Sniffen *et al.*, 1992; O'Connor *et al.*, 1993) has undergone various analytical improvements, error corrections, and other modifications to incorporate new research findings into the framework. This has allowed for the improvement of model accuracy and precision in the prediction of the most limiting nutrient, i.e. metabolizable protein (MP) or metabolizable energy (ME) allowable milk (Van

Amburgh *et al.*, 2009; 2012; 2013). Recent enhancement of the model has allowed for better prediction of AA supply, requirements and limiting AA's, thus allowing diets to be balanced for AA's, to provide an optimal profile of absorbable essential AA's (EAA) to meet the EAA requirement of the dairy cow with reduced dietary CP levels (Schwab, 2012). This has been shown to be a successful strategy to improve the efficiency of N utilisation for milk and milk protein synthesis in dairy cows (Tucker, 2014), thus improving production and profitability. Refinement of the model to be more sensitive to MP supply, during the development of CNCPS version 6.1. (Van Amburgh *et al.*, 2007; Tylutki *et al.*, 2008) has since allowed the model to be more robust in the assessment of the most limiting nutrient on farm, and in doing so has allowed users to formulate diets at CP levels below 16 % without detriment to productive performance (Van Amburgh e*t al.*, 2009; 2012).

It is widely known that for production to be maximised the AA profile of MP needs to match the tissue needs for AA (Tucker, 2014). Metabolizable protein consists of protein from three sources, dietary protein escaping ruminal degradation, ruminally synthesised microbial crude protein (MCP), and endogenous protein, with MCP contributing 50 % to 80 % to MP (Storm & Ørskov, 1983). Unlike the NRC (2001) which uses a regression approach to estimate the duodenal flow of AA, independent of the AA composition of MCP, the CNCPS uses a factorial approach which requires accurate knowledge of the AA composition of MCP (Sok e*t al.*, 2017). Although accurate knowledge of the AA composition of MCP is imperative to the accurate prediction of AA supply, the CNCPS and derivative models currently utilise a static AA profile of mixed bacteria obtained from literature (Storm *et al.*, 1983; Clark *et al.*, 1992; Volden & Harstad, 1998), of which most of the data is ovine in source (Fessenden *et al.*, 2017; Sok *et al.*, 2017). In addition, the contribution of protozoa to microbial protein production has been historically disregarded, however, this has been corrected for in the latest CNCPS, version 7.0, with the inclusion of protozoa to the microbial sub-model (Higgs, 2014). Unfortunately, the use of static AA profiles for both the bacteria and protozoa, limits the accuracy with which these models can predict AA supply to the duodenum as this data is likely not applicable to dairy cows. Although the study of Clark *et al.* (1992) provided the mean AA composition of mixed bacteria which has been used in a static manner in formulation, this study also highlighted large variation in the AA composition of the ruminal microbes in response to various factors, to name a few, diet composition; feed intake; source of crude protein; and potentially the use of feed additives. In so doing Clark *et al.* (1992) discounted the idea of the AA profile of ruminal bacteria as being constant, showing variability particularly for the EAA's Lysine (Lys) and Methionine (Met), which are regarded as the two first limiting AA's under most dietary conditions for lactating dairy cows. Therefore, if nutritionists wish to more accurately predict the AA supply available to the dairy cow, models utilising a factorial approach need to be more dynamic, and allow for the incorporation of knowledge on how the AA composition of the different fractions of MCP can vary under different dietary conditions.

Dietary additives have been incorporated into dairy rations for many years as modulators of ruminal fermentation to enhance production efficiency, however, since the European Union (EU) ban on ionophores as growth promoters, as of January 2006 (EC, 2003), alternative feed additives are being increasingly utilised in the dairy industry. These dietary additives could potentially affect the AA composition of the microbially synthesised CP, the yield of MCP through the alteration of ruminal fermentation and passage rates, and the proportions of the microbial fractions flowing to the duodenum. However, little published research is available on this topic, whilst much of the research on the effect on ruminal fermentation has been inconsistent, and so requires further validation.

For this reason, the primary objective of this research trial was to examine the effects of different feed additives, namely; a buffer (Acid Buf 10), a direct-fed microbial (DFM) product (Achieve$^{FE}$), and an ionophore (Rumensin®), on the AA profile of both ruminal fluid-associated bacteria (FAB) and particle-associated bacteria (PAB), and microbial protein synthesis, when fed to lactating dairy cows. With the secondary objective of examining the effects of these additives on ruminal fermentation dynamics. The information gathered will have the potential to enhance our current understanding as to how feed additives influence ruminal fermentation and the microbial AA supply to the lactating dairy cow. With the ultimate goal being the incorporation of such information into new versions of feed formulation programmes, thus allowing for the more accurate and precise prediction of AA supply and requirements, potentially improving the duodenal ratios of EAA with less dietary CP, increased N efficiency and reduced environmental impact.

The hypotheses for this study were:

$H_0$: Calcareous marine algae, a direct-fed microbial and ionophores are not capable of altering the amino acid profile of the ruminal bacteria associated with both the fluid and particulate phases of the rumen.

$H_1$: Calcareous marine algae, a direct-fed microbial and ionophores are capable of altering the amino acid profile of the ruminal bacteria associated with both the fluid and particulate phases of the rumen.

$H_0$: Calcareous marine algae, a direct-fed microbial and ionophores are not capable of altering ruminal microbial protein synthesis.

$H_1$: Calcareous marine algae, a direct-fed microbial and ionophores are capable of altering ruminal microbial protein synthesis

$H_0$: Calcareous marine algae, a direct-fed microbial and ionophores are not capable of favourably altering ruminal fermentation dynamics.

$H_1$: Calcareous marine algae, a direct-fed microbial and ionophores are capable of favourably altering ruminal fermentation dynamics.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Introduction

The recent report of the FAO/SOFA (2016) stated that by 2050 global food demand will have increased by 60 %, particularly in regions with high rates of undernourishment and adverse climatic conditions. Dairy cattle play a significant role in human nutrition through the provision of protein and calcium rich milk and by-products, thus advancement in the nutrition of dairy cattle holds the key to enhancing the productive performance of these animals so as to meet global demand. Nutrition of ruminants is governed by microbial fermentation, with the symbiotic relationship between the ruminant and the inherent ruminal microbiome, allowing ruminants to utilise fibrous plant materials as their primary feed source (Hungate, 1966). Unfortunately, ruminal fermentation is inherently inefficient (Callaway *et al.*, 2003) but can potentially be improved in several ways. The primary means by which nutritionists have been able to maximise efficiency has been through the manipulation of ruminal fermentation via the modification of the ruminal microbial consortium. This has for the most part been accomplished with the use of feed additives, which are non-nutritive dietary compounds which bring about desirable production responses through altered ruminal or post-ruminal metabolism, improved health, and enhanced nutrient utilisation. For decades antibiotic ionophores, were widely used in the animal industry as the feed additive of choice, and gained popularity from being both cost-effective and improving the efficiency of beef and dairy production (McGuffey *et al.*, 2001). However, in the 1990s the appearance of residues and antibiotic resistant strains of bacteria (Gustafson & Bowen, 1997) which could potentially pose a threat to human health caused concern. Under the eye of scrutiny, with a general public which is becoming increasing aware and outspoken with regards to animal welfare, food safety, and public health, ionophores were banned as growth promoters in the EU as of January 2006 (EC, 2003) and although still legal in many countries worldwide there is a movement towards eradicating the use of ionophores. The consumers demand for safe, nutritious food, produced with the environment in mind, combined with the ruminant nutritionist's interest in improving the efficiency of energy and protein utilisation has sparked interest in exploiting natural products such as DFM's, exogenous enzymes, and various plant extracts as alternative feed additives.

## 2.2 The ruminant digestive system

### 2.2.1 Anatomy

The digestive anatomy and physiology of the ruminant is markedly different to that of the monogastric animal, owing to thousands of years of evolution which has enabled the ruminant to utilize forages and fibrous roughages as food sources (Van Soest, 1994). This specialised digestive system involves the microbial fermentation of the ingested feed prior to exposure to the animals' own digestive enzymes, this unique feature allows the ruminant to extract and absorb nutrients from fibrous plant sources which otherwise would not be available to mammalian enzymes.

The ruminants' digestive anatomy is characterised by the marked expansion of the oesophageal region into a multi-chambered forestomach, comprising of the rumen, reticulum and omasum. Ingested feed passes through this series of chambers, where it undergoes microbial digestion, before arriving at the abomasum, which is known as the true stomach and is the first glandular portion of the ruminant gastrointestinal tract (Frandson *et al.*, 2009). Hereafter digesta passes through to the small intestine and is subjected to chemical digestion and the subsequent absorption of nutrients. From this point and onward the gastrointestinal tract of the ruminant parallels that of the monogastric.

The rumen and reticulum are collectively referred to as the reticulorumen, owing to their functional and anatomical relatedness, and are partially separated by the reticuloruminal fold. The reticulorumen is a large,

hollow, muscular organ, occupying the left side of the abdominal cavity, which by adulthood comprises about 85 % of the total stomach capacity (McDonald *et al.*, 2011). The typical capacity of this organ in adult cattle ranges from 110 L to 235 L depending on the individuals' size (Frandson *et al.*, 2009). This organ, as well as the omasum, is lined with non-glandular, non-mucus producing, keratinised, stratified squamous epithelium (van Soest, 1994). The mucosal surface of the rumen is lined with finger-like projections known as papillae, these vary in shape and size (1 cm – 1.5 cm in length) and are found primarily in the ventral regions of the rumen (Van Soest, 1994). These papillae serve to increase the absorptive surface area of the rumen, allowing for the absorption of the end-products of fermentation. Papillae distribution, size, and number differ among animals of the same species and are closely related to the forage to concentrate ratio of the diet, feeding habits, forage availability, and digestibility (Ishler *et al.*, 1996).

Briefly, the omasum is a small, ovoid organ which lies to the right of the reticulorumen, it is characterised by the presence of many layers of muscular tissues that lie in sheets. Although its role has not been fully elucidated it is known to reduce particle size (Ishler *et al.*, 1996) and absorb excess water from digesta. In cattle the omasum probably serves as an absorptive organ, with 30 % to 60 % of the water, 40 % to 69% of the VFAs, and considerable amounts of ions, particularly sodium ($Na^+$) and potassium ($K^+$) being absorbed (Van Soest, 1994).

### 2.2.2 Ruminal environment

The ruminal ecosystem operates as a continuous culture system, and can be referred to as a biological fermentation unit. It is vital for the ecological conditions, which serve as regulators of the microbial population, within this "fermentation vat" to be maintained within narrow, well-defined limits, to ensure the maintenance of normal microbial growth and metabolism.

The reticulorumen provides a warm, moist, buffered, substrate- rich, anaerobic environment ideal for the growth of anaerobic bacteria, protozoa, and fungi. The reticulorumen operates as a continuous culture system as there is a more or less continual supply of substrate, removal of end products of fermentation (by either absorption, eructation, or passage), and the passage of undigested feed and waste products (McDonald *et al.*, 2011; Nagaraja, 2016).

The ruminal contents are heterogeneous, comprising of a complex mass of digesta of various densities. Typically, the ruminal contents are in the form of stratified layers displaying both ventral-to-dorsal, and anterior-to-posterior differences. Despite strong contractions of the rumen to turnover and mix rumen contents, mixing is incapable of completely randomising the distribution of the particulate matter. A typical rumen presents with a gas dome in the dorsal sac, below which a dense, fibrous, floating mat sits, buoyed by gas production above the lower liquid phase, in which fine feed particles and microbial cells are suspended. However, both the structure and composition of the ruminal contents are markedly influenced by the diet. As previously mentioned, the digesta is comprised of feed particles of various densities, particles with low densities (i.e. forages and hay) form part of the floating mat, whereas heavier particles (i.e. grains) sediment to the bottom of the rumen. Thus, when fed a high concentrate, or pelleted diet the rumen contents are more homogenous and viscous with the absence of a rumen mat (Van Soest, 1994).

The gas dome of the rumen is composed primarily of carbon dioxide ($CO_2$) (~ 65 %) and methane ($CH_4$) (~ 35 %), the proportions of which vary based on fermentation and the rumen ecology (Nagaraja, 2016). Small amounts of other gases namely, nitrogen ($N_2$), oxygen ($O_2$), and hydrogen ($H_2$) are also present. Gases are eliminated from the rumen by three main pathways; eructation, exhalation, and absorption across the rumen wall (Van Soest, 1994).

The general conditions of the rumen are detailed below, it is important to note that numerous homeostatic mechanisms are in place to ensure that these conditions are maintained.

Temperature within the reticulorumen is carefully regulated and remains close to that of the animals, at 36 ºC to 42 ºC (McDonald *et al.*, 2011; Nagaraja, 2016). A moisture – rich environment is necessary for microbial growth, with water that the animal drinks and saliva being the only source of moisture to the rumen,

baring that in the feed. Ruminal contents contain on average 850 g water/kg to 930 g water/kg (McDonald *et al.*, 2011).

The ruminal milieu is anaerobic and highly reduced due to the absence of oxygen, presence of the highly-reduced end-products of fermentation, and the propensity of the ruminal microbes to utilise electron carriers with a very low potential. The redox potential is typically within the range of -150 mV to -350 mV (Clarke, 1977). Previous research (Broberg, 1957ab, 1958) has shown that the redox potential of the rumen milieu is fairly constant across various diets and post-prandially. The research of Baldwin & Emery (1960) demonstrated that the microbial population of the rumen was capable of maintaining a constant redox potential, even in the presence of a strong, oxidising agent, thus supporting their hypothesis that the redox potential of the ruminal contents is constant and stabilised by factors other than the substances present. A possible explanation is that the native facultative rumen microbes utilise highly oxidative compounds which enter the rumen as hydrogen acceptors, thus maintaining the low redox potential.

Ruminal pH is a function of the production and absorption of volatile fatty acids (VFA's), the buffering capability of the saliva, feed acidity (fibre content), water flux across the rumen wall, and water efflux to the lower gastrointestinal tract (Erdman, 1988). Rumen pH is subject to substantial variation due to a multitude of factors and can range from 5.5 to 7.4 (Erdman, 1988), although typically the pH of the rumen falls within 5.5 to 6.5 (McDonald *et al.*, 2011). Rumen pH is lower, i.e. more acidic, on high concentrate diets and higher, i.e. more basic, on roughage-based diets. Rumen pH is subject to diurnal variation, with a post- prandial drop in pH typically being observed (Cruywagen *et al.*, 2015), with the pH of the rumen slowly recovering after the nadir has been reached. It is imperative that rumen pH be maintained within a narrower range to ensure maintenance of the preferred microbial community structure of the rumen, digestion and overall animal health.

The ruminal milieu is rich in ions, such as those of hydrogen ($H^+$), sodium ($Na^+$), potassium ($K^+$), chloride ($Cl^-$), phosphate ($PO^{3-}_4$), and bicarbonate ($HCO^{3-}$), which along with VFA's, glucose and lactate influence the osmolality of the rumen contents (Van Soest, 1994). Ruminal osmolality normally ranges from approximately 240 mOsm/kg to 265 mOsm/kg for cattle on roughage-based diets, and 280 mOsm/kg to 300 mOsm/kg for cattle fed concentrate diets (Garza *et al.*, 1989). A post-prandial rise in the osmolality of the ruminal fluid has been reported (Garza *et al.*, 1989), the rate and extent of this rise is dependent on the diet, quantity of feed consumed in a given time, water intake, and ruminal microbial activity, as a result of the dissolution of minerals from the ingested feed and water, and the production of VFA's from ruminal fermentation (Dijkstra *et al.*, 1993). Having said this the osmolality of the rumen rarely exceeds 400 mOsm/kg (Russell, 2002) and is regulated by copious amounts of isotonic saliva entering the rumen, rapid absorption of water from hypotonic solutions, and the iso-osmotic absorption of water along with VFA's, ions, and other solutes present in the ruminal contents (Van Soest, 1994). Generally, the osmotic pressure of the ruminal contents is in equilibrium with that of the plasma and interstitial fluids of the animal, owing to the efflux of ions between them.

### 2.2.3 Rumen function

The rumen serves primarily as a fermentation unit, allowing the ruminant to utilise the complex carbohydrates found in plant material by subjecting the ingested material to a host of microorganisms which produce the enzymes (i.e. cellulase) necessary to digest and extract nutrients from these feedstuffs. This microbial fermentation provides the animal with energy in the form of VFA's, and protein from the digestion of MCP in the lower gastrointestinal tract. Apart from operating as a fermentation unit the rumen performs other inter-related functions which contribute to the digestion of feed, absorption of nutrients, and the maintenance of homeostasis within the rumen.

The rumen plays an important role in the buffering of its contents, in order to maintain the pH of the ruminal milieu within a narrow- range. This is mediated by balancing the production and absorption of the end-products of microbial fermentation, namely the principal VFA's (acetate, propionate, butyrate), as well as other by-products of microbial metabolism (i.e. lactate). Lactate and VFA's are theoretically capable of reducing the rumen pH to 2.5 to 3.0 (McDonald *et al.*, 2011) owing to their low acid dissociation constant ($pK_a$) values of 3.9 and 4.8 to 4.9, respectively (Krause & Oetzel, 2006). As mentioned previously the rumen is lined with small projections, known as papillae, which serve to absorb VFA's, when the VFA concentration

in the rumen is increased, an increased absorption rate is required in order to maintain optimal rumen pH and osmotic pressure. This is realised by an increase in the absorptive surface area of the rumen via increased papillae size and numbers (Ishler *et al.*, 1996). Rumen buffering is also achieved by the copious amounts of saliva which flow into the rumen upon mastication and swallowing of the feed, adult cattle can secrete up to 180 L to 200 L (Van Soest, 1994; Frandson *et al.*, 2009) of saliva daily. This saliva is rich in mineral ions, $Na^+$, $K^+$, $PO^{3-}_4$, and $HCO^{3-}$ all of which provide buffering capacity to neutralise the acids produced (Ishler *et al.*, 1996).

The rumen is a strong, muscular organ, which undergoes a series of complex muscular contractions (Frandson *et al.*, 2009). This rumen motility serves to mix the rumen digesta, promote rumen turnover by the provision of sufficient force to move liquid digesta and fine particles through to the omasum, reduce particle size, expel gases from the rumen, and cause regurgitation of digesta for rumination. These contractions which affect the either the entire reticulorumen or only part of the organ, primarily serve to mix the ruminal contents, this increases the contact of digesta with saliva to ensure sufficient buffering, it also promotes microbial colonisation, and thus fermentation, of the feed particles by ensuring that the digesta and microbes are in close contact, lastly it also increases contact between the rumen epithelia and digesta for the absorption of nutrients across the rumen wall. Turnover of rumen contents is promoted thus allowing for the undigested feed particles, liquid digesta, and microbial protein to flow through the reticulo-omasal orifice into the omasum for enzymatic digestion in the lower gastrointestinal tract. Rumen motility promotes rumination, this re-chewing of the coarser, fibrous material, allows for a reduction in particle size, extraction of soluble contents, enrichment of the fibre content of the bolus, and stimulates saliva production (Van Soest, 1994). This reduction in particle size further enhances microbial accessibility to potentially digestible nutrients, thus promoting the extraction of nutrients. Gases, namely $CO_2$ and $CH_4$, are a by-product of microbial fermentation and build up in the rumen. These gases must be expelled from the rumen by eructation in order to maintain homeostasis and prevent bloat (Ishler *et al.*, 1996), this is by way of the rumen contractions which move these gases into the cranial part of the rumen from where they are forced into the oesophagus for eructation.

## 2.3 The rumen microbiome

### 2.3.1 Overview

The rumen houses a consortium of microorganisms which interact to form a complex microbiota which is responsible for the ruminant's ability to convert typically indigestible fibrous plant material into digestible compounds, such as soluble sugars, VFA's, and microbial proteins which can be utilised by the microbes and the host, to give rise to the final milk or meat product. Although animal variation in microbial populations is seen, studies suggest a core microbiome in the bovine rumen (Jami & Mizrahi, 2012). The microbial populations comprising this microbiome can be described as occurring in four different compartments; Compartment 1: The liquid phase, which is greatest in volume and contains the lowest concentration of microbes, which are the fluid-associated microbes, this compartment is markedly influenced by dilution rate and the supply of soluble substrates; Compartment 2: Is intermediate of compartments 1 and 3, it contains a mixture of fluid-associated microbes which have associated with the solid phase, and loosely adherent particle-associated microbes, here microbial matter, nutrients and fermentation end-products are shuttled between compartments 1 and 3; Compartment 3: Contains firmly adherent particle-associated microbes, breakdown of fibrous feed occurs here; and Compartment 4: Contains the microbes which adhere to the rumen wall (Czerkawski, 1986; Dehority, 2003). The rumen microbiome clearly plays an essential role in ruminant nutrition, thus the alteration of specific classes or species of microbes, in terms of number or activities, could potentially lead to improvements in animal production, production yields and animal wellbeing. Knowledge of the intricacies of the microbial population inhabiting the rumen is imperative if one wishes to succeed in altering this microbiome to the benefit of producers (Weimer, 1998).

### 2.3.2 Factors influencing the microbiome

Owing to the dependence of the ruminant on the microbial ecosystem for its survival, it is essential to maintain an optimal ruminal ecosystem balanced in acidity, nutrient availability and fermentation products, to ensure microbial functionality and the growth of desirable microbial species, ultimately optimising digestion.

Many factors such as the diet composition, level of feed intake, rate of passage, rumen pH and osmolality can influence the ruminal environment and hence the microbial ecosystem.

Properties of the ration, i.e. chemical, physical, and structural, are important determinants of the extent of fermentation and microbial populations present, as these properties influence substrate availability, bacterial colonisation of the feed particles, and rate of passage. The primary determinant of the microbial population is substrate (Van Soest, 1994), as various microbes have preferences for specific substrates, diet composition is a key factor influencing the microbial population. Bacterial flora have been shown to be highly diversified and complex on diets high in nutrient rich, forages (Bryant & Burkey, 1953). Cellulolytic bacteria and fungi predominate on rations high in roughages, whereas amylolytic bacteria predominant on rations rich in concentrates (i.e. grains) (Bryant & Burkey, 1953). Regarding the protozoa, populations are most diverse when diets of higher digestibility, 40 % to 60 % concentrate based, are fed (Bryant & Small, 1960). Particle size of the ration is another major factor influencing the ruminal ecosystem, as it specifically effects bacterial colonisation and rate of passage. Because enzymes act on the surface of feed particles particle size is an important factor affecting fermentation rate (Hungate, 1966). Research has demonstrated that on pelleted diets, especially with high feed intakes, protozoal numbers are reduced (Christiansen e*t al.*, 1964) owing to the increased rate of passage which exerts a "sweeping out" action of the protozoa (Hungate, 1942). The grinding of fibrous feeds, destroys the 3-dimensional structure necessary for bacterial colonisation, it also increases the rate of passage of particles out of the rumen, whilst decreasing the pH due to being highly fermentable, thus reducing the cellulolytic bacterial populations (Yang *et al.*, 2001). Bacterial colonisation of feed particles is a prerequisite for fibre degradation and it has been shown that bacterial attachment to feed particles is mediated by the production of bacterial slime, with slime production being stimulated by increased availability of soluble carbohydrates which are primarily provided by fine concentrate particles (Cheng e*t al.*, 1977).

Ruminal pH is one of the most variable factors influencing both microbial populations and VFA production, thus maintenance of the ruminal pH within narrow limits is essential for the maintenance of a desirable rumen environment and desirable bacterial species. Microbes differ in the pH range at which they function optimally, fibrolytic bacteria are most active in a pH range of 6.2 to 6.8, with their numbers and activities, as well as those of methanogenic bacterial species, declining when pH drops below 6.0. Whereas amylolytic bacteria are most active in the pH range 5.2 to 6.0 (Ishler *et al.*, 1996). Ruminal fibre digestion has been shown to be inhibited by low ruminal pH (pH < 6.0) due to the retardation of the growth of fibrolytic bacteria (Calsamiglia *et al.*, 2008). Protozoal populations are also sensitive to acidity, these microbes function optimally at pH 6.5, and whilst they can withstand lower pH values for short time periods, if pH is out of range for an extended period of time populations will decline (Hungate, 1966). Low ruminal pH is associated with the accumulation of lactic acid, owing to the increase in numbers of the acid tolerant lactate-producing bacteria, *Streptococcus bovis* and lactobacilli, and a decrease in lactate-utilising bacteria, *Megasphaera elsdenii* (Russell & Hespell, 1981). The efficiency of microbial protein synthesis (MPS) has also been shown to be influenced by rumen pH, with reduced efficiency reported when ruminal pH falls below 5.5 (Calsamiglia *et al.*, 2008).

Maintenance of ruminal osmolality within a predefined range is important for microbial survival, as there is a limit to which certain microbial species can withstand increased turgor pressure. Research has indicated that gram-negative bacterial species are more sensitive to shifts towards higher ruminal osmolality than are gram-positive bacteria (Mackie & Theron, 1984). This is likely due to differences in the cross-linkage of bacterial cell wall peptidoglycan, with thicker cell walls and more cross-linkage allowing the cell to counteract increases in turgor pressure thus preventing osmotic lysis (Hsu & Fahey, 1990).

### 2.3.3 The rumen bacteria

The majority of the ruminal microbes belong to the Eubacteria, the true bacteria, these microorganisms densely populate the rumen with bacterial numbers ranging from $10^8$ to $10^{11}$ cells per ml or gram of rumen contents (Ishler e*t al.*, 1996; McDonald e*t al.*, 2011; Nagaraja, 2016). Overall the bacteria contribute 40 % to 90 % of the total microbial mass, with this value being reflective of the digestibility of the diet (Nagaraja, 2016). The rumen bacteria are typically thought of in terms of 22 predominant species, however with the advent of cultivation-independent techniques, namely 16S ribosomal ribonucleic acid (rRNA) gene sequencing and deoxyribonucleic acid (DNA): DNA hybridisation, it has been shown that the number of bacterial species

found in the rumen has been greatly underestimated to date (Krause & Russell, 1996a). The ruminal bacterial population can be grouped based on morphology, distribution within the rumen, or substrate affinity. Morphologically the ruminal bacteria are grouped into three main shapes, being rod, spherical or spiral shaped, with the majority of the ruminal bacteria being classified as rod- shaped organisms (Ishler e*t al.*, 1996; Nagaraja, 2016). Ruminal bacteria are often referred to in terms of being gram-negative or gram-positive, based on differences in the peptidoglycan layer surrounding the cell, the majority of ruminal bacteria, 80 % to 90 % of the population, are classified as gram-negative, with the remaining 20 % to 30 % of the population classified as being gram-positive (Nagaraja, 2016). From a ruminant nutritionist's viewpoint is it more beneficial to group the ruminal bacteria based on distribution within the rumen, i.e. to classify the ruminal microbes according to their association to each of the four aforementioned ruminal "compartments". Thus, the ruminal bacteria can be classified as those species which float freely in the liquid phase, i.e. Fluid-associated bacteria (FAB); those which are either loosely or firmly adherent to the particulate matter, i.e. Particle-associated bacteria (PAB); and of lesser importance are those which associate with the epithelial cells of the rumen wall. The FAB constitute a minor fraction of the total bacterial population, with studies showing that the FAB do not exceed more than 20 % of the total bacterial mass in the rumen (Legay- Carmier & Bauchart, 1989; Yang *et al.*, 2001). Numerous marker – based studies have demonstrated that the majority of the ruminal bacteria are associated with the particulate matter, values obtained have been in the range of either 50 % to 55 % (Merry & McAllan, 1983; Olubobokun & Craig, 1990) or 70 % to 80 % (Forsberg & Lam, 1977; Craig *et al.*, 1987a; Yang e*t al.*, 2001). Due to the predominance of PAB in the ruminal contents it has been postulated that the PAB are the major microbial fraction present in the duodenal contents (Faichney, 1980). Lastly ruminal bacteria can be grouped based on substrate affinity (See Table 2.1) however, much overlap of bacterial species is seen as most ruminal species have the ability to utilise more than one substrate (Ishler *et al.*, 1996).

**Table 2.1** Grouping of the predominant rumen bacterial species according to type of substrate fermented (Sources: Ishler *et al.*, 1996; McDonald *et al.*, 2011; Dehority, 2003)

| **Group** | **Bacterial species** |
|---|---|
| Major Cellulolytic Species | *Fibrobacter succinogenes*<br>*Ruminococcus flavefaciens*<br>*Ruminococcus albus*<br>*Butyrivibrio fibrisolvens* |
| Major Hemicellulolytic Species | *Prevotella ruminicola*<br>*Ruminococcus flavefaciens*<br>*Ruminococcus albus*<br>*Butyrivibrio fibrisolvens* |
| Major Pectinolytic Species | *Butyrivibrio fibrisolvens*<br>*Prevotella ruminicola*<br>*Lachnospira multiparus*<br>*Succinivibrio dextrinosolvens* |
| Major Amylolytic Species | *Streptococcus bovis*<br>*Ruminobacter amylophilus*<br>*Succinimonas amylolytica*<br>*Selenomonas ruminantium*<br>*Prevotella ruminicola*<br>*Butyrivibrio fibrisolvens* |
| Major Lactate-utilising Species | *Megasphaera elsdenii* |
| Major Proteolytic Species | *Butyrivibrio fibrisolvens*<br>*Streptococcus bovis*<br>*Prevotella ruminicola*<br>*Ruminobacter amylophilus* |

The cellulolytic bacteria account for a considerable proportion of the ruminal bacteria, with cellulose digestion proceeding primarily via relatively few bacterial species, which have been shown to adhere directly to the surface of the particulate matter (Weimer, 1998; Michalet-Doreau *et al.*, 2001). The rumen houses three main cellulolytic species, *Fibrobacter succinogenes*, *Ruminococcus albus*, and *Ruminococcus flavefaciens*,

which are able to digest cellulose at rate constants of 0.05 $h^{-1}$ to 0.10 $h^{-1}$, faster than almost all other cellulolytic species (Weimer, 1996). Each of these major cellulolytic species produces a mixture of fermentation end-products that is fairly characteristic of the species (Weimer, 1998). Bryant & Burkey (1953) demonstrated that the percentage of cellulolytic bacteria in the diet varied based on the crude fibre content of the ration. Of the amylolytic species, *S. bovis* and *Selenomonas ruminantium*, are of importance as these species are typically implicated in the production of large amounts of lactic acid (Dehority, 2003), particularly on rations rich in concentrates which lead to substantial increases in the number of these species (Bryant & Burkey, 1953). Proteolysis has been exhibited in a broad taxonomic distribution amongst the more versatile rumen bacteria (Weimer, 1998), with many of the carbohydrate fermenting bacteria being implicated owing to their ability to produce small amounts of proteinase enzymes (Dehority, 2003). The most active species being *Butyrivibrio fibrisolvens*, *S. bovis* and *Prevotella ruminicola*, with the latter being historically thought to contribute significantly to amino acid degradation (Weimer, 1998). However, it has since been discovered that several species of specialist microbes known as "Hyper-ammonia producers" (HAP) are present in the rumen and are able to hydrolyse small peptides and deaminate AA's at more rapid rates than the proteolytic species (Russell *et al.*, 1988), currently four species have been identified *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, *Clostridium aminophilum*, and *Fusobacterium necrophorum* (Russell *et al.*, 1988; Nagaraja, 2016).

As mentioned earlier from the ruminant nutritionist's viewpoint it is more beneficial to group the ruminal bacteria into two main groups, those associated with the liquid phase (i.e. FAB) and those associated with the particulate phase (i.e. PAB). It has been demonstrated that the ruminal bacteria are not equally distributed throughout the rumen, with the distribution of bacterial species differing within the FAB and PAB (Minato *et al.*, 1966). Evidence of this has been shown by the differences seen in the chemical composition of the FAB and PAB across studies. Typically, PAB are higher in organic matter (OM), polysaccharide content, and lipids, whereas FAB are higher in ash content, total N, and RNA (Merry & McAllan, 1983; Martín-Orúe *et al.*, 1998; Rodríguez *et al.*, 2000). These marked differences in chemical composition are reflective of differences in the nutrition, stage of growth (Czerkawski, 1976; Smith & McAllan, 1974; Cecava *et al.*, 1990), growth rates (Rodríguez *et al.*, 2000), enzymatic (Michalet-Doreau *et al.*, 2001) and metabolic activities (Williams & Strachan, 1984), and variation in the bacterial composition of the different populations found within each fraction (Czerkawski, 1976). Differences in the function of the two populations are reflected by the higher fibrolytic enzymatic activity observed in the PAB, compared to the higher enzymatic activity of those enzymes involved in the degradation of soluble non-structural carbohydrates (NSC) seen in the FAB populations (Legay-Carmier & Bauchart, 1989; Michalet-Doreau e*t al.*, 2001). Owing to their large mass within the rumen (Craig *et al.*, 1987b) and fibrolytic activities (Michalet-Doreau e*t al.*, 2001), the PAB population appear to play a significant role in the digestion of forages. Research has shown that the chemical composition of these microbial fractions can be altered by diet composition (Martín-Orúe *et al.*, 1998; Yang *et al.*, 2001), and changes in the ruminal environment, with the PAB being most affected by dietary composition (Legay-Carmier & Bauchart, 1989).

### 2.3.4 The rumen protozoa

The protozoa were the first rumen organisms to be discovered owing to their conspicuous size and active motility (Nagaraja, 2016). The rumen protozoa are Eukaryotic and number about $10^5$ to $10^6$ cells per gram of ruminal contents across diets (Ishler *et al.*, 1996; McDonald *et al.*, 2011), much less than bacteria, however, owing to their large size their mass may be $10^3$ times that of bacteria, thus total protozoal mass in the rumen may equate that of the bacteria (NRC, 1985). Morphologically the rumen protozoa are divided into flagellates and ciliates (Hungate, 1966), with the ciliates being the predominant protozoal group within the rumen. Ciliated protozoa are highly specialised single cells which are divided into two board groups, holotrichs and oligotrichs, based on differences in morphological structure and functionality (Nagaraja, 2016). The holotrichs are relatively simple (Hungate, 1966) and are members of the genera *Isotricha* and *Dasytricha* (Russell & Hespell, 1981). While oligotrichs are morphologically more complex organisms, this group contains several genera, of which *Entodinium* is the principal genus (Russell & Hespell, 1981; Nagaraja, 2016). Entodiniomorphs are more numerous in the rumen than the holotrichs, with each contributing 75 % to 90 % and 10 % to 25 % of the total protozoal population, respectively (Nagaraja *et al.*, 2016). Both the holotrichs and entodiniomorphs display diurnal variation in population size, typically the holotrichid population displays

a two- to four-fold increase in numbers within one to two hours of the commencement of feeding (Purser, 1961; Abe *et al.*, 1981) where after the population gradually declines to pre-feeding numbers. In contrast, the entodiniomorph population displays a post-prandial decrease in numbers until 6 to 8 hours post-feed, due to dilution of the ruminal contents, thereafter numbers gradually increase to pre-feed levels (Purser & Moir, 1959). The ciliated protozoa actively participate in ruminal digestion, as the protozoa possess a full complement of enzymes necessary to enable them to ferment the major components of feedstuffs (Nagaraja, 2016). Most entodiniomorph species are able to ingest small plant particles and digest some if not all of the major polysaccharide components (i.e. cellulose, hemicellulose, starch and pectin) of these plant materials (Dehority, 2003), entodiniomorphs are also capable of using soluble sugars and proteins as substrates (Nagaraja, 2016). In contrast, holotrichs utilise soluble sugars as a primary energy source (Dehority, 2003) but are also able to utilise starch, pectin and proteins (Nagaraja, 2016) with the end products of fermentation being acetate, butyrate lactate, hydrogen and $CO_2$ (Howard,1959). Various studies have also shown that the ciliate protozoa actively ingest bacteria as a source of protein and are quite specific as to which species they can ingest (Dehority, 2003). Due to the fastidious growth requirements of protozoa they have proved difficult to culture *in vitro* thus knowledge of their biochemical activities are limited. Overall protozoa have been implicated in the digestion of fibre, oxygen scavenging to maintain anaerobiosis, and the stabilization of ruminal fermentation (Ishler *et al.*, 1996; Nagaraja, 2016).

The chemical composition of the protozoa differs significantly from that of bacteria, this reflects not only the differences in growth rates and specific nutritional characteristics (Bates *et al.*, 1985) but may also be indicative of distinct metabolic functions as shown by Martin *et al.* (1993). Typically, protozoa have been shown to contain more OM (Storm & Ørskov, 1983; Martin e*t al.*, 1994), as much as 2.4 to 12 times greater than that of bacteria (Olubobokun *et al.*, 1988), greater proportions of unsaturated fatty acids (Or-Rashid *et al.*, 2007) and less N and CP than bacteria (Olubobokun *et al.*, 1988; Martin e*t al.*, 1994). Protozoal protein is similar in biological value (BV) to that of bacterial protein, but is of increased digestibility (McNaught *et al.*, 1954), however, the benefit of this may be limited as ciliated protozoa are preferentially retained in the rumen, thus contributing much less than bacteria to the post-ruminal microbial protein supply. Research has estimated the protozoa to represent between 21 % to 25 % (Yáñez – Ruiz *et al.*, 2006) of the total microbial N flowing to the duodenum.

Overall whilst the protozoa are clearly an integral part of the microbial population found in the rumen, and contribute to ruminal fermentation, their benefit to the ruminant is controversial, as positive, negative and nil responses have been reported upon defaunation of the rumen (Williams & Coleman, 1988). The effects of the absence or presence of the rumen protozoa on the ruminant may be dependent on the diet, population size and species of protozoa present (Nagaraja, 2016).

### 2.3.5 The rumen fungi

Discovered only in 1975 (Nagaraja, 2016) as members of the ruminal microbial population are the anaerobic fungi. These small flagellated organisms are present as a minor group in the rumen, with marker studies estimating fungi to contribute 8 % to 10 % of the total microbial mass (Ishler *et al.*, 1996; Nagaraja, 2016). The prevalence of fungi is diet-dependent with the fungal concentration being positively correlated to the concentration of fibre in the ration (Bauchop, 1981). The functionally important fungi belong to the phylum *Chytridomycota* and are referred to as Chytridomycete, currently six genera have been isolated all of which belong to the family *Neocallimastigaceae* (Firkins & Yu, 2015). The relative contribution of fungi to ruminal digestion is not known, and it remains unclear as to whether the fungi are functionally important. Research has provided evidence of the role of fungi in fibre digestion (Bauchop, 1981) and the overall fermentation of carbohydrates in the rumen. Fungi have been shown to produce enzymes such as, cellulases, hemicellulases, pectin lyases and amylases, all of which are necessary to break down the principal polysaccharides found in forages, as well as phenolic esterases which assist in the breakdown of the cross-linkages between hemicellulose and lignin, thus increasing fungal accessibility to hemicellulose. Structurally fungi possess thalli which are able to penetrate deep into feed particles, breaking them apart and increasing the surface area

available for degradation (Russell, 2002). Ruminal fungi have been shown to be able to utilise a wide-range of mono- and di-saccharides, with acetate, lactate, hydrogen, $CO_2$ and trace amounts of ethanol and formate as end products of metabolism (Russel, 2002; Nagaraja, 2016).

### 2.3.6 The rumen methanogens

The rumen methanogenic archaea are a special class of microorganisms which differ phylogenetically from eubacteria, protozoa and fungi. Methanogens constitute approximately 2 % to 4 % of the bacterial population of the rumen (Nagaraja, 2016), with numbers ranging from $10^5$ to $10^8$, per millilitre or gram of rumen contents (Dehority, 2003; Nagaraja, 2016). The methanogens serve to regulate the overall fermentation within the rumen by utilising the end-products of fermentation, namely $H_2$ and $CO_2$, or alternatively formate, as energy sources. The methanogens utilise the electrons derived from the $H_2$ and formate to reduce $CO_2$ to $CH_4$, which is expelled from the animal through eructation. Research has shown that the methanogens consist of seven species belonging to five genera (Nagaraja, 2016). With the most important methanogens being *Methanobrevibacter ruminantium*, *Methanomicrobium mobile* and to a lesser extent *Methanosarcina barkeri* (Dehority, 2003). Physically the methanogens lack peptidoglycan, have unusual lipid structures, are non- or weakly motile, and non-spore forming (Dehority, 2003; Nagaraja, 2016). Methane production, in cattle, has been shown to result in a 2 % to 12 % loss of feed energy, particularly on diets rich in forages (Johnson & Johnson, 1995), for this reason and the fact that methane is considered a greenhouse gas, much of the current research in animal science is focussed on the mitigation of enteric methane production, either through manipulation of the ruminal populations or the use of chemical inhibitors of the methanogens (Weimer, 1998).

### 2.3.7 Amino acid composition of microbes

The ruminant relies not only on rumen undegradable protein (RUP) but also the microbes flowing through to the duodenum as a protein source. Microbial CP has been shown to contribute more than 50 % to the duodenal CP flow (Clark *et al.*, 1992), thus knowledge of the AA composition and distribution of the ruminal bacteria and protozoa are important in terms of meeting the protein, specifically the AA, requirements of the ruminant. The extensive review of Clark *et al.* (1992) indicated substantial differences in the AA composition of the rumen bacteria, but many have attributed these differences to variations in the techniques used to isolate and analyse the microbes for AA's. However, upon refinement of the dataset to include only data from trials on cattle fed various diets but utilising the same isolation and assay techniques the same conclusion was reached.

**Table 2.2** Amino acid composition of mixed ruminal bacteria (g/100 g of AA) (Source: Clark *et al.*, 1992[1])

| Amino Acid | Mean | Minimum | Maximum |
|---|---|---|---|
| Arginine | 5.1 | 3.8 | 6.8 |
| Histidine | 2.0 | 1.2 | 3.6 |
| Isoleucine | 5.7 | 4.6 | 6.7 |
| Leucine | 8.1 | 5.3 | 9.7 |
| Lysine | 7.9 | 4.9 | 9.5 |
| Methionine | 2.6 | 1.1 | 4.9 |
| Phenylalanine | 5.1 | 4.4 | 6.3 |
| Threonine | 5.8 | 5.0 | 7.8 |
| Valine | 6.2 | 4.7 | 7.6 |
| Alanine | 7.5 | 5.0 | 8.6 |
| Aspartic acid | 12.2 | 10.9 | 13.5 |
| Glutamic acid | 13.1 | 11.6 | 14.4 |
| Glycine | 5.8 | 5.0 | 7.6 |
| Proline | 3.7 | 2.4 | 5.3 |
| Serine | 4.6 | 3.4 | 5.4 |
| Tyrosine | 4.9 | 3.9 | 7.7 |

[1] Average composition of 441 bacterial samples from animals fed 61 dietary treatments in 35 treatments

Prior to and since the publication of Clark *et al.* (1992), the AA composition of both pure cultures of ruminal bacteria and the microbial fractions (i.e. FAB, PAB and protozoa) have been shown to be fairly constant (Purser & Buechler, 1966; Storm & Ørskov, 1983; Martin e*t al.*, 1996; Fessenden *et al.*, 2017) even

under diverse dietary conditions. Differences in the AA composition of the FAB and PAB (Yang *et al.*, 2001; Boguhn *et al.*, 2006; Sok e*t al.*, 2017) have been observed, although they are less pronounced than the differences seen between bacteria and protozoa (Sok *et al.*, 2017). Typically, the ruminal protozoa contain greater proportions of the EAA's, lysine (Lys), leucine (Leu), phenylalanine (Phe), isoleucine (Ile), and tyrosine (Tyr) (Purser & Buechler, 1966; Shabi *et al.*, 2000; *Sok et al.*, 2017). Whereas the ruminal bacteria typically contain greater proportions of alanine (Ala), glycine (Gly), proline (Pro), serine (Ser), threonine (Thr) and valine (Val) (Shabi *et al.*, 2000; Sok *et al.*, 2017). These significant differences in AA composition of the bacteria and protozoa, especially with regards to the EAA's, point out the importance of acknowledging the presence of protozoa in duodenal MCP flow if one is to correctly predict the AA duodenal flow. The FAB and PAB show slight differences in AA composition, with the proportion of AA's being lesser in FAB as compared to PAB (Rodríguez *et al.*, 2000). Fluid-associated bacteria have generally been shown to contain higher proportions of Ala, Thr, Lys, Met, Val, and aspartic acid (Asp), whereas PAB have been shown to contain higher proportions of arginine (Arg), Leu, Phe, Pro, cysteine (Cys), and glutamic acid (Glu) (Rodríguez *et al.*, 2000; Yang *et al.*, 2001; Boguhn e*t al.*, 2006; Sok *et al.*, 2017).

The differences seen in the AA composition of FAB and PAB are not always consistent (Martin *et al.*, 1996) and may reflect differences in stage of growth and the varying bacterial species composition present in different populations (Czerkawski, 1976; Rodríguez *et al.*, 2000). Factors such as level of feed intake, diet composition, and dietary factors (i.e. grain processing, forage-to-concentrate ratio, and particle length) have been researched in terms of effect upon the AA composition of the microbial fractions, however, results have been conflicting. As previously mentioned, much of the research has shown the AA composition to be remarkably constant even under diverse dietary conditions, however, some researchers have observed changes in the AA composition based on diet composition (Shabi *et al.*, 2000; Boguhn e*t al.*, 2006), dietary factors, specifically forage-to-concentrate ratio (Yang *et al.*, 2001), and level of feed intake (Rodríguez e*t al.*, 2000). The *in vitro* work of Boguhn *et al.* (2006) reported that the AA profile within each microbial fraction could be altered by diet composition, as when total mixed ration's (TMR's) were fed with similar nutrient and energy contents but based on differing dietary ingredients significant changes in AA composition were noted, as opposed to when TMR's based on the same feed ingredients were fed. The work of Yang *et al.* (2001) showed that an increased forage-to-concentrate ratio (55:45 *versus* 35:65) lead to increases in the proportions of Cys and Tyr, and decreases of Met and Val in bacteria. Whilst forage particles shorter in length (6.08 mm *versus* 7.59 mm) increased the content of Arg and decreased that of Met. Rodríguez *et al.* (2000) noted that with increased levels of feed intake the concentration of AA's present in bacteria decreased, with the concentration of Arg and Met being significantly increased and those of Ala and Gly significantly decreased. They explained the variation as being due to altered species composition, and variation in the physiological state of the cells associated with changes in growth and turnover rates, which can alter cell wall to protoplasm ratios and thus the proportions of some AA's.

Consideration must also be given to the fact that not all microbial protein is of the same protein quality and digestibility. The original work of McNaught *et al.* (1954) determined that although the BV of the bacteria and protozoa was similar, 81 and 80, respectively, that true digestibility (74 and 90, respectively) and net protein utilisation (60 and 73, respectively) differed between the two microbial fractions, with protozoa surpassing bacteria, this work was later confirmed (Bergen *et al.*, 1968). As presented the AA composition of each of the microbial fractions differs, therefore, knowledge of factors which induce variation in the relative contribution of each fraction to the total microbial outflow is imperative as these changes will give rise to differences in the AA profile of microbial protein flowing to the duodenum (Dijkstra *et al.*, 1997).

### 2.3.8 Microbial interactions

The ruminal microbial population is rich in diversity and complexities, which exist due to numerous microbial interactions. An understanding of the intricate microbial interactions between and within microbial species as well as those with the host are paramount as these interactions significantly influence the productive performance of ruminant animals (Firkins & Yu, 2015).

### 2.3.8.1 Ruminant-microbe interactions

Under normal conditions the interaction between the ruminal microbial consortium and the ruminant can be described as being symbiotic (Nagaraja, 2016). Not only does the ruminant provide the microbes with a continuous supply of substrates, but it maintains the ruminal environment within the narrow limits necessary for the maintenance of optimal microbial growth and metabolism. The host maintains the anaerobic state, pH, temperature and osmolality of the ruminal environment via the series of mechanisms previously described. Mastication, rumination and ruminal contractions enlarge the surface area of feed particles and increase the contact of microbes with fresh substrate, thus enhancing the accessibility of the microbes to substrate. In return, the microbes provide energy, in the form of VFA's, protein and vitamins to the host. The microbes, owing to their fibrolytic capabilities, allow the ruminant to utilise fibrous feedstuffs as nutrient sources, and also allow for the ruminant to synthesise AA's and protein from sources of non-protein nitrogen (NPN).

### 2.3.8.2 Microbe-microbe interactions

The fermentation of feedstuffs within the rumen is the result of the coordinated activities of an assortment of microorganisms, all of which are competing for substrates, thus a particular species of microbe can be involved in several types of interactions at any given time (Russell & Hespell, 1981). Numerous microbial interactions have been observed within the rumen, however, for the purpose of this review only the most important interactions will be discussed, namely commensalism, amensalism and predation.

Commensalism is an association which is beneficial to one of the microorganisms without affecting the other (Russell & Hespell, 1981). An important example being that of cross-feeding, in which the hydrolysis products of one microbe are utilised by another, this ultimately results in a relatively more complete utilisation of feed into final fermentation products (Nagaraja, 2016). Considerable, cross- feeding of cellulose hydrolysis products has been observed in the rumen, with the cellulolytic species *F. succinogenes* providing soluble sugars which *S. ruminantium* can utilise (Russell & Hespell, 1981). The utilisation of end-products of rumen fermentation can also be classified as commensalism, an example being the interspecies hydrogen transfer between the rumen methanogens and the carbohydrate fermenting species, in which the methanogens metabolise the hydrogen and $CO_2$, produced from carbohydrate fermentation, to form $CH_4$ (Russell & Wallace, 1988).

Amensalism occurs when inhibitory or toxic substances are produced by certain microbes to the detriment of the other (Russel & Hespell, 1981), thus allowing the toxin-producing microbe to outcompete the other for substrate. This relationship is typically seen within species which have the same substrate affinities. Research has shown inhibition between strains of *F. succinogenes* and *R. flavefaceins*, and between *R. albus* and *R. flavefaceins*, with the inhibition likely being brought about by the production of a bacterocin-like compound (Odenyo *et al.*, 1994).

The predatory role of ciliated protozoa on bacteria is well documented, with most of the research done by Coleman *et al.* (1972) as well as Coleman *et al.* (1975; 1979). The Entodiniomorphs are highly active and have been shown to not only engulf bacteria but to cannibalise other protozoa (Russell, 2002). Generally, predation is thought to be non-specific, however, certain Entodiniomorphs have been shown to be selective in their engulfment of bacteria, engulfing cellulolytic bacteria more rapidly than others (Russell & Hespell, 1981). These engulfed bacteria serve as a source of N for the protozoa (Russell & Hespell, 1981) and promote microbial protein turnover (Russell, 2002).

## 2.4 Nutrient metabolism in dairy cows

### 2.4.1 Energy metabolism

Carbohydrates are the primary energy source in dairy cow diets and typically comprise 60 % to 80 % of the total dietary dry matter (DM) (NRC, 2001; Hutjens, 2008). Carbohydrates can be differentiated as either structural (e.g. lignin, cellulose, hemi-cellulose, and pectin) or non-structural (e.g. starch, sugars, and organic acids) and serve primarily as an energy source to the dairy cow and the ruminal microbial population, but are also important in maintaining the health of the gastrointestinal tract. Non-structural carbohydrates, in particular, are highly digestible and serve as a major source of energy for high-yielding dairy cows (NRC,

2001). Carbohydrates undergo microbial fermentation which hydrolyses complex polymers to small saccharides which are then fermented into numerous intermediates, with the final fermentation products of importance being the VFA's. The main VFA's, in descending order of abundance, are acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate (Van Soest, 1994). Although the proportions of ruminal VFA's are relatively stable across diets (Ishler *et al.*, 1996) the proportions of VFA's are influenced by diet composition (Van Soest, 1994) such as the forage-to-concentrate ratio and forage particle size (Sutton, 1985), frequency of feeding (Russell & Hespell, 1981), pH (Ishler e*t al.*, 1996) and the status of the methanogen population (Van Soest, 1994).

These VFA's serve as the principal source of ME, providing 60 % to 80 % of the energy requirement of the cow (Hutjens, 2008). Acetate is the primary VFA, predominating on high forage diets, and can represent 55 % to 70 % of the total production of VFA (Hutjens, 2008). Acetate travels via systemic blood to various organs and tissues where it serves as a source of energy and fatty acids (McDonald *et al.*, 2011). Propionate is produced primarily from the fermentation of NSC and can represent from 15 % to 30 % of the total VFA production (Hutjens, 2008), through the conversion of this VFA to glucose in the liver energy is provided to various body tissues, as is reduced coenzymes for fatty acid synthesis, and glycogen synthesis (McDonald *et al.*, 2011). Butyrate is a ketogenic VFA, comprising 5 % to 15 % of the total VFA's produced, it provides energy to the rumen wall and it largely converted to the ketone, β-hydroxybutyrate (BHBA) during its absorption through the ruminal epithelium. This VFA serves as an energy source particularly to skeletal and heart muscle (McDonald e*t al.*, 2011) and is involved in fatty acid synthesis in adipose and mammary gland tissue (Ishler *et al.*, 1996).

Other sources of energy for ruminants include dietary fats, fatty acids mobilised from the breakdown of adipose tissue, and AA's. Although the latter would typically only serve as energy source when the animal is required to break down body tissue to maintain essential bodily functions (McDonald e*t al.*, 2011).

### 2.4.2 Nitrogen metabolism

#### 2.4.2.1 Overview

Nitrogen (protein) metabolism in ruminants is a complex, dynamic process, with dairy cows consuming dietary CP so as to supply the ruminal microbial consortium with the required N for growth, and to obtain AA's necessary for maintenance, growth, reproduction and lactation. Ruminal N metabolism can be divided into two distinct actions, the first being protein degradation followed by MPS (Bach e*t al.*, 2004). Ruminally synthesized MCP, along with ruminally undegraded dietary CP and to a lesser extent endogenous CP contribute to the passage of MP, defined as the true protein digested postruminally and the resultant AA's absorbed, reaching the small intestine (NRC, 2001). Ruminant protein digestion in the small intestine and absorption of AA's and peptides is analogous to that in the non-ruminant. Dietary protein is separated into rumen degradable protein (RDP), comprised of true protein N and NPN, and rumen undegradable protein (RUP). True protein is degraded to peptides and AA's which are either deaminated to ammonia-N or incorporated into microbial protein. Non-protein nitrogen includes N present in DNA, RNA, ammonia, AA's and small peptides, of which the N present in the latter three is utilised for microbial growth (Bach *et al.*, 2004).

Approximately 60 % to 70 % of dietary protein (Hutjens, 2008) is subjected to microbial degradation in the reticulorumen, the first step being the attachment of bacteria to the feed particles followed by activity of microbial proteases (Brock e*t al.*, 1982). Of the ruminal microbial population, approximately 70 % to 80 % of the microbes attach to the undigested particulate matter (Craig *et al.*, 1987a) of which 30 % to 50 % have proteolytic activity (Prins *et al.*, 1983). This extensive microbial enzymatic action leads to the hydrolysis of the peptide bond to produce peptides and AA's (NRC, 1985), following proteolysis liberated peptides may be further degraded by peptidases into AA's, which can either be incorporated into microbial protein or deaminated to VFA's, $CO_2$ and ammonia (Tamminga, 1979; NRC, 1985). The rate and extent to which ruminal protein degradation occurs has been shown to vary greatly (Russell & Hespell, 1981) and is largely dependent upon ruminal microbial proteolytic activity, microbial access to the protein and rumen turnover (NRC, 1985). In brief, ruminal pH, ration type, ruminal passage and dilution rate affect protein degradation through the alteration of the ruminal microbiome, whilst susceptibility to enzymatic action and degradation of protein

depends primarily on the type of protein, more specifically the tertiary structure of the protein and the solubility of the protein (NRC, 1985; Bach *et al.*, 2004).

Dietary protein serves to supply the ruminal microbes with N for maintenance and growth, with ammonia being the primary N source for the majority of ruminal microbes (Owens & Bergen, 1983). Studies across a variety of diets have shown that 50 % to 80 % of bacterial N is derived from ammonia (Leng & Nolan, 1984), with the ruminal microbial population having no absolute requirement for AA's (NRC 2001). However, it has been reported that some microbial species require other nitrogenous compounds, i.e. peptides and AA's for more efficient, rapid growth (Allison, 1982) through the provision of carbon skeletons which can be used for energy production or for the synthesis of new microbial AA's (Bryant, 1973), and by serving as precursors for the synthesis of branched-chain fatty acids which are growth factors to a number of bacterial species (Allison *et al.*, 1958). In particular, when low quality diets are fed, peptides and AA's could be of greater importance as on these types of rations up to 40 % of the bacterial N has been shown to come from sources other than ammonia (Nolan & Stachiw, 1979). Typically, microbes which degrade structural carbohydrates utilise ammonia as their main N source, whereas microbes which degrade NSC utilise ammonia, peptides and AA's (Russell *et al.*, 1992) to meet their higher maintenance requirements.

### 2.4.2.2 Microbial protein synthesis

The ruminal microbial consortium has a significant capacity to synthesise microbial protein, with MCP serving as the primary source of metabolizable protein, accounting for 50 % to 80 % of the total absorbable protein (Storm & Ørskov, 1983), depending on several dietary and animal factors. Microbial protein, post-ruminally, supplies 40 % to 80 % of the ruminant's daily AA requirement (Sniffen & Robinson, 1987), with Clark *et al.* (1992) stating that the passage of microbial AA's to the small intestine averaged 1101 g/d, whilst Sok *et al.* (2017) proposed that the hydrolysis of 1 kg of true microbial protein yields 1.16 kg of free AA's. For this reason, it is imperative that the factors influencing MPS are understood and that simple, reliable methods for measuring MPS are available.

#### 2.4.2.2.1 Factors influencing microbial protein synthesis

In view of the fact that the ruminant receives the majority of its daily AA requirements from ruminally synthesised MCP, it is imperative to understand and quantify factors affecting MCP synthesis, efficiency, and flow to the duodenum, as well as how these may be manipulated so that more accurate and sensitive protein feeding systems can be developed. Detailed discussion of these topics, goes beyond the scope of this review but comprehensive reviews on the topic of N metabolism and microbial protein can be found (Stern & Hoover, 1979; Leng & Nolan, 1984; Sniffen & Robinson 1987; Clark *et al.*, 1992).

A continuous supply of fermentable carbohydrates, ammonia, peptides, AA's and various other nutrients are required to optimise the utilisation of energy for MPS. Several factors influence MPS, with the MCP present in the small intestine being a function of microbial efficiency, resulting from the synthesis of microbial mass and ruminal washout (Sniffen & Robinson, 1987). Of the factors influencing MPS the amount and source of both carbohydrates and proteins are probably the most important to consider, as these influence the synchrony at which ruminal carbohydrate and protein digestion occurs, and at which nutrients become available (NRC, 2001; Bach *et al.*, 2004) thus affecting the efficiency of MPS. The *in vitro* research of Stern *et al.* (1978) demonstrated that dietary energy does not exclusively affect the utilisation of degraded dietary N but instead that the type and rate of availability of carbohydrates was a major factor. Carbohydrates serve as an energy source for the synthesis of peptide bonds, with ruminal MPS being dependant on a continuous supply of adequate quantities and type of carbohydrates. Readily fermentable carbohydrate sources, such as starch and sugars, have been shown both *in vivo* and *in vitro* to be more effective in promoting microbial growth than other carbohydrate sources (Stern & Hoover, 1979), thus increasing the level of NSC and/or substituting more readily degradable carbohydrates for those which are less degradable supports maximum microbial yield (NRC, 2001). Ruminal MPS requires an adequate supply of N for maximal efficiency, with most microbial N being derived from ammonia-N and/or preformed AA's. Whilst the concentration of dietary N is an important factor to consider, N source and extent of ruminal degradability are pivotal in determining the efficiency of MPS (Stern & Hoover, 1979). The asynchronous rate of degradation and release of nutrients can be described in two scenarios, the first being when the rate of protein degradation exceeds the rate of carbohydrate

fermentation resulting in an excess of N, here energy availability may limit N utilisation, with the excess N being lost as ammonia. The second scenario is when the rate of carbohydrate fermentation exceeds the rate of protein degradation, thus leading to a deficiency of available ammonia, AA's or peptides, which may lead to the uncoupling of ruminal fermentation, resulting in fermentation without useful energy production (Stern & Hoover, 1979; Bach *et al.*, 2004). This asynchronous release of nutrients results in the inefficient utilisation of fermentable substrates, thus by improving the synchronisation of energy and N, increases in MCP synthesis, efficiency of MPS, and passage of microbial N to the small intestine can be observed (Clark *et al.*, 1992; NRC, 2001).

As reviewed by Stern & Hoover (1979), Sniffen & Robinson (1987) and Clark *et al.* (1992) various other factors influence MPS and flow to the small intestine, these include the following. 1) Feed intake, with increasing dry matter intake (DMI) increasing MCP yield and flow of microbial N and AA to the small intestine. 2) Forage to concentrate ratio, with increasing levels of carbohydrate decreasing the efficiency of microbial growth due to uncoupled fermentation, with the optimal ratio for maximum microbial yield being 70:30 (Mathers & Miller, 1981). 3) Feeding frequency and 4) dilution rate which is a function of the abovementioned factors, with increases in the dilution rate of ruminal fluid and particulate fractions leading to a greater efficiency of MPS. Other lesser studied factors include the fat content of the diet, dietary sulphur and the effect of feed additives.

#### 2.4.2.2.2 Measurement of microbial protein synthesis

Owing to the nutritional importance of the microbial protein quantitative measurements of microbial protein supply are necessary. Various methods have been utilised *in vivo* to quantify microbial protein flow to the duodenum, traditionally these techniques are based on the determination of a single, inert microbial marker, which may be either an internal marker such as RNA, diaminopimelic acid (DAPA) or adenosine triphosphate (ATP), or an external marker such as the isotopes $^{15}N$, $^{35}S$ and $^{32}P$, which are thought to characterise the microbial components (Stern & Hoover, 1979). These methods require the use of post-ruminally cannulated animals which are doubtfully representative of their intact counterparts and require more care, in addition these methods are laborious, expensive and imprecise (Gonzalez-Ronquillo *et al.*, 2003) preventing extensive *in vivo* studies on MPS (Chen & Gomes, 1992).

Urinary excretion of purine derivatives (PD) is a non-invasive, indirect measurement of ruminal microbial synthesis, originally proposed by Topps and Elliott in 1965, which has the benefit of avoiding the use of cannulated animals and measurements related to digesta and microbial marker kinetics (Martín-Orúe *et al.*, 2000). The principal behind this method is that the duodenal flow of nucleic acids and their derivatives, which are essentially of microbial origin (Topps & Elliot, 1965; Chen & Gomes, 1992; Tas & Susanbeth, 2007), are significantly digested and absorbed in the small intestine, with the absorbed purine bases being catabolised and proportionally excreted in the urine and milk as PD (Topps & Elliot, 1965; Valadares *et al.*, 1999; Tas & Susanbeth, 2007). A major assumption of this method is that the majority of dietary nucleic acids are completely degraded within the rumen (McAllan & Smith, 1973) and therefore that the PD excreted originate exclusively from the degradation of microbial nucleic acids in the small intestine (McAllan, 1980; Tas & Susanbeth, 2007). In cattle the daily excretion of PD is linearly correlated with the quantity of microbial purines absorbed, and thus reflects ruminal MCP synthesis (IAEA, 1997). In cattle, the two primary PD excreted in urine are allantoin (AL) and uric acid, with allantoin being the chief PD contributing 0.80 or more of total PD, unlike in sheep, hypoxanthine and xanthine are absent (Chen *et al.*, 1990). Research has found urine to be the main excretory route for PD, accounting for approximately 0.85 of all absorbed PD, with mammary excretion being the most significant non-renal route of PD excretion, accounting for less than 0.03 of urinary PD excretion (Tas & Susanbeth, 2007).

Limitations to this method and potential sources of error arise from the assumptions that all purines are of microbial origin, as feedstuffs which contain relatively high amounts of nucleic acids could potentially contribute to the duodenal flow of purines, and that the ratio of purine to total N in the mixed microbial population is constant despite knowledge that this ratio differs between FAB and PAB and varies with bacterial species, strain and growth stage. Other potential sources of error include alterations to the partitioning of PD between milk and urine, and the endogenous contribution of PD to urinary excretion (Chen & Gomes, 1992;

Tas & Susanbeth, 2007). Nevertheless, despite providing slightly lower estimates of duodenal MCP flow than direct measurements, a strong positive linear relationship between microbial purine flow to the duodenum and urinary output of PD has been observed (Moorby *et al.*, 2006). In addition, Gonzalez-Ronquillo *et al.* (2004) reasoned that owing to its significantly lower coefficient of variation (CV) that the PD method has a greater sensitivity to detect treatment differences than conventional methods. Therefore, this method is ideal for the estimation and comparison of differences in intestinal flow of MCP from the rumen between dietary treatments (Chen & Gomes, 1992; Martín-Orúe *et al.*, 2000).

To estimate MCP flow quantification of daily urinary PD excretion is required, which entails total urine collection. However, total urine collection is laborious, impractical and can cause discomfort in the animal. As an alternative, researchers (Chen *et al.*, 1992; Valadares *et al.*, 1999) have proposed the use of the PD to creatinine (CR) ratio in spot urine samples as an indicator of daily urine PD excretion (Chen *et al.*, 1995). Urinary excretion of CR, however, is a function of body weight (BW) (Susmel *et al.*, 1995) thus urine CR concentrations may vary with changes in BW. To account for this Chen & Ørskov (2004) developed the PD to CR index (PDC) which allows for comparison amongst cows by correcting the PD to CR ratio for individual animal metabolic BW. Total urine volume from spot urine samples can be estimated by measuring urine specific gravity (SG) as it has been shown to have a close relationship with urine volume (Burgos *et al.*, 2005). By estimation of urine volume and knowledge of the AL concentration in urine it is possible to estimate actual MCP synthesis (Chen & Gomes, 1992). The research of Valadares *et al.* (1999) and Chizzotti *et al.* (2008) found that daily PD excretion estimated by spot urine sampling did not differ from total urine collection, and that this simplified technique may be used to estimate daily MCP flow from the rumen in cattle under farm conditions.

## 2.5 Diet formulation for the dairy cow

In recent years attention has been drawn towards protein nutrition in the dairy industry for two primary reasons. Firstly, purchased feeds, particularly protein-rich feeds and supplements are a major expense to the producer (Klausner *et al.*, 1998) with the efficiency of utilisation of dietary protein being limited to only 25 % to 35 % (Sinclair *et al.*, 2014), which translates to substantial losses of high cost N. Secondly, the N not captured by the animal is excreted, leaching into the environment and contributing significantly to environmental N pollution (NRC, 1993 2003). For these reasons improving the efficiency of N utilisation, thereby reducing N excretion has become a focal point of nutrition models, in order to maintain the sustainability of dairy farms (Dinn *et al.*, 1998; Van Amburgh *et al.*, 2012). Development of ruminant nutritional models began in the 1980s with the advent of the digital age. These models can be defined as "an integrated set of equations and coefficients that predict animal requirements for maintenance, growth, pregnancy, and lactation, and supply of nutrients available to meet those requirements as the result of rumen fermentation, intestinal digestion and metabolism of the feeds consumed in each unique production situation" (Tedeschi e*t al.*, 2015). Various models exist today with two of the most prominent being the equations described in the Nutrient Requirements of Cattle (NRC, 2001), and the CNCPS (Fox *et al.*, 2004) along with derivative models.

Significant progress has been made in dairy nutrition by moving from a CP-based requirement system to a MP system (NRC, 2001; Firkins *et al.*, 2007), however, as reviewed in the NRC (2001) the efficiency of use of MP by dairy cattle is influenced by its content of EAA's, thus models capable of accurately predicting the EAA composition of duodenal protein are required if nutritionists are to advance protein nutrition (NRC, 2001). The AA's available for absorption in the duodenum are supplied by the dietary protein escaping ruminal degradation (i.e. RUP), ruminally synthesised MCP, and endogenous protein, and it is imperative that the AA profile of the MP matches the tissue needs for AA if production is to be maximised (Tucker, 2014). Considering that MCP accounts for 50 % to 80% of the total MP (Storm & Ørskov, 1983), for nutritionists to enhance the effectiveness of the MP system improved accuracy and precision in the prediction of microbial N flow to the duodenum is required (Firkins *et al.*, 2007). The CNCPS was developed to include an AA sub-model which uses a factorial approach to predict the daily metabolizable EAA requirements and supply (O'Connor *et al.*, 1993), with the daily supply of absorbed EAA's based on the sub-modes described by Russell e*t al.* (1992) and Sniffen e*t al.* (1992); and the daily requirements based on the sub-model described by Fox e*t al.* (1992; 2004). The CNCPS surpasses most nutritional models due to its continual evolution since its release

in 1992, as described in a series of four publications (Fox *et al.*, 1992; Russell, *et al.*, 1992; Sniffen *et al.*, 1992; O'Connor *et al.*, 1993). Recently the development of CNCPS has focussed on improving the prediction of AA requirements and supply for lactating dairy cattle, this has led to numerous changes within the model since its original release. One of the main changes has been the update of the AA profiles in the feed library in version 6.5 (Tylutki *et al.*, 2008; Van Amburgh *et al.*, 2013), which has allowed for an increased supply of EAA's. In the version 6.5 the AA requirement computed from the tissue and milk protein AA content was also revised, enhancing the ability of the model to accurately predict the supply and requirement for AA's. Originally the model utilised two separate equations to describe the efficiency of absorbed AA utilisation for maintenance and lactation (Fox *et al.*, 2004), however, the research of Lapierre *et al.* (2007) questioned the biological correctness of this assumption and found a combination of the two into one equation to be more biologically sound, this combined efficiency of utilisation was adopted in CNCPS version 6.5.

Owing to the significant contribution of MCP to the duodenal flow of MP it is imperative that accurate estimation of available AA's from MCP is achievable, this requires reliable estimation of; ruminal MCP yield; the chemical and AA composition of the MCP; and digestibility of microbial AA's (O'Connor *et al.*, 1993). The microbial growth model described by Russell *et al.* (1992) demonstrated that ruminal MCP yield could be reliably estimated using a Michaelis-Menton kinetic model (O'Connor *et al.*, 1993), with the CNCPS determining ruminal MPS on the rate of ruminal carbohydrate fermentation, ruminal carbohydrate availability and ruminal passage rate (Bateman *et al.*, 2001). For this reason, prediction errors in models regarding the ruminal digestibility and passage rate of feedstuffs can lead to errors in the estimation of not only the passage of dietary CP to the duodenum but also ruminal MPS and passage. Since the release of CNCPS various updates have allowed for increased sensitivity and ability of the model to predict MP, in brief these have included; new fractionation schemes; adjustment of protein and carbohydrate ruminal degradation rates (Van Amburgh *et al.*, 2007); re-assignment of pool characterisation (Lanzas e*t al.*, 2007b); passage rate assignment (Van Amburgh *et al.*, 2010); and changes to equations for the calculation of rumen outflows and post-ruminal digestion (Van Amburgh *et al.*, 2015).

The above-mentioned changes have influenced the predictive ability of the model in estimating MPS and yield, but additional reliable estimates of the protein composition, particularly the AA composition, of the ruminal microbes are required to determine the quality of ruminally synthesised MCP (O'Connor *et al.*, 1993). When a factorial approach is used to estimate duodenal AA flow, accurate knowledge of the proportions of the different protein fractions, i.e. RUP, bacterial, protozoal and endogenous CP, flowing to the duodenum are important, as is the AA content of each. The estimation of total duodenal passage of individual AA's is calculated as the sum of the individual AA's from each fraction (Sok *et al.*, 2017). The CNCPS divides the ruminal microbiome into two groups, those bacteria which ferment structural carbohydrates and those fermenting NSC, reflecting differences in N utilisation and growth efficiency (Russell *et al.*, 1992). In terms of the AA composition of bacteria, CNCPS differentiates between cell wall and non-cell wall fractions, of which the majority of the AA's are found in the non-cell wall material (Chalupa, 1972). A description of the AA content of each fraction can be found in O'Connor e*t al.* (1993). Typically, the CNCPS and derivative models use the static AA profile of mixed bacteria described in literature by Storm *et al.* (1983), Clark *et al.* (1992) and Volden & Harstad (1998), of which most of the data was ovine in source and described only the fluid-associated bacterial fraction (Sok e*t al.*, 2017) despite the PAB having been shown to represent a larger fration of the bacteria passing to the duodenum than the FAB (Faichney, 1980).

Until recently ruminal protozoa have only been accommodated in previous versions of the CNCPS by reducing the theoretical maximum growth yield of bacteria, owing to their preferential predation of the ruminal bacteria (Russell *et al.*, 1992), with no regard for the contribution of the protozoa to digestion and microbial protein production (Higgs & Van Amburgh, 2016). However, protozoa have been shown to play a significant role in terms of nutrient digestion and cycling within the rumen (Firkins *et al.*, 2007), and to contribute up to 21 % to 25 % of the total microbial N flowing to the duodenum (Yáñez-Ruiz *et al.*, 2006). Protozoa also differ from bacteria in AA content as previously discussed (Refer to section *2.3.7 Amino acid composition of microbes*). For these reasons, aspects of the growth and metabolism of protozoa were included in CNCPS version 7.0. Overall, the remodel of the entire gastrointestinal tract in version 7.0 has allowed for a more dynamic, holistic approach which allows for the prediction of post-absorptive components of N metabolism such as urea recycling and amino acid supply.

In order to more accurately predict the AA composition of the duodenal flow, i.e. the AA supply to the dairy cow, models need to accurately estimate the source of N as endogenous N, feed N, bacterial N and protozoal N as all have different AA concentrations, profiles and digestibility. The enhancements of the CNCPS version 7.0 allow for each N fraction to be accounted for and appear to allow for the more accurate prediction of total N flows than previous versions. However, the limitation still remains that this and other models use static AA profiles for the different microbial fractions thus limiting the ability of these models to be more dynamic and allow for the incorporation of varying AA profiles as well as the differential passage of microbes under different dietary conditions.

## 2.6 Modern dairy diets: Acidity and its consequences

Modern dairy diets are formulated to be highly digestible and are composed primarily of readily fermentable carbohydrate sources as opposed to forages, the ruminant's natural food source, in order to meet the energy demands for lactation and reproduction of high-yielding, genetically superior dairy cows. Such diets typically contain 50 % to 65 % concentrate, with the remaining proportion of the diet being comprised of forages, which are often silage based (Waldo & Jorgensen, 1981; Staples & Lough, 1989, Allen 1997). This shift towards high fermentable cereal grain and by-product-based diets, which are inherently more acidic and lower in physically effective fibre, combined with the high feed intakes of the modern dairy cow, 4 % or more of body weight (Staples & Lough, 1989), drives milk production but this is often to the detriment of the cow. Such highly fermentable diets, consumed at high levels, result in the rapid production and hence accumulation of VFA's, and $H^+$ in the rumen which drives down the pH of the ruminal milieu (Slyter & Rumsey, 1976). The ruminant possesses inherent buffering capabilities, primarily that of the mineral rich saliva, which owing to the presence of bicarbonate and hydrogen phosphate ions (Maekawa *et al.*, 2002) neutralises the acid build-up, however, on highly-fermentable diets, low in physically effective fibre, these buffering capacities are overwhelmed and the animal itself it not able to maintain ruminal pH within the physiological threshold. Once the ruminal pH declines to critical levels, i.e. below 5.8, rumen fermentation is altered due to a population shift in the ruminal microbiome towards elevated numbers of amylolytic and lactic acid producing bacteria (LAB), such as *S. bovis*, with a simultaneous decline in the activity and numbers of the fibrolytic bacteria (Calsamiglia *et al.*, 1999). Excess rumen acidity has a multitude of negative effects on the dairy cow, and can result in inconsistent DMI (Allen, 1997), poor feed and fibre utilisation, diarrhoea (Maekawa *et al.*, 2002), liver abscesses (Allen, 1997), decreased protein digestion (Calsamiglia *et al.*, 1999), a decline in microbial protein and fibre digestibility (Pitt *et al.*, 1996) and milk fat depression (Staples & Lough, 1989, Xu *et al.*, 1994). One of the most significant impacts of a rumen pH consistently below 5.5 is the onset of metabolic disorders, most commonly sub-acute rumen acidosis (SARA) which is known to have a significant impact on animal health and productivity (Krause & Oetzel, 2006).

## 2.7 Dietary Buffers

### 2.7.1 Introduction

Since the 1960s, dietary buffers have routinely been added to high concentrate diets to compensate for the inability of the endogenous buffering mechanisms, to maintain ruminal pH within the physiologically sound threshold (Russell & Chow, 1993). A true buffer can be defined as a salt of a weak aid or base which is capable of neutralising acids that are either present in feedstuffs, or produced by the process of nutrient fermentation, metabolism, and digestion (Chalupa & Schneider, 1985), without raising the pH. In other words, a buffer increases the resistance of ruminal fluid to changes in pH (Le Ruyet & Tucker 1992). Examples of true buffers include sodium bicarbonate ($NaHCO_3$), limestone ($CaCO_3$), bentonite and sodium sesquicarbonate (Staples & Lough, 1989). Other compounds, namely magnesium oxide (MgO) and calcium magnesium carbonate, are commonly referred to as buffers but are in fact alkalinizing agents as they function to neutralise but not prevent the acidity brought about by the excessive production and accumulation of VFA's and can bring about an increase in pH (Staples & Lough, 1989). Benefits of dietary buffers include; reduced ruminal acidity (Erdman, 1988); provision of a more favourable ruminal milieu for microbial activity (Harrison *et al.*, 1989) thus preventing the overgrowth of undesirable acid-tolerant gram-positive bacteria, i.e. lactobacilli (Garry, 2002) which can further drive down ruminal pH; and promotion of the growth of the more desirable gram- negative bacterial species which produce more desirable VFA's. All of which improve the performance

of animals on high-concentrate diets (Cruywagen *et al.*, 2004). Studies have also demonstrated the ability of buffers to alleviate the symptoms of SARA, in particular milk fat depression, by the positive effects seen on feed intake and milk fat percentage (Erdman, 1988; Enemark, 2008). Although typically fed individually, combinations of various dietary buffers have been investigated, with positive effects on milk production, milk fat percentage and DMI being documented (Hutjens, 1991).

### 2.7.2 Sodium bicarbonate

Despite several chemicals having been evaluated for their buffering abilities and proven to be effective as buffers of ruminal fluid (Herod *et al.*, 1978), sodium bicarbonate has remained the buffer of choice and has been routinely added to concentrate rich, commercial dairy cow diets (Xu *et al.*, 1994) since the early 1960's when it first appeared in dairy cattle feeding experiments (Staples & Lough, 1989). The popularity of sodium bicarbonate as a ruminal buffer is attributed to the compound's high water solubility and acid-dissociation constant which is close to that of the optimal physiological pH of rumen fluid (Enemark, 2008; Marden *et al.*, 2008). These attributes result in the high-buffering capacity observed with sodium bicarbonate (Le Ruyet & Tucker, 1992, Mesgaran *et al.*, 2013) allowing this exogenous buffer to effectively increase and stabilize ruminal pH postprandially (Erdman, 1988) and thus, at least, maintain the productivity of high-yielding dairy cows when high-concentrate diets are fed.

Much research has been conducted to elucidate and validate the effects of sodium bicarbonate on rumen fermentation and productive performance in lactating dairy cows. Comprehensive meta-analyses and reviews have been published, Erdman (1988), Staples & Lough (1989) and Hsu & Murphy (2005), all of which have found the supplementation of sodium bicarbonate to have positive effects on feed intake, milk yield and milk components, particularly milk fat, when fed at levels of approximately 1.1 % of dietary DM regardless of stage of lactation. However, these positive outcomes have not been unanimously observed across literature. The three abovementioned publications evaluated copious research papers and each independently came to the conclusion that beneficial responses were only observed in studies where maize-silage was the main forage source in concentrate-rich rations, with positive results being inconsistent or absent in studies were rations contained non-maize silages or had a forage content greater than 30 %. Briefly, Staples & Lough (1989) reviewed 28 maize-silage based experiments and determined that cows supplemented with sodium bicarbonate produced on average 0.8 kg/d to 0.9 kg/d more milk, with a 0.16 % to 0.30 % higher fat content, which translated to, on average, an additional 1.4 kg to 1.9 kg of 4 % fat-corrected milk (FCM) per day, for early- and mid- lactation cows, respectively. These favourable intake and production responses are a direct result of the alteration of the ruminal milieu, with sodium bicarbonate being repeatedly shown to be effective in stabilising the ruminal pH (Erdman, 1988; Marden *et al.*, 2008; Mesgaran *et al.*, 2013) and to favourably alter ruminal VFA patterns. In response to supplementation, one typically observes an increase in the ruminal acetate to propionate ratio (Erdman *et al.*, 1982; Erdman, 1988; Hsu & Murphy, 2005) which has been shown to be attributed to a reduction of the molar percentage of propionate as opposed to an increase in the molar percentage of acetate (Erdman *et al.*, 1982), and it is this shift in VFA patterns which makes sodium bicarbonate effective at alleviating milk fat depression (Erdman & Sharma, 1989; Hsu & Murphy, 2005). Other beneficial effects of sodium bicarbonate reported include a strengthened reducing power of the ruminal milieu (Marden *et al.*, 2008) and increased apparent digestibility of DM, neutral detergent fibre (NDF) and acid detergent fibre (ADF) (Solorzano *et al.*, 1989; Erdman, 1988; Marden *et al.*, 2008).

The concern with sodium bicarbonate is that its ruminal impact is short-lived and thus may ineffectually buffer the rumen against the continuous production of VFA's (Van Soest, 1994). The pKa of sodium bicarbonate is 6.25 (Enemark, 2008, Marden *et al.*, 2008), thus when the pH of the ruminal fluid decreases below 6.0 the buffering capacity of the compound will become limited and further declines in pH may not be averted (Russell, 1998). The *in vitro* work of Le Ruyet & Tucker (1992), demonstrated that sodium bicarbonate increased both the ruminal pH and buffering capacity of the rumen fluid sharply, with the effect being most pronounced within the first 12 hours of incubation. Hence the study concluded that sodium bicarbonate would be more beneficial in preventing the temporary post-prandial rise in ruminal fluid $H^+$ concentration, whereas buffers with slower release rates would be more successful at longer term buffering of the rumen.

### 2.7.3 Magnesium oxide

Magnesium oxide is another commercially used mineral buffer, although it can best be described as an alkalizing or neutralizing agent as it doesn't conform to the definition of a true buffer. Nevertheless, MgO has shown to be effective at preventing the sharp post-prandial decline in ruminal pH (Erdman *et al.*, 1982; Erdman, 1988), although the *in vitro* research Le Ruyet & Tucker (1992) suggested that despite being capable of stabilising the ruminal acid-base status, the efficacy of MgO may be lost due to passage out of the rumen as the buffering capacity of the mineral only peaked at 24 hours of incubation. Many researchers have studied the effects of MgO inclusion, at levels of 0.4 % to 0.8 % of dietary DM, in lactating dairy cows fed potentially acidotic, concentrate-rich, maize silage-based rations and found MgO inclusion to increase milk yields by 0.6 kg/d (Erdman *et al.*, 1982), peak milk yield by 2.6 kg (Teh *et al.*, 1985), milk fat content and FCM yields (Erdman *et al.*, 1982; Thomas *et al.*, 1984). The review of Staples & Lough (1989) concluded that the supplementation of MgO to lactating dairy cows receiving high energy diets may increase milk yield if dietary magnesium (Mg) is deficient, or increase the milk fat content and hence FCM if dietary fibre is deficient. Research has also demonstrated that the addition of this mineral increases the concentration of total VFA's (Erdman *et al.*, 1982; Teh *et al.*, 1985), increases the molar percentage of acetate while decreasing that of propionate, resulting in an increase in the acetate to propionate ratio (Erdman *et al.*, 1982; Thomas *et al.*, 1984; Teh *et al.*, 1985), improves DM digestibility, and N balance (Erdman *et al.*, 1982). The mode of action of MgO is yet to be elucidated and it is unknown whether the improvements it brings about are due to the mineral's proven alkalinizing properties (Le Ruyet & Tucker, 1992), the alleviation of a dietary Mg deficiency (Staples & Lough, 1989) or improvements in digestibility (Erdman *et al.*, 1980; Erdman *et al.*, 1982). As seen with sodium bicarbonate the addition of MgO has minimal effects on feed intake and milk production performance in moderate to high forage diets (Erdman, 1988), and these two buffering agents appear to be by nature additive in effects (Erdman e*t al.*, 1982).

### 2.7.4 Acid Buf

#### 2.7.4.1 Introduction

Acid Buf (Celtic Sea Minerals, Cork, Ireland) is a dietary rumen buffer, more specifically it is a natural buffer derived from the calcified skeletal remains of the seaweed, *Lithothamnium calcareum*, harvested off the Irish and Icelandic coasts. Calcareous marine algae (CMA) is rich in highly bioavailable minerals with calcium (Ca) predominating at levels of 300 g/kg, the calcium carbonate occurs in three different calcium structures, namely calcite (65 %), and its polymorphs, aragonite (23 %) and vaterite (12 %) (Cruywagen *et al.*, 2015). Other major minerals include Mg at levels of 55 g/kg and potassium (K) at 7 g/kg, with several trace minerals included in varying amounts, as presented in Table 2.3, according to Celtic Sea Minerals (2016). The unique honeycomb structure of CMA ensures a slow release in the acidic environment of the rumen thus conditioning the rumen and neutralising significantly more acid than most conventional buffers over a longer time period. Acid Buf is typically included in lactating dairy cow rations at a level of 0.3 % to 0.4 % of dietary DM, or 80 g/cow/d to 90 g/cow/d, however, diet acidity and heat stress should be considered when determining dietary inclusion levels, with inclusion levels increasing with increasing diet acidity or heat stress. Claimed advantages for lactating cows include better neutralisation of rumen acids, extension of rumen buffering, enhanced fibre digestibility, enhanced milk yield and components, and a reduction in methane production. Acid Buf has achieved widespread use, as the preferred rumen buffer, in South Africa, America and certain regions of Europe (Gouws *et al.*, 2016) despite the lack of research on the product, with currently only three published research papers on dairy cattle available, of which results were unclear (Bernard *et al.*, 2014; Cruywagen *et al.*, 2015; Wu *et al.*, 2015).

**Table 2.3** Mineral composition of Acid Buf (Celtic Sea Minerals, 2016)

| Mineral | Quantity (mg/kg) | Mineral | Quantity (mg/kg) |
|---|---|---|---|
| Boron (B) | 10 mg/kg | Manganese (Mn) | 50 mg/kg |
| Cobalt (Co) | 0.1 mg/kg | Molybdenum (Mo) | 0.2 mg/kg |
| Copper (Cu) | 10 mg/kg | Phosphorous (P) | 500 mg/kg |
| Iodine (I) | 30 mg/kg | Selenium (Se) | 1.8 mg/kg |
| Iron (Fe) | 800 mg/kg | Zinc (Zn) | 10 mg/kg |

### 2.7.4.2 Effects on ruminal fermentation

Research on CMA, principally Acid Buf, has focussed on elucidating the effects of this alternative buffer on the various rumen parameters when incorporated into high concentrate diets. *The in vitro* research of Calitz (2009) and Mesgaran *et al.* (2013) utilised mixed rumen microbes, to establish the acid buffering capacity of various combinations of several non-organic buffering compounds. Calitz (2009) reported that Acid Buf alone had a higher ($P < 0.05$) buffering capacity than sodium bicarbonate alone in terms of the milliequivalents of acid required to reduce the pH by one unit, however, although not significantly different from the buffering capacity of Acid Buf, the highest buffering capacity was observed with a combination of Acid Buf and sodium bicarbonate (80 mg Acid Buf + 120 mg sodium bicarbonate). This study also showed that whilst pH initially increased more with the addition sodium bicarbonate than Acid Buf, that after time pH declined rapidly compared to Acid Buf, which confirms that the impact of sodium bicarbonate is short-lived, as previously discussed. Calitz (2009) postulated that Acid Buf alone is highly effective in preventing acute pH declines, even more so than sodium bicarbonate, when incorporated in high concentrate diets, but that a blend of Acid Buf and sodium bicarbonate could be just as efficacious. The *in vitro* work of Mesgaran e*t al.* (2013) supported Calitz (2009) in that Acid Buf was capable of increasing the pH of the medium, but concluded that the best buffering capacity was always displayed by sodium bicarbonate. Both buffering agents displayed lower acidogenic values, indicating that both are capable of maintaining ruminal fluid pH, but as with Calitz (2009) the best buffering efficiency was observed with a combination of Acid Buf and sodium bicarbonate. The results of this *in vitro* research have been corroborated in various *in vivo* studies, in which Acid Buf has been included either alone or in combination with sodium bicarbonate, at levels of 0.35 % to 0.50 % of dietary DM, to concentrate-rich, TMR's, formulated to be potentially acidotic. The pH of ruminal fluid was shown to be either increased or maintained within physiological limits in feedlot steers (Montañez-Valdez e*t al.*, 2012), non-productive dairy cows (Bilik *et al.*, 2014), and lactating dairy cows (Beya, 2007; Cruywagen *et al.*, 2007; Calitz, 2009; Cruywagen e*t al.*, 2015). Calitz (2009) demonstrated that Acid Buf was capable of maintaining pH within ideal physiological limit, whilst Beya (2007), Cruywagen *et al.* (2007) and Cruywagen *et al.* (2015) observed no effect ($P > 0.05$) of Acid Buf or sodium bicarbonate on mean or maximum pH values. Beya (2007) and Cruywagen *et al.* (2015) did, however, observe a lower ruminal pH nadir for the unbuffered, control diet (5.14 and 5.19, respectively) and sodium bicarbonate buffered diet (5.37 and 5.37, respectively) as opposed to the diet buffered with Acid Buf (5.42 and 5.42, respectively), but this was only significant in the study of Cruywagen *et al.* (2015). These researchers also reported that across all treatments, buffered or not, the ruminal pH decline after the morning feed was unavoidable, but that the rate of pH decline was significantly reduced with the addition of a buffer. Whilst it was shown that sodium bicarbonate was able to sustain pre-feeding ruminal pH levels for approximately one hour longer (Cruywagen *et al.*, 2015) than either Acid Buf or the control, owing to the immediate solubility of this mineral, this treatment was not able to sustain a higher ruminal pH for as long as the Acid Buf treatment, which has a slow release feature (Beya, 2007).

Perhaps of more importance than maximum, mean, and nadir ruminal pH values is the length of time for which ruminal pH remains below 5.5. The studies of Beya (2007), Cruywagen *et al.* (2007) and Cruywagen *et al.* (2015) by continuous pH monitoring, unanimously observed a clear treatment effect on ruminal acidity ($P < 0.05$), especially from midday to midnight when ruminal pH fell below pH 5.5 for a longer period of time for the control treatment. Beya (2007) and Cruywagen *et al.* (2015) reported that Acid Buf reduced the time period pH was suboptimal from 13 hours and 13.8 hours, respectively, to just 4 hours, and was more effective than sodium bicarbonate, which displayed suboptimal pH for 8.7 hours and 7.5 hours, respectively. These results prove that Acid Buf alone can reduce the time ruminal pH is below pH 5.5 ($P < 0.05$). This finding is notable as it has been suggested that the length of time that ruminal pH is suboptimal may be a more critical factor than the relationship between mean daily ruminal pH and the optimal pH (De Veth & Kolver, 2001), and that SARA is caused when ruminal pH consistently remains below pH 5.5 for prolonged periods of time (Krause & Oetzel, 2006). Cruywagen *et al.* (2004) also proposed that milk production may be optimised by preventing a significant decline in ruminal pH in the evening hours, during which inherent buffering capacity is reduced by the reduction in bicarbonate flow from saliva.

The inclusion of Acid Buf to potentially acidotic rations has not yet been shown to have an appreciable effect on total VFA production (Farren *et al.*, 2003; Calitz, 2009; Bilik *et al.*, 2014) although Cruywagen *et al.*

(2015) did report increased ($P$ = 0.01) total VFA concentrations on diets buffered with Acid Buf. Volatile fatty acid concentrations have been shown to be affected by dietary buffers, with Cruywagen *et al.* (2015) reporting increases ($P$ < 0.01) in acetate concentrations versus the control and sodium bicarbonate, although acetate concentrations have been reported to be higher with a combination of Acid Buf and sodium bicarbonate as opposed to Acid Buf alone (Calitz, 2009). Acid Buf has been reported to decrease propionate concentrations (Cruywagen *et al.*, 2015), which is not uncommon in buffered, high-concentrate diets as has been observed with sodium bicarbonate (Erdman *et al.*, 1982), and has a tendency to increase butyrate concentrations (Cruywagen *et al.*, 2015). As with sodium bicarbonate, Acid Buf has been observed to maintain more favourable acetate to propionate ratios, which theoretically could reduce methane production (Calitz, 2009; Cruywagen *et al.*, 2015), whilst others report no significant effect of Acid Buf on the molar proportions of acetate, propionate, butyrate, or the ratio of acetate to propionate (Beya, 2007; Bilik *et al.*, 2014) and have gone so far as to say that calcareous seaweed extracts have no significant effect on ruminal fermentation (Montañez-Valdez *et al.*, 2012).

Typically, the effects of buffers on ammonia-N are not consistent if apparent at all, this holds true for Acid Buf which as of yet has not been shown to have a significant effect on rumen ammonia-N concentrations, when fed to lactating dairy cows on potentially acidotic TMR's (Beya, 2007; Cruywagen *et al.*, 2015). It would beneficial to further research the effect of CMA on ruminal ammonia concentrations as if this buffer is shown to decrease the levels of ammonia, one can expect increases in bacterial N flow and improved efficiency of bacterial protein synthesis.

Owing to the improvements in ruminal pH, overgrowth of acid-tolerant lactobacilli is prevented when buffers are fed (Enemark, 2008), thus one would expect Acid Buf to reduce concentrations of lactic acid in ruminal fluid. This has been reported by Cruywagen *et al.* (2015) who observed that a potentially acidic diet buffered with each Acid Buf and sodium bicarbonate had lower lactic acid concentrations as opposed to the control treatment ($P$ < 0.01). Contrary to this, Bilik *et al.* (2014) stated that Acid Buf did not appear to have an impact on ruminal lactic acid concentrations.

### 2.7.4.3 Effects on animal productive performance

Of the limited research available on calcareous marine extracts, much is focussed on the effects on animal productive performance as a result of the improvement of ruminal pH and fermentation. Owing to the pH stabilization of the ruminal milieu, increases in DMI are typically observed when sodium bicarbonate is fed (Erdman *et al.*, 1982; Staples & Lough, 1989, Bernard *et al.*, 2014), however, these increases in intake have not been observed when feeding CMA to lactating dairy cows on potentially acidotic rations (Calitz, 2009; Bernard *et al.*, 2014; Cruywagen *et al.*, 2015). To date no differences have been observed in the BW, body condition score (BCS) or BW change of cows fed Acid Buf as opposed to control cows (Bernard *et al.*, 2014; Wu e*t al.*, 2015). Research in feedlot lambs also showed no differences in average daily gain, start or end weights when fed Acid Buf (Gouws *et al.*, 2016).

Research on the effects of Acid Buf on energetics and serum metabolites and enzymes has been limited. Wu *et al.* (2015) reported that Acid Buf had no effect on calculated energy balance, with Bernard *et al.* (2014) reporting that with the exception of a reduction in serum glucose levels when fed buffers, no other differences were observed in the concentrations of serum metabolites or enzymes. Research on the effect of calcified seaweed extracts on digestibility, is almost non-existent with only two published papers of which one reported no effect of these extracts on fibre digestibility when fed to Holstein steers on high concentrate diets (Montañez-Valdez e*t al.*, 2012), and the other increased fibre digestibility when fed to lactating Holstein dairy cows (Cruywagen *et al.*, 2007).

Owing to the widespread use of Acid Buf in the dairy industry the majority of research on CMA has focussed on the effects on milk production and composition. When included in concentrate rich, potentially acidotic TMR's at levels of 0.35 % DM to 0.4 % DM (i.e. 80 g/cow/d to 90 g/cow/d) lactating Holstein dairy cows have been reported to respond with increased daily milk yields, yields of 4 % FCM, yields of energy-corrected milk (ECM) and increased milk fat contents with a concomitant increase in milk fat yields, with the increases exceeding those of experimental animals fed diets buffered with sodium bicarbonate (Beya, 2007; Cruywagen *et al.*, 2007; Cruywagen *et al.*, 2015). The research of Beya (2007) illustrated that Acid Buf was

capable of increasing milk production by 4 kg/d as compared to the control and by 2.5 kg/d more than sodium bicarbonate, with a 25 % and 15 % improvement in fat content as compared to the control and sodium bicarbonate, respectively. However, the research trials of Calitz (2009), Bernard *et al.* (2014) and Wu *et al.* (2015) reported no response of Acid Buf on milk yield and milk fat content, it must be noted that in these trials diets were all well buffered, owing to the significant inclusion of forages high in inherent buffering capacity, and thus would not have been expected to decrease ruminal pH levels and affect ruminal fermentation significantly enough to compromise milk production and quality, to effect a response to the dietary addition of buffers. Typically, no treatment effects are observed for milk protein content, milk lactose content, solids-not-fat (SNF) and total milk solids, upon the inclusion of dietary buffers (Beya, 2007; Calitz, 2009; Bernard *et al.*, 2014; Cruywagen *et al.*, 2015). However, the addition of Acid Buf has been shown to increase milk fat, milk protein and milk lactose yields, more so than sodium bicarbonate, as a result of the increase in milk production (Beya, 2007; Cruywagen *et al.*, 2015). One study (Cruywagen *et al.*, 2004) demonstrated an increase in milk protein content by 0.6 % in early lactation dairy cows when increasing the inclusion level of Acid Buf from 0.125 % DM to 0.3 % DM, however, this has not been reported when feeding at levels higher than 0.3 % of dietary DM. In terms of efficiency, Acid Buf has been shown to improve the efficiency of feed conversion into milk (i.e. ECM/ DMI) more effectively than sodium bicarbonate (Cruywagen *et al.*, 2015). The studies of Bernard *et al.* (2014) and Wu *et al.* (2015) found dairy efficiency to exhibit and interaction of treatment by week, with Acid Buf numerically increasing efficiency relative to the unbuffered or sodium bicarbonate treatments only after week six and seven of each study. It has been suggested that when fed both pre- and post-partum Acid Buf assists cows in transitioning better into lactation (Wu *et al.*, 2015)

To conclude, the reported positive influence of Acid Buf appears to be related to its ruminal buffering capacity which enhances feed intakes and fibre digestibility with the concomitant rise in VFA production, leading ultimately to an increase in the efficiency of milk production and milk yield without compromising milk quality. Ultimately research has indicated that Acid Buf, fed at levels of 80 g/d to 90 g/d (0.35 % of dietary DM to 0.4 % of dietary DM) has a greater impact than sodium bicarbonate, at levels of 180 g/d or 0.8 % of dietary DM, on ruminal pH, milk production and composition, and is a safe, efficacious alternative to conventional buffers.

### 2.7.5 Bottom-line

Overall dietary buffers have been shown to be beneficial to not only high-yielding dairy cows, but rather any cattle placed on concentrate-rich diets, which challenge their inherent ability to buffer the production of VFA's in the rumen. When evaluating research on dietary buffers it is important to keep in mind that the response to feeding buffers is dependent on both the type of forage(s) fed and the physical structure of these (Krause & Oetzel, 2006), as well as the forage to concentrate ratio of the experimental diet, as typically only diets which threaten to overwhelm the acid-base balance of the animal will produce a positive response to dietary buffers. Although dietary buffers are of use, buffers should not be routinely used in an attempt to compensate for suboptimal feeding management. When deciding on the optimal buffer for one's herd needs one needs to factor in the benefit to cost factor, in this case Acid Buf may surpass conventional buffers such as sodium bicarbonate, as it achieves the same benefits but at half the inclusion level of sodium bicarbonate (Beya *et al.*, 2007) with the additional benefit of not only buffering the transient post-prandial rise in ruminal pH but too stabilizing the ruminal acid-base status. Whilst Acid Buf is a promising exogenous buffer more research is required to fully elucidate the effects this buffer has on ruminal fermentation, digestibility and production responses.

## 2.8 Ionophores

### 2.8.1 Introduction

Originally developed as coccidiostats for poultry (Richardson *et al.*, 1976) in the early 1970's, ionophores, particularly, monensin and lasalocid, were shown to affect ruminal fermentation. Subsequently monensin and lasalocid gained approval from the Food and Drug Administration, in 1975 and 1982 respectively, for use in cattle to promote growth and improve feed efficiency in cattle (Russell & Strobel, 1989). These two ionophores dominated the market, as Rumensin® and Bovatec®, but by the end of the 1990's were joined by several other ionophores which were approved for use in various livestock production systems

(Feed Additive Compendium, 2000). The benefits derived by cattle from the biological action of these carboxylic polyethers can be classified as follows (Bergen and Bates, 1984); 1) increased efficiency of energy metabolism of the ruminal microbiota and/or the animal; 2) improved N metabolism of the ruminal microbiota and/or the animal and 3) abatement of digestive disorders resulting from abnormal rumen fermentation. Each of these biological actions provides nutritional and metabolic advantages to the animal allowing for improvements in production efficiency (McGuffey *et al.*, 2001). For lactating dairy cattle, claims that supplementation with ionophores have led to improved milk production, production efficiency, and immune responses have been reported in several countries worldwide, including Australia, Argentina, New Zealand and South Africa (Bagg, 1977). These improvements in productive performance and efficiency and the resultant decrease in morbidity and mortality bring about significant economic benefits and reduce the impact of animal production on the environment (McGuffey *et al.*, 2001).

### 2.8.2 Mode of action

Ionophores are highly lipophilic anti-microbial compounds, produced by the naturally occurring *Streptomyces cinnamonensis*, and are fed orally as salts (Duffield *et al.*, 2008a). Ionophores are toxic to several bacterial, protozoal and fungal species (Russell & Strobel, 1989). Owing to their highly lipophilic nature and ability to interact stoichiometrically with metal ions, ionophores are capable of rapidly penetrating biological membranes (Pressman, 1976; Ipharraguerre & Clark, 2003) subsequently modifying the normal ion flux and disrupting essential ion gradients (Pressman, 1976) and in doing so giving rise to the toxic effect observed. Whilst ionophores share a common mode of action, there are differences among the various ionophores in ion proclivity (i.e. cation specificity) and the capacity to achieve effective rumen concentrations. Ionophores either exchange a monovalent cation (i.e. $Na^+$ or $K^+$) for a proton ($H^+$), such as monensin, or a divalent cation for two protons such as lasalocid (Pressman, 1976).

The primary transport system in bacterial cytoplasmic membranes is the $Na^+$/ $K^+$ ATPase system (deVoe, 1974), this enzymatic system plays a central role in various cellular functions such as osmoregulation, excitation and solute transport (Bergen & Bates, 1984). Ruminal bacteria are dependent upon ion gradients for the uptake of nutrients and establishment of a proton motive force (Van Kessel & Russell, 1992), by dissipating these cation and protein gradients, ionophores bring about their detrimental effects on the bacterial cells (Bergen & Bates, 1984). At the cellular membrane interface of the bacterial cells, the monensin-$Na^+$ complex becomes solubilized in the phospholipid bilayer (McGuffey *et al.*, 2001), and subsequently serves as an ion-selective mobile carrier (Bergen & Bates, 1984; Russell & Strobel, 1989; Ipharraguerre & Clark, 2003), exchanging extracellular cations, i.e. $Na^+$, for intracellular protons (McGuffey *et al.*, 2001; Callaway *et al.*, 2003). As the monensin mediated alterations in ion flux progress, bacterial cells experience an efflux of intracellular $K^+$ from the cells and an influx of extracellular $Na^+$ and $H^+$, culminating in a rise in intracellular acidity (Russell & Strobel, 1989; McGuffey *et al.*, 2001) and owing to the depletion of intracellular $K^+$, a decrease in MPS (McGuffey e*t al.*, 2001). Ruminal bacteria attempt to counteract the cytoplasmic acidification and re-establish ion gradients by activating the Na/K and $H^+$ ATPase systems (Callaway *et al.*, 2003; Ipharraguerre & Clark, 2003), which expel intracellular $H^+$ at the expense of one ATP per proton (McGuffey *et al.*, 2001). As a result, the expenditure of energy for maintenance functions is increased (Ipharraguerre & Clark, 2003) resulting in the uncoupling of ATP hydrolysis from growth, eventually diminishing intracellular energy, thus compromising the ability of bacteria to grow and reproduce (Bergen & Bates, 1984; Callaway *et al.*, 2003) which ultimately leads to cellular death (Russell & Strobel, 1989).

### 2.8.3 Effects on the ruminal microbial populations

In the anaerobic conditions of the rumen, microbes obtain the required nutrients and energy for growth by fermenting ingested carbohydrates and proteins. Whilst certain fermentation end-products, such as VFA's and microbial protein, serve as major nutrient sources for the ruminant, others such as heat, methane and ammonia represent a loss of feed energy and protein (Owens & Goetsch, 1988). Through their mode of action, ionophores target specific ruminal bacterial populations thereby altering the ruminal microbial consortium, so as to bring about their multitude of effects. The various ruminal bacteria vary in sensitivity to the detrimental effects of ionophores and because ionophores exert their effects at the cellular membrane level, they are most effective against bacterial species which are more permeable to macromolecules (Russell, 1996; Ipharraguerre

& Clark, 2003). For this reason, gram-positive bacterial species are most susceptible to ionophores as these bacteria lack the highly impermeable lipopolysaccharide layer (Ipharraguerre & Clark, 2003) seen in gram-negative bacterial species which gives these bacteria a selective survival advantage (Chen & Wolin, 1979; Russell & Strobel, 1989). Thus, ionophores can easily penetrate the porous peptidoglycan layer surrounding gram-positive bacteria and dissolve into the cytoplasmic membrane thus disrupting this intricate system (Bergen & Bates, 1984). By chance the gram-positive bacterial species inhibited by ionophores, namely *S. bovis*, *R. albus*, *R. flavefaceins* and *B. fibrisolvens*, produce acetate, butyrate, lactate, formate, hydrogen and ammonia as end products of fermentation (Russelll & Strobel, 1989; Ipharraguerre & Clark, 2003; Weimer *et al.*, 2008), most of which are coupled to the production of methane and other energetically wasteful processes. Whereas, the ionophore resistant gram-negative populations, namely *M. elsdenii*, *F. succinogenes*, and *S. ruminantium*, which produce succinate and propionate and are engaged in more energy efficient fermentation pathways, are enriched upon supplementation (Chen & Wolin, 1979; Russell & Strobel, 1989; Ipharraguerre & Clark, 2003). Although tempting one should not generalise the supressing effects of monensin to only gram-positive bacterial species, as the effects on the various populations within the ruminal microbiome are likely complex (Weimer *et al.*, 2008). Research has shown that on certain diets, i.e. high starch, that monensin does not supress populations of classical gram-positive bacterial species (Weimer *et al.*, 2008), that ruminal bacterial species vary considerably with respect to initial resistance shown and ability to develop resistance, and that there is considerable dichotomy between gram-positive and gram-negative bacterial cell types (Russell & Houlithan, 2003).

Ionophores, specifically monensin, are known to have a "protein sparing effect" (Russell & Strobel, 1989) which arises from the observed ability of ionophores to reduce the populations and activities of gram-positive, proteolytic and obligate AA fermenting ruminal bacteria (Chen & Russell, 1991; Russell, 1996; Wang e*t al.*, 2015) which utilise AA's and peptides as energy sources for growth (Yang & Russell, 1993a). Monensin has been shown both *in vitro* (Yang & Russell, 1993a) and *in vivo* (Yang & Russell, 1993b) to reduce the most probable number of obligate AA fermenting, ammonia producing ruminal bacteria by approximately 10-fold. Of particular interest is a specific group of obligate AA fermenters which possess a high specific activity for ammonia production, and are believed to be capable of deaminating more than 25 % of feed protein (Krause & Russell, 1996b; Callaway *et al.*, 2003). These aptly named hyper-ammonia producers (HAP), i.e. *P. anaerobius*, *C. sticklandii* and *C. aminophilum*, were identified in 1988 (Russell, 1988, Russell & Strobel, 1989) and although only found to be present in small numbers, 2 % to 10 % of total counts, were discovered to have specific activities for ammonia-N production 20-fold greater than previously studied ammonia-N producing bacteria (Russell, 1988, Chen & Russell, 1989; Russell & Strobel, 1989). This particular group of bacteria have been shown to be highly sensitive to monensin (Yang & Russell, 1993b; Callaway *et al.*, 2003; Wang *et al.*, 2015).

Ionophore supplementation has been shown to affect other ruminal microorganisms but data is scarce and often contradictory. Ruminal protozoa have been shown to be sensitive to ionophores *in vitro* (Hino, 1981) and *in vivo* (Richardson *et al.*, 1978; Guan *et al.*, 2006) but no change in protozoal numbers, *in vivo*, has been reported (Dinius *et al.*, 1976; Benchaar *et al.*, 2006b). It has been postulated that the degree of sensitivity may vary with species (McGuffey *et al.*, 2001) and that effects may be diet dependent (Guan *et al.*, 2006). Ruminal fungi have been shown to be sensitive to ionophores *in vitro* (Stewart e*t al.*, 1987) however, *in vivo* research has been limited, but has shown variations in special and strain sensitivity (McGuffey *et al.*, 2001). Although it may be beneficial to reduce the fungal population, owing to their proteolytic nature and substantial production of hydrogen (Russell & Strobel, 1989) it must be kept in mind that fungi play a role in fibre digestion, thus any negative effects induced by ionophores on the fungi may lead to a reduction in ruminal fibre digestion (McGuffey *et al.*, 2001). To date there is no evidence that ionophores directly affect ruminal methanogens (Weimer *et al.*, 2008).

### 2.8.4 Effects on ruminal fermentation

Alterations of the microbial populations within the greater ruminal consortium, are responsible for the observed impact of monensin on ruminal fermentation and digestion (Bergen & Bates, 1984). The most universally observed effect of ionophores, specifically monensin, on ruminal fermentation is enhanced propionate production, with increases in the molar proportions of propionate being observed in several *in vitro*

studies utilising mixed cultures of ruminal microbes (Richardson *et al.*, 1976; Busquet *et al.*, 2005; Castillejos e*t al.*, 2006) and confirmed by numerous *in vivo* studies, when fed at levels of 250 mg/d to 350 mg/d (Yang & Russell, 1993b; Ali-Haïmoud *et al.*, 1995; do Prado *et al.*, 2015; Wang *et al.*, 2015). The rise in molar proportions of propionate is generally not accompanied by an increase in total VFA concentrations, but is owed solely to a shift in ruminal VFA patterns, although some have reported increases in total VFA concentrations *in vitro* (Richardson *et al.*, 1976; Chalupa *et al.*, 1980; Busquet *et al.*, 2005; Castillejos e*t al.*, 2006). This enhanced propionate production can be explained in part by the replacement of gram-positive bacterial species with gram-negative species (McGuffey *et al.*, 2001), however a substantial portion of the effect is as a result of ionophore-mediated alterations in metabolism within the gram-negative population (Bergen & Bates, 1984). With the rise in molar proportions of propionate comes the concomitant decline in the molar proportions of acetate and butyrate which has been observed both *in vitro* (Richardson *et al.*, 1976; Busquet e*t al.*, 2005; Castillejos *et al.*, 2006) and *in vivo* (Yang & Russell, 1993b; do Prado *et al.*, 2015; Wang e*t al.*, 2015) on both concentrate and forage rich diets, although results in pasture-based trials have not always been so clear (Richardson *et al.*, 1976). The effect of monensin on valine and the branched chain VFA's (BCVFA's) have not been as consistent, valine has responded variably whilst BCVFA's have typically shown a decrease in concentration (Busquet e*t al.*, 2005; Castillejos *et al.*, 2006) or remained unchanged (Yang & Russell, 1993b). These shifts in ruminal VFA patterns lead to reduced lipogenic VFA (i.e. acetate) to glucogenic VFA (i.e. propionate) ratios which are seen consistently across literature (Yang & Russell, 1993b; Erasmus *et al.*, 2005; Martineau *et al.*, 2007; do Prado *et al.*, 2015). This reduction in the ratio is as a result of the ability of monensin to inhibit AA deamination, via a reduction in the growth and activity of the ionophore sensitive proteolytic and obligate AA fermenting bacteria (Yang & Russell, 1993b; Russell, 1996) thereby lessening the wasteful breakdown of protein and AA's of feed origin as observed by a decrease in ruminal ammonia-N concentrations (Ali-Haïmoud *et al.*, 1995; Guan *et al.*, 2006; Martineau *et al.*, 2007; Wang *et al.*, 2015). However, previous research has shown that this effect on ruminal ammonia-N accumulation is not always statistically significant (Yang & Russell, 1993b). Ruminal pH is typically unchanged in response to supplementation with monensin (Ali-Haïmoud *et al.*, 1995; Guan *et al.*, 2006; Wang *et al.*, 2015), with responses only being pronounced in trials based on high-concentrate, potentially acidotic diets (Guan e*t al.*, 2006), or when experimental animals were in an adaptive phase. The effect of ionophores on ruminal pH is mediated via a reduction in ruminal lactic acid concentrations, due to the ability of ionophores to decrease populations of the prolific LAB species *S. bovis* whilst not affecting lactic acid utilising bacteria (LUB) species (i.e. *M. elsdenii* and *S. ruminantium*) (Ipharraguerre & Clark, 2003). These ionophore resistant bacterial species may also contribute to the increased ruminal propionate concentrations by augmenting propionate formation from lactate (Bergen & Bates, 1984). These modifications of ruminal fermentation result in the increased efficiency of N and energy utilisation, however, it is important to note that these modifications of the VFA patterns are dependent on diet composition and the level of ionophore administered, as at lower doses responses are reduced and the overall effect on ruminal fermentation is limited (Broderick, 2004; Benchaar e*t al.*, 2006b).

### 2.8.5 Effects on digestibility

Ionophores have been shown to affect the digestibility of the various dietary fractions, specifically it has been shown that these anti-microbial compounds shift the site of digestion from the rumen to the hindgut (McGuffey *et al.*, 2001). Fibre digestion, NDF and ADF, has been shown to decrease in response to monensin when included in *in vitro* cultures (Busquet e*t al.*, 2005; Castillejos *et al.*, 2006) due to the inhibition of ionophore-sensitive cellulolytic bacteria. However, *in vivo* studies have shown total tract fibre digestion to be largely unaffected by ionophores (Ali-Haïmoud *et al.*, 1995; Benchaar *et al.*, 2006a, b; Yang e*t al.*, 2007; do Prado *et al.*, 2015). This may be explained firstly by the ability of the enriched fibrolytic ruminal bacteria, e.g. *F. succinogenes*, to offset the diminished ionophore-sensitive ruminococci populations (McGuffey *et al.*, 2001), and secondly by the enhancement of post-ruminal digestion to the extent that the decreased ruminal digestibility is compensated for (Ali-Haïmoud *et al.*, 1995). Although ruminal digestion of DM, OM, starch, dietary N and fibre has been shown to decline, by 18.7 %, 8.2 % to 16 %, 11.9 %, 11 % and 20.3 %, respectively (Ali-Haïmoud *et al.*, 1995; Yang *et al.*, 2007), no effect on total tract apparent digestibility (TTAD) was observed due to an increase in post-ruminal digestion (Ali-Haïmoud *et al.*, 1995). Some researchers have reported increases in the TTAD of CP (Plazier *et al.*, 2000; Benchaar *et al.*, 2006b; Martineau *et al.*, 2007) NDF and ADF (Plazier *et al.*, 2000) but these responses appear to be diet dependant with increases in the

TTAD of CP being observed when experimental diets are rich in concentrates, and increases in TTAD of fibre only being observed in forage-based diets (Plazier *et al.*, 2000; McGuffey *et al.*, 2001).

### 2.8.6 Effects on nitrogen utilisation

Ionophore supplementation has been shown to have a profound impact on ruminal N retention (Callaway *et al.*, 2003), with retained N being shown to increase when expressed as either a percentage of N intake or as a percentage of N absorbed (Poos *et al.*, 1979). *In vitro* research has demonstrated that monensin reduces proteolysis, deamination, ruminal ammonia production and accumulation, and microbial N (Whetstone *et al.*, 1981; Busquet e*t al.*, 2005). The "protein sparing effect" observed when monensin is fed can be attributed primarily to an inhibition of ruminal AA deamination rather than proteolysis, as evidenced by the accumulation of small peptides and AA-N *in vitro* (Busquet e*t al.*, 2005) and *in vivo* (Wang *et al.*, 2015), with a simultaneous decline in ruminal ammonia-N concentrations. Ruminal AA degradation is nutritionally wasteful, often leading to excess ammonia production, which cannot be fully utilized by the microbes, with this excess ammonia-N representing a loss of dietary N (Yang & Russell, 1993a). This monensin induced reduction of dietary AA degradation diverts the peptides and AA's which are spared from deamination and allows for monensin resistant bacterial species to convert these nutritional building blocks into microbial protein (Yang & Russell, 1993b). The primary means by which ruminal AA catabolism is decreased is by the previously discussed reduction in populations and activities of gram-positive, ionophore-sensitive obligate AA fermenting bacterial species, particularly the HAP species, which utilise AA's and peptides as energy sources for growth (Yang & Russell, 1993a, b). Although the efficiency of ruminal bacterial protein synthesis is generally unchanged (Ali-Haïmoud, *et al.*, 1995), net microbial growth has been shown to be reduced by monensin (Whetstone *et al.*, 1981) which can be attributed to a reduction in ruminal bacterial protein synthesis (McGuffey e*t al.*, 2001). Ali-Haïmoud *et al.* (1995) reported that monensin improved flows of non-ammonia N (NAN) and dietary N (i.e. RUP) but decreased the proportion of duodenal N which originated from bacteria, when fed to lactating dairy cows. In this study, flows of total essential and non-essential AA's to absorption sites in the duodenum were also reported to increase significantly, by 17.2 %, as did the apparent intestinal absorption and digestion of total AA's. This increase in dietary N reaching the small intestine might explain the observed improvements in N digestibility and retention when feeding ionophores (Poos e*t al.*, 1979), but the improvement in N utilisation may also arise from enhanced glucose synthesis brought about from elevated ruminal propionate which spares AA's (Van Maanan e*t al.*, 1978). When evaluating the magnitude of proteolytic and deaminative inhibition caused by ionophore antibiotics, one must consider the degree to which ionophores can modify the relative proportions of bacterial N and RUP in the duodenal digesta. The extent of the effect is related to the nature of the dietary protein itself, namely the solubility, extent to which it can be degraded ruminally, and the propensity to liberate ammonia-N (Dinius, 1978; Ali-Haïmoud e*t al.*, 1995).

### 2.8.7 Effects on energy utilisation

Ionophores have been shown to have a positive effect on energy utilisation. The observed improvement in the efficiency of energy metabolism (Duffield *et al.*, 2008a) is mediated primarily by modification of ruminal fermentation, specifically alteration of the VFA patterns. As previously mentioned, monensin, enhances the production of propionate with a concomitant decline in molar proportions of acetate and butyrate. This shift in VFA patterns, to a more reduced, highly efficiently utilised gluconeogenic VFA (Richardson *et al.*, 1976), and a decrease in the ratio of lipogenic to glucogenic VFA's is a hallmark of increased energy availability to the animal (Russell & Strobel, 1989; Callaway *et al.*, 2003). Propionate is known to have a higher enthalpy than acetate, thus upon oxidation it is capable of providing the animal with more available feed energy which can be directed towards production (Russell & Strobel, 1989). Research has shown that the increased feed efficiency observed when feeding monensin, can be attributed to alterations in ruminal VFA and gas production, which favourably modify ruminal fermentation efficiency (Chalupa *et al.*, 1980; Phipps *et al.*, 2000). The *in vitro* research of Richardson *et al.* (1976) and Whetstone e*t al.* (1981) demonstrated that fermentation efficiency could be increased by 5.6 % and 4.7 %, respectively, this considerable energy savings was brought about by a shift in the ratio of acetate: propionate: butyrate from 60: 30: 10 to 52: 40: 8, thus increasing the gross energy (GE) retained (Richardson e*t al.*, 1976). The *in vivo* work of Richardson e*t al.* (1978) on both pasture and concentrate fed cattle, demonstrated that upon feeding monensin, total VFA energy

produced ruminally increased per kg DM consumed from 3.57 MJ/kg DM to 4.76 MJ/kg DM, representing a 33 % increase in ruminal digestible energy.

The production of methane, an unavoidable by-product of ruminal OM fermentation, is a necessary reductive step required for disposal of reducing equivalents, i.e. $H_2$; $CO_2$ and formate, produced by gram-positive bacterial species (Owen & Goetsch, 1988; Ipharraguerre & Clark, 2003), in order to maintain overall ruminal fermentation. This is a highly energy inefficient process, with one litre of methane produced equating to a loss of 39.5 kJ of feed energy (Guan *et al.*, 2006), thus resulting in a significant loss of gross feed energy of 2% to 12%, particularly on diets rich in forages (Johnson & Johnson, 1995). In order to maintain fermentation balance the increase in propionate production seen by monensin must be accompanied by a reduction in methanogenesis (McGuffey *et al.*, 2001). Monensin supresses methanogenesis, not by reducing the population of methanogens, as these archaea are resistant to ionophores (Chen & Wolin, 1979), but instead indirectly by decreasing the availability of $H_2$ and formate, the primary energy substrates for the methanogenic archaea (McGuffey *et al.*, 2001; Weimer *et al.*, 2008). By reducing the populations of ionophore sensitive, gram-positive bacterial species and protozoa, cross-feeding of nutrients is limited (Bergen & Bates, 1984), with the enriched gram-negative bacterial species diverting $H_2$ to more efficient end products (McGuffey *et al.*, 2001). Methanogenesis has been shown to be reduced by 15 % to 40 % *in vitro* (Chalupa e*t al.*, 1980) and has been confirmed *in vivo* in steers (Guan *et al.*, 2006) in which enteric methane emissions, expressed as L per kg DMI and as a percentage of GE, were significantly reduced by 30 % and 27 %, on low- and high-concentrate diets, respectively. Odongo e*t al.* (2007) reported that when fed long-term to lactating dairy cows, monensin decreased methane production by 7 % to 9 %. Although promising, the decrease in methanogenesis, is often short-lived, with an adaptive response being observed, this response may be influenced by diet composition, with restoration of methane emissions occurring sooner on forage-based diets (Guan *et al.*, 2006).

Propionate is the principal precursor of glucose in ruminants, thus by enhancing the production of propionate the supply of glucogenic substrate to the liver is increased, stimulating the production of glucose via gluconeogenesis (Ipharraguerre & Clark, 2003; Duffield e*t al.*, 2008a). The shift of starch digestion from the rumen to the lower gastrointestinal tract is also beneficial as it increases the amount of carbon from starch being absorbed directly as glucose as opposed to as VFA's, which is a more efficient use of energy (Ali-Haïmoud e*t al.*, 1995). These improvement in glucose status, promote insulin secretion thereby reducing lipid mobilisation and supply of fat to the liver (Duffield e*t al.*, 2008a). This leads to an improvement in energy metabolism, as observed by blood metabolites, which is particularly beneficial to early lactation cows, as it leads to an improved transition into lactation with a decreased risk of metabolic disorders (Duffield *et al.*, 2008a).

### 2.8.8 Effects on animal productive performance & health

Based on the meta-analysis of 36 papers and 77 trials in dairy cattle, Duffield e*t al.* (2008b) reported that monensin decreases DMI by 0.3 kg, this correlates with the review of Ipharraguerre & Clark (2003) in which 14 experiments were evaluated and found the mean decrease in DMI to be 0.3 kg. Although Duffield *et al.* (2008b) found these results to be highly consistent, and could not identify any variables to influence the effect of monensin on DMI, variation in the DMI response has been observed. Whilst some have reported decreases in DMI (Benchaar e*t al.*, 2006a; Gandra *et al.*, 2010), many have reported no change in DMI (McGuffey *et al.*, 2001; Benchaar *et al.*, 2006b; Odongo *et al.*, 2007; do Prado *et al.*, 2015) with some observing numerically lower but non- significant decreases in DMI (Phipps e*t al.*, 2000; Erasmus *et al.*, 2005). Wagner e*t al.* (1999) demonstrated that DMI was unaffected in early-lactation cows but depressed in mid- to late-lactation cows, thus illustrating that there is variability in response over the lactation period. This can be explained as follows, in early-lactation dairy cows are in a negative energy balance, due to high production and low intakes, thus the additional energy provided by monensin supplementation is used to improve production and abate body reserve losses, whereas in mid- to late-lactation cows are in a positive energy balance and eat to maintenance requirements, thus DMI is reduced owing to more energy being available per unit of feed consumed (Tedeschi *et al.*, 2003). Dry matter intake has also shown to be influenced by the level of supplementation and diet, with Guan *et al.* (2006) illustrating that DMI was unchanged when fed to animals on forage-based diets but depressed when fed to those on concentrate-based diets.

Dairy cows fed monensin typically lose less body condition in early lactation, and maintain higher body condition throughout lactation (McGuffey *et al.*, 2001). The meta-analysis of Duffield *et al.* (2008b) determined that supplementation with monensin increased body condition by 0.03 points and BW by 0.06 kg/d in lactating dairy cows. This slight increase in body condition is consistent with the increase in energy and protein supply to the cow.

The reported effects of monensin supplementation on milk productive performance and milk composition are inconsistent. The extensive meta-analysis of Duffield *et al.* (2008b) reported that monensin increases milk yield by 0.7 kg/d, which is in agreement with the reviews of Ipharraguerre & Clark (2003) who found that although the response in milk yield was variable across studies, overall a 1.5 kg/d increase could be observed, and McGuffey e*t al.* (2001) who reported a 1.3 kg/d increase. The herd level randomised clinical trial of Dubuc *et al.* (2010) included 47 herds of dairy cows and too found milk production to be increased by 0.9 kg/d but did report a significant effect of stage of lactation on the response, as significant improvements in milk production were observed only for those cows less than 150 days in milk (DIM). Others reporting increases in milk production include Phipps *et al.* (2000) who reported at various doses, an increase in milk yield of 0.8 kg/d to 2.8 kg/d, and Gandra *et al.* (2010) who observed an increase of 0.66 kg/d, equating to a 2.7 % increase in milk production. However, both of these studies noted that at increasing doses, particularly, above that of the manufacturer's specifications that the increase in milk production lessens. In contrast many researchers have not observed any effect on milk production in response to monensin (Erasmus *et al.*, 2005; Martineau *et al.*, 2007; Odongo *et al.*, 2007; do Prado *et al.*, 2015). Although Duffield *et al.* (2008b) concluded that the failure of some studies to represent this increase was due to a lack of statistical power, there are certain factors to consider which can modify the response. These factors have been highlighted in the reviews of Kennelly & Lien (1997) and Duffield *et al.* (2008b) and include 1) The herd (Lean *et al.*, 1994); 2) stage of lactation (Dubuc *et al.*, 2010); 3) BCS (Duffield *et al.*, 1999), with increases in milk production observed when cows have increased BCS at the initiation of monensin treatment, 4) Genetic merit (Van der Werf *et al.*, 1998); 5) level of supplementation and 6) diet composition, as increases in milk production have been shown to be greater in pasture-based herds (+ 1.5 kg/d) as opposed to herds on low forage diets (+ 0.7 kg/d) (Ipharraguerre & Clark, 2003; Duffield *et al.*, 2008b).

The milk component with the most variable magnitude or response to monensin supplementation across studies is milk fat content and yield (Duffield e*t al.*, 2008b). Meta-analysis has shown that milk fat content typically declines in response to monensin (0.13%) whilst milk fat yield is unaffected (Duffield e*t al.*, 2008b), when increases in milk fat yield are observed these can typically be attributed to increases in milk yields (Gandra *et al.*, 2010). Other studies reporting these effects are the reviews of McGuffey e*t al.* (2001) and Ipharraguerre & Clark (2003), who both reported a 4.5 % decline in milk fat content, the extensive herd level study of Dubuc *et al.* (2010) who reported a significant decline of 0.18 % over an entire lactation, and the individual studies of Phipps e*t al.* (2000), Broderick (2004), Benchaar *et al.* (2006b), Odongo e*t al.* (2007) and Yang *et al.* (2007). This reduction in milk fat content can be attributed to the decline in ruminal acetate and butyrate, in response to monensin, as these VFA's are lipogenic precursors required for the synthesis of fatty acids in the mammary gland (Ipharraguerre & Clark, 2003; Van der Werf *et al.*, 1998), as well as to an inhibition of biohydrogenation of long-chain fatty acids, as indicated by the observed reduction in short-chain fatty acids and increases in the concentration of trans-C18:1which is a potent inhibitor of de novo synthesis of milk fat (Ipharraguerre & Clark, 2003; Benchaar e*t al.*, 2006b). Meta-analysis reported that milk protein percentage is decreased by 0.03 % and milk protein yield by 0.016 kg/d (Duffield *et al.*, 2008b), whilst many have observed a decrease in milk protein content (McGuffey *et al.*, 2001; Odongo *et al.*, 2007; Dubuc e*t al.*, 2010) others have not observed an effect on milk protein (Benchaar *et al.*, 2006b; Martineau e*t al.*, 2007; Gandra *et al.*, 2010; do Prado e*t al.*, 2015). It has been suggested that the decline in milk protein content, could be due to dilution from increased milk production (Phipps *et al.*, 2000; Dubuc *et al.*, 2010). Responses of both milk fat and protein to monensin are highly variable across research, with factors which may influence the effect including 1) Mode of supplementation 2) Stage of lactation 3) dose and 4) diet composition and feeding system (Duffield e*t al.*, 2008b; Dubuc *et al.*, 2010; Gandra *et al.*, 2010). Milk lactose, somatic cell count (SCC), total solids and milk protein fractions are typically unaffected as are the yields of these milk constituents (Erasmus e*t al.*, 2005; Gandra e*t al.*, 2010; do Prado e*t al.*, 2015).

When supplemented with sodium monensin, lactating dairy cows typically display an improvement in milk production efficiency, expressed as the quotient of milk yield (kg) and DMI (kg). The meta-analysis of Duffield e*t al.* (2008b) reported that milk production efficiency increased by 2.5 % owing to the approximate 2 % increase observed in both DMI and milk yields. The review of Ipharraguerre & Clark (2003) determined that the efficiency of milk production increased by 11.4 % and 3.6 %, on high-forage and high-concentrate diets, respectively. Others to observe this include Gandra e*t al.* (2010) and Phipps e*t al.* (2000), who reported an increase in efficiency ($P < 0.05$) at all levels of monensin supplementation, and a net energy (NE) efficiency increase of 5% although this was not significant.

Other benefits of feeding monensin to lactating dairy cows, include decreased risk of metabolic disorders particularly ketosis in early lactation, owing to the improvement in energy balance. The incidence of abomasal displacement, acidosis and multiple other illnesses have also shown to be reduced (McGuffey *et al.*, 2001; Ipharraguerre & Clark, 2003).

### 2.8.9 Bottom-line

Ionophores have recently been subjected to public scrutiny which lead to these in feed antibiotics being banned as growth promoters in the European Union (Castillejos *et al.*, 2006). In order to appease the public much of the recent research in the animal nutrition has been focussed on alternate feed additives, however, owing to their cost- effectiveness, proven and predictable benefits for both beef and dairy cattle, they remain the "gold standard" against which all emerging feed additives are evaluated. With all of the observed improvements in animal performance and health representing a secondary effect caused by ionophore-mediated alteration of the ruminal microbiome and fermentation, to increase the supply of nutrients to the dairy cow. A multitude of factors influence the animal response to ionophore supplementation but typically positive responses are observed at doses of 240 mg/d to 335 mg/d, and in cows at greater risk of negative energy balance (Ipharraguerre & Clark, 2003).

## 2.9 Direct-Fed Microbials

### 2.9.1 Introduction

Direct-fed microbials (DFM's) are dietary supplements which have been both utilised in ruminant production and a topic of research for over thirty years (McAllister *et al.*, 2011). Direct-fed microbials fall under the definition of probiotics, which are defined as "a live microbial feed supplement, which may beneficially affect the host animal by improving its intestinal microbial balance" (Fuller, 1989; Krehbiel *et al.*, 2003). However, the term probiotic is all-encompassing, describing viable microbial cultures, culture extracts, enzyme preparations, crude extracts, or assorted combinations of these products (Yoon & Stern, 1996; Krehbiel *et al.*, 2003; Seo *et al.*, 2010). Direct-fed microbials have been more precisely defined, by the USDA (1989), as microbial-based feed additives which contain "live, naturally occurring microorganisms" (AlZahal *et al.*, 2014). Direct-fed microbials are known to interact symbiotically with the host, giving rise to an optimally regulated microbial milieu throughout the gastrointestinal tract (Seo *et al.*, 2010) which translates to improvements in animal health and productivity. Originally used primarily in young ruminants to promote the establishment of multifarious ruminal and intestinal microbiomes thereby fostering ruminal maturation and preventing the establishment of intestinal enteropathogens (Krehbiel e*t al.*, 2003; Chaucheyras-Durand *et al.*, 2008), DFM's are now commonly incorporated in cattle feeds. Reported benefits of DFM's include; improved milk production (Krehbiel *et al.*, 2003); increased daily gain and feed efficiency in feedlot cattle (Krehbiel *et al.*, 2003); enhanced production efficiency; improved health (Nocek *et al.*, 2003) and immunity (McAllister *et al.*, 2011).

### 2.9.2 Types of Direct-Fed Microbials

Microorganisms utilised in the animal feed industry as DFM products are regulated with only those microbial organisms which have been identified as Generally Recognised As Safe (GRAS) being included in commercially available DFM's. The various DFM's can be classified into sub-categories according to microbial species type and basic mode of action (Kalebich & Cardoso, 2017). These sub-categories are 1) Bacterial DFM's, which include LAB and LUB amongst others; 2) Yeast DFM's; and 3) Fungal DFM's

(McAllister e*t al.*, 2011; Kalebich & Cardoso, 2017). Bacterial DFM's consist of inherent ruminal bacteria which have the potential to alter ruminal fermentation (McAllister *et al.*, 2011) and favourably affect the post-ruminal gastrointestinal tract (Seo *et al.*, 2010). The primary focus has been on those bacterial species which can be classified as either LUB or LAB. Species of LUB include *M. elsdenii, Propionibacterium freudenreichii* and *S. ruminantium* (Krehbiel *et al.*, 2003; Seo *et al.*, 2010; McAllister *et al.*, 2011) all of which bring about their effect by enhancing ruminal lactic acid metabolism (McAllister e*t al.*, 2011). Lactic acid producing bacterial species, namely of the genera *Lactobacillus*, *Bifidobacterium*, *Streptococcus* and *Enterococcus* (Seo *et al.*, 2010) may potentially have beneficial effects in both the rumen, by promoting the adaptation of the inherent ruminal microbes to the presence of lactic acid (Yoon & Stern, 1996; Krehbiel *et al.*, 2003) and in the lower gastrointestinal tract (Seo *et al.*, 2010). When used in conjunction LAB and LUB complement each other to stabilise the ruminal milieu (Seo *et al.*, 2010), thereby leading to the enhancement of animal production (McAllister *et al.*, 2011). Other bacterial strains which have been investigated include a strain of *Prevotella bryantii* which ferments starch into end-products other than lactic acid (Chiquette e*t al.*, 2008), and the fibrolytic ruminal bacteria *R. albus* and *R. flavefaciens* to potentially increase fibre digestion, although success to date has been limited (Chiquette *et al.*, 2007). The most prominent yeast DFM is *Saccharomyces cerevisiae*, this microbe is not an inherent inhabitant of the ruminal microbiome, but is one of the most extensively researched and utilised DFM's in dairy production systems (McAllister *et al.*, 2011). Fungal DFM's typically included in ruminant diets are *Aspergillus oryzae* and *Aspergillus niger*, however, these are typically included as crude enzyme extracts and not whole cells, and are thus not true DFM's (McAllister *et al.*, 2011). Most commercially available DFM products used in cattle production systems include at least one lactic acid producing bacterial strain (McAllister e*t al.*, 2011), either alone or most commonly with *S. cerevisiae* (AlZahal e*t al.*, 2014).

### 2.9.3 Mode of action

Numerous mechanisms by which DFM's lead to improvements in gastrointestinal tract health and animal performance, in ruminants, have been proposed by researchers. These have included modulation of ruminal fermentation, enhanced ruminal fibre digestion, and the enrichment of the ruminal microbiome with desirable bacterial species. Post-ruminally actions such as the competitive exclusion of pathogens, production of antibacterial compounds, stimulation of enzyme production, enhanced nutrient absorption and immune stimulation have been suggested (Krehbiel *et al.*, 2003; Seo *et al.*, 2010; McAllister e*t al.*, 2011). Much of the research has focussed on how DFM's affect ruminal fermentation rather than their post-ruminal effect, however, overall the exact mode of action of DFM's remains to be fully elucidated (Krehbiel *et al.*, 2003; AlZahal *et al.*, 2014).

Yeast-based DFM's are the most studied and utilised DFM's in the dairy industry (AlZahal e*t al.*, 2014), and fall into two categories active dried yeasts (ADY) and yeast cultures. The difference being that ADY by definition must contain more than 15 billion live yeast cells per gram (Malekkahi *et al.*, 2016), whereas yeast cultures are derived via yeast fermentation and thus contain fermentation by-products, these products are not dependent on live yeast cells for their physiological effects (Callaway & Martin, 1997; Malekkahi *et al.*, 2016). Several mechanisms have been proposed as to how live yeast-based DFM's exert their effects, these have primarily included microbial stimulation and modulation, oxygen sequestration, and pH modulation (Chaucheyras- Durand *et al.*, 2008; Seo *et al.*, 2010; McAllister *et al.*, 2011). Yeast supplementation has repeatedly been shown to increase ruminal bacterial numbers (Newbold *et al.*, 1995; Seo e*t al.*, 2010), microbial activity (Erasmus e*t al.*, 1992) and metabolism (Miller-Webster *et al.*, 2002). This microbial stimulation, particularly of cellulolytic and LUB species, has been proposed to be brought about by the provision of soluble growth factors, such as organic acids, vitamins and AA's (Callaway & Martin, 1997; Seo *et al.*, 2010) either from the yeast itself or from micronutrients contained in the yeast culture (Robinson & Erasmus, 2009). Oxygen sequestration is another mechanism by which growth of the ruminal microbes is stimulated, yeast cells are suggested to remove dissolved oxygen from the rumen (Rose, 1987), as evidenced by a reduction in redox potential of ruminal fluid (Marden e*t al.*, 2008). As a result, a ruminal milieu which is conducive to the growth of the strict anaerobic, cellulolytic bacterial species is sustained (Jouany *et al.*, 1999), thus maintaining metabolic activity (Seo *et al.*, 2010) and stimulating the initial rate of cellulolysis (Callaway & Martin, 1997; Sullivan & Martin, 1999), thus leading to improvements in fibre digestibility (McAllister e*t al.*, 2011). Yeast supplementation has been demonstrated to cause shifts in ruminal bacterial populations,

typically increases in population sizes of the primary fibrolytic species, i.e. *F. succinogenes* and *R. albus*, and primary lactic acid utilisers, i.e. *S. ruminantium a*nd *M. elsdenii*, are observed. Although much of this research has been done *in vitro* (Newbold *et al.*, 1995; Callaway & Martin, 1997) these microbial shifts have been corroborated by *in vivo* trials (Pinloche *et al.*, 2013; Malekkahi *et al.*, 2016). Yeasts have also been reported to stimulate ciliate Entodiniomorphid protozoa, which are known to engulf starch particles, utilise lactate, and compete with amylolytic bacteria for substrate (Williams & Coleman, 1997). *Saccharomyces cerevisiae* is reported to metabolise lactic acid (McAllister *et al.*, 2011) and to compete with starch-utilising bacteria for substrate (Kalebich & Cardoso, 2017), thus preventing the accumulation of lactic acid in the rumen, leading to the stabilisation of ruminal pH and improved ruminal fermentation. However, this mode of action is not fully supported, with the review of Robinson & Erasmus (2009) finding no support that yeasts allow ruminal microbes to effectively metabolise the end-products of starch fermentation. Additionally, yeasts are aerobic thus the extent to which they can actively metabolise lactic acid under anaerobic ruminal conditions remains questionable (McAllister e*t al.*, 2011).

Fungal DFM's, are fed with the primary aim of increasing ruminal fibre and or starch digestion, however, these products may alter feed utilisation via several mechanisms (McAllister *et al.*, 2001). Although these crude extracts often contain a number of viable fungal cells, owing to the aerobic nature of *Aspergillus* species it is unlikely that their influence on ruminal fermentation is as a result of a direct effect on metabolism or growth (McAllister *et al.*, 2011). Bacterial stimulation by *A. oryzae* has been observed both *in vitro* (Beharka & Nagaraja, 1998) and *in vivo* (Yoon & Stern, 1996; Higginbotham *et al.*, 2004), as evidenced by increased bacterial numbers, particularly fibrolytic bacterial species such as *R. albus* and *F. succinogenes*. Bacterial stimulation by fungal DFM's likely results from pH stabilisation by increasing the growth rates of LUB, such as *M. elsdenii*, *S. ruminantium* and *Selenomonas lactilytica* (Beharka & Nagaraja, 1998), enhanced nutrient digestion and absorption due to increased production of enzymes (Higginbotham e*t al.*, 1994), provision of growth factors such as AA's, organic acids and B-vitamins (Wiedmeier e*t al.*, 1987; Seo e*t al.*, 2010), and an overall more stable ruminal milieu (Higginbotham e*t al.*, 2004; Seo e*t al.*, 2010).

The most commonly utilised bacterial DFM's, are classified as LAB and LUB species. The proposed mechanism of action for both is similar (Kalebich & Cardoso, 2017) and as previously mentioned, these are often administered in combination or with yeast cultures, owing to their complimentary nature to achieve the most beneficial results (McAllister e*t al.*, 2011). Lactic acid producing bacteria bring about their effect by promoting the adaptation of the ruminal microbiome to the presence of lactic acid by providing a constant supply (Yoon & Stern, 1995; Seo *et al.*, 2010), and by stimulating the growth of LUB by providing lactic acid as a substrate (Seo *et al.*, 2010) allowing for stabilisation of ruminal pH (Nocek *et al.*, 2002; Seo e*t al.*, 2010;). Complementing LAB, the LUB enhance ruminal lactic acid metabolism (McAllister *et al.*, 2011), metabolising lactose, glucose and maltose to produce greater quantities of propionate (McAllister *et al.*, 2011; Kalebich & Cardoso, 2017). It has been hypothesised that LAB may have post-ruminal benefits such as enhancement of the gut microbial populations, due to the competitive exclusion of pathogens and production of antibacterial compounds; improved diet digestibility; and enhanced immune function (Seo e*t al.*, 2010; McAllister e*t al.*, 2011). The exact mechanism by which LAB and LUB improve animal performance and health is yet to be fully elucidated, however, it is likely due to the ability of these bacterial DFM's, either alone or in combination, to moderate lactic acid metabolism and thereby improve ruminal functionality (AlZahal e*t al.*, 2014).

### 2.9.4 Effects on ruminal fermentation

Variable success has been observed in the ability of DFM products to favourably modify ruminal fermentation with the associated beneficial effects on animal performance. Variable effects of DFM's on ruminal fermentation may reflect differences amongst studies in dosage, feeding times and frequency, ration composition, strain of DFM and number of viable cells, and the physiological state of the animal (Piva *et al.*, 1993; Seo e*t al.*, 2010; Kalebich & Cardoso, 2017).

Various yeast-based DFM's have been shown to affect rumen fermentation, however, the responses are complex and not fully elucidated, as reviewed by Chaucheyras-Durand *et al.* (2008) and Desnoyers *et al.* (2009). Recently, Desnoyers *et al.* (2009) undertook an extensive meta-analysis on the influence of yeast cultures, from a variety of commercial preparations, on ruminal fermentation parameters and performance in

dairy cattle. It was concluded that supplementation with *S. cerevisiae*-based yeast products lead to increases ($P < 0.05$) in ruminal pH (0.03 units) and tended ($P < 0.10$) to reduce ruminal lactic acid concentrations (0.9 mM) with these effects being shown to be more pronounced in dairy cattle fed diets composed of increasing levels of concentrate and consuming a higher level of DM. This study also reported that increasing levels of dietary NDF, lessened the positive pH response. The stabilising effect of yeasts on ruminal pH may be dependent on not only the diet composition but also the strain of yeast (McAllister *et al.*, 2011), for this reason the effect of yeasts on ruminal pH stabilisation has been variable. Several studies have reported no effect of yeast cultures on ruminal pH (Erasmus *et al.*, 1992; Putnam *et al.*, 1997; Robinson & Garret, 1999; Erasmus *et al.*, 2005; Hristov e*t al.*, 2009), whereas some studies have, unexpectedly, reported a decline in ruminal pH (Harrison *et al.*, 1988; Piva *et al.*, 1993). However, as detailed below, many have reported increases in ruminal pH upon yeast-based supplementation. Upon feeding a live yeast (*S. cerevisiae*, $10^{10}$ cfu/g DM) to early lactation dairy cows, Marden e*t al.* (2008) observed an elevated ruminal pH, strengthened reducing power of the ruminal milieu, and a 67 % reduction in mean lactic acid concentration ($P < 0.001$), overall illustrating the pH stabilisation effect of yeasts. These effects of live yeast were corroborated by Pinloche e*t al.* (2013) who reported increased ruminal pH, a reduction in the post-prandial pH decline, reduced redox potential of the ruminal milieu and reduced levels of lactic acid. Thrune e*t al.* (2009) fed an ADY (*S. cerevisiae*, $10^{10}$ cfu/d), to late-lactation dairy cows fed once daily and found mean, nadir, and maximum ruminal pH levels to be increased ($P < 0.05$) with supplementation, and possibly more importantly that the time ruminal pH was suboptimal, below 5.6, was reduced ($P < 0.05$). These results indicate that yeasts are capable of limiting the reduction in ruminal pH typically linked to the increased production of ruminal VFA's (Desnoyers e*t al.*, 2009).

Bacterial DFM's have been shown to reduce the time ruminal pH is below the threshold for SARA, to reduce lactic acid concentrations and to increase ruminal propionate concentrations, with the response being dependant on the particular species or combination of species utilised (Krehbiel e*t al.*, 2003). The LUB, *M. elsdenii* has been shown to lessen fluctuations in ruminal pH and reduce the time ruminal pH is below 5.6 (Aikman *et al.*, 2011). Because this bacterial species can utilise lactate, its accumulation is limited and significant reductions in ruminal pH are averted (Seo *et al.*, 2010). Various combinations of bacterial DFM's, or bacterial DFM's and yeasts have been studied with varying responses. In a mid-lactation dairy study, Raeth-Knight e*t al.* (2007) did not observe a change in mean ruminal pH when feeding a combination of *Lactobacillus acidophilus* and *P. freudenreichii*. However, upon feeding a combination of *Lactobacillus plantarum* and *Enterococcus faecium* Nocek *et al.* (2002), reported an increase in the pH nadir within the typical diurnal cycle or ruminal pH. As a side note, when feeding a combination of LUB and LAB species one must keep in mind the individual characteristics of each bacterial strain with regard to the prevailing microbiome (Krehbiel e*t al.*, 2003). In a Latin square design, Chiquette *et al.* (2015) evaluated the effect of *E. faecium* alone or in combination with either *S. cerevisiae* or *Lactococcus lactis* during adaptation and induced SARA. Results showed that cows supplemented with a combination of either *E. faecium* and *S. cerevisiae* or *L. lactis* were superior to *E. faecium* alone in maintaining greater mean ruminal pH values during both adaptation and SARA challenge. Fungal DFM's based on *A. oryzae* are reported to give rise to a more stable ruminal milieu, however, most studies observe no beneficial effect of supplementation on ruminal pH (Yoon & Stern, 1996; Higginbotham e*t al.*, 2004).

Supplementation of various DFM's on total and individual ruminal VFA concentrations, as well as VFA patterns has been highly variable. The meta-analysis of Desnoyers *et al.* (2009) found yeast supplementation to increase total rumen VFA concentrations on average by + 2.17 mM ($P < 0.05$), with the positive effect increasing with increased DMI and dietary CP levels, but to have no influence on the ratio of acetate to propionate. Yeast supplementation has often been shown to have no effect on total VFA concentrations (Piva *et al.*, 1993; Putnam *et al.*, 1997; Hristov *et al.*, 2009). However, in accordance with Desnoyers *et al.* (2009) a rise in total VFA concentration has been observed both *in vitro* (Sullivan & Martin, 1999; Miller-Webster *et al.*, 2002) and *in vivo* (Enjalbert *et al.*, 1999; Marden *et al.*, 2008). The mechanisms by which VFA production is enhanced with yeast supplementation are yet to be fully elucidated, but appear to be associated with changes in the ruminal microbial population (Poppy e*t al.*, 2012), more specifically the increased activity of the anaerobic microflora (Newbold e*t al.*, 1996). The ruminal acetate to propionate ratio is often decreased with yeast supplementation, owing primarily to the increase in propionate (Harrison *et al.*, 1988; Enjalbert *et al.*,

1999; Erasmus *et al.*, 2005) with either a reduction in acetate (Harrison e*t al.*, 1988) or no change (Enjalbert e*t al.*, 1999). The other ruminal VFA's vary in their response to supplementation but often no change is observed (Carro *et al.*, 1992; Thrune *et al.*, 2009; Hristov *et al.*, 2009). Both bacterial (Raeth-Knight *et al.*, 2007; Chiquette *et al.*, 2015) and fungal (Higginbotham *et al.*, 1994; Yoon & Stern, 1996; Higginbotham *et al.*, 2004) based DFM's have been shown to have little effect on total VFA concentrations or the proportion of individual VFA's, however, bacterial DFM's based on *M. elsdenii* and Propionibacterium species (Seo *et al.*, 2010) seem to alter fermentation patterns in favour of propionate, as evidenced by a reduction in the acetate to propionate ratio (Aikman e*t al.*, 2011; Weimer *et al.*, 2015).

Typically, supplementation with DFM's leads to no change or a reduction in ammonia-N concentrations. Erasmus e*t al.* (1992) observed a 10 % decline in ruminal ammonia- N concentrations when feeding a yeast culture to lactating Holstein dairy cows, others to report a decline include Harrison *et al*. (1988); Enjalbert *et al.* (1999) and Malekkahi *et al.* (2016). Whereas many have reported no change in ruminal ammonia-N in response to yeast supplementation (Putnam *et al.*, 1997; Erasmus *et al.*, 2005; Thrune *et al.*, 2009). Bacterial DFM's do not typically affect ammonia-N as observed by Raeth-Kinght *et al.* (2007), the same can be said for fungal DFM's which are usually unable to elicit a response (Yoon & Stern, 1996; Higginbotham *et al.*, 2004). However, fungal DFM's based on *A. oryzae* do have a tendency to increase ruminal ammonia-N levels due to their inherent proteolytic activity (Arambel e*t al.*, 1987).

Overall results of supplementation with yeast and bacterial DFM's suggest an improvement in ruminal fermentation (Desnoyers *et al.*, 2009; Kalebich & Cardoso, 2017).

### 2.9.5 Effects on digestibility

On the whole, feeding lactating dairy cattle DFM's is alleged to improve nutrient digestibility (Kalebich & Cardoso, 2017), however, this beneficial effect is not consistently reported. A comprehensive meta-analysis on commercial yeast cultures determined that yeast supplementation increased ($P < 0.01$) total tract OM digestibility (TTOMD) by 0.8 %, on average, with this positive effect increasing with the percentage of concentrate and NDF in the ration (Desnoyers *et al.*, 2009). Others have reported improved TTOMD (Marden e*t al.*, 2008) and ruminal OM digestibility (OMD) (Yoon & Stern, 1996), as well as improved digestibility of the fibre fractions (Erasmus *et al.*, 1992; Marden e*t al.*, 2008) and CP (Erasmus e*t al.*, 1992; Yoon & Stern, 1996). However, many have failed to observe an effect of yeast supplementation on either total tract or ruminal nutrient digestibility (Harrison *et al.*, 1988; Putnam *et al.*, 1997; Hristov e*t al.*, 2009). The study of AlZahal e*t al.* (2014) showed that by feeding transition cows a combination of *E. faecium* and *S. cerevisiae*, starch digestibility could be improved upon. The effect of bacterial DFM's on nutrient digestibility has been mixed, with Nocek e*t al.* (2002) reporting that feeding a combination of *L. plantarum E. faecium* increased the rate of DM digestibility (DMD) of specific ingredients, whereas Raeth-Knight *et al.* (2007) observed no change in the apparent digestibility of DM, CP, NDF or starch when feeding a combination of *L. acidophilus* and *P. freudenreichii*. Fungal based DFM's typically increase the cellulolytic bacterial population within the rumen (Yoon & Stern, 1996), thus one would expect an increase in fibre digestibility when feeding these additives, but often only a minimal improvement in nutrient digestibility is observed (Takiya e*t al.*, 2017).

### 2.9.6 Effects on nitrogen and energy utilisation

As previously detailed depending on the species or combination of species, DFM's can enhance ruminal VFA production and more specifically propionate production. Lactic acid utilising bacterial species, particularly *Propionibacterium* species, are able to reduce lactic acid concentrations within the rumen, by metabolising this substrate to generate greater concentrations of propionate (McAllister e*t al.*, 2011) thus enhancing the level of glucogenic fuel provision. This is of particular benefit to dairy cows in early lactation as at this point in the lactation cycle propionate serves as the primary precursor for gluconeogenesis (Reynolds e*t al.*, 2003). This results in an enhancement of hepatic glucose production (Stein *et al.*, 2006) and ultimately an increase in the efficiency of energy utilisation (Krehbiel *et al.*, 2003; Seo e*t al.*, 2010). Furthermore, by increasing ruminal propionate concentrations there is a reduction in free hydrogen available for methanogenesis (Seo *et al.*, 2010), thus reducing the energy loss to this wasteful process (Kalebich & Cardoso, 2017) and promoting energy retention (Wolin, 1960; Luan *et al.*, 2014).

Information on the effects of DFM's on ruminal N metabolism and the utilisation of N are on a whole limited and often contradictory. Research based on ADY suggest that these products are capable of altering the N metabolism of ruminal microbes (Chaucheyras-Durand *et al.*, 2008). As previously discussed, various DFM's have been shown to increase ruminal microbial populations, particularly the fibrolytic and cellulolytic populations, these bacterial species have a high preference for ammonia as their N source (Bryant 1973). Thus, any DFM which enhances the growth of these bacterial species, would likely increase the overall utilisation of ruminal ammonia resulting in the more efficient conversion of ruminal ammonia-N into microbial protein thereby enhancing the overall utilisation of dietary N and decreasing urinary N losses (Hristov *et al.*, 2009).

### 2.9.7 Effects on animal productive performance and health

Direct-fed microbials have been reported to improve DMI and enhance milk productive performance in dairy cows, and to increase feed efficiency and average daily gains in feedlot cattle (Seo e*t al.*, 2010; Krehbiel *et al.*, 2003). However, the observed effects on animal performance have been inconsistent.

Comprehensive meta-analyses on the influence of *S.* cerevisiae-based yeast cultures on productive performance in dairy cows have concluded that these DFM's are capable of increasing DMI. Desnoyers *et al.* (2009) reported a 0.44 g/kg BW increase in DMI, whilst Poppy e*t al.* (2012) observed an increase of 0.62 kg/d in early lactation cows. Various mechanisms have been proposed to explain how yeasts may stimulate DMI, with the oldest being that yeasts may be able to grow for a short period of time within the rumen thus enhancing fibre digestion and DMI (Fuller, 1989). The positive effect of yeast supplementation on DMI tends to increase linearly with yeast dose and is also increased by the proportion of concentrates in the diet (Desnoyers *et al.*, 2009). The positive effect of yeast based DFM's on DMI is more pronounced in studies with early-lactation cows, with increases of 1.2 kg/d; 1.4 kg/d; and 0.9 kg/d observed by Williams *et al.* (1991); Erasmus *et al.* (1992) and Putnam e*t al.* (1997), respectively. Research with mid- to late-lactation cows, generally results in no change in DMI (Erdman & Sharma, 1989; Piva *et al.*, 1993) with the meta-analysis of Poppy e*t al.* (2012) identifying a significant decline in DMI in late-lactation cows of 0.78 kg/d. Fungal DFM's can potentially improve DMI through the stabilisation of the ruminal environment and increases in fibre digestion (Williams & Newbold, 1990) however, this effect is not often observed in studies with most being unable to detect an effect on DMI (Sievert & Shaver, 1993; Takiya *et al.*, 2017). The effect of bacterial-based DFM's on DMI are unclear, with a lack of effect being typically observed (Raeth-Knight e*t al.*, 2007). Two of three studies each evaluating the effect of various combinations of *S. cerevisiae* and strains of *E. faecium* have observed no effect of DFM supplementation on DMI (AlZahal *et al.*, 2014; Chiquette *et al.*, 2015), whereas Nocek e*t al.* (2003) did observe an increase ($P < 0.05$) in DMI in the post-partum period.

Supplementation with DFM's generally has no effect on BW, body weight change or body condition, with the exception that owing to an increase in DMI in the early post-partum period, body energy reserves are utilised less rapidly (Dann *et al.*, 2000) thus reducing the substantial BW losses typical of this stage of lactation.

Various meta-analyses have shown a positive effect of yeast based DFM's on milk productive performance. The extensive study of Desnoyers *et al.* (2009) analysed findings from 110 research papers evaluating at least 11 different commercial preparations of yeast cultures, and found supplementation to increase milk yield by 0.8 kg/d. The more selective reviews of Erasmus & Robinson (2009) and Poppy *et al.* (2012) reported an increase in milk production of 0.9 kg/d and 1.18 kg/d, respectively. Poppy *et al.* (2012) also observed a 1.61 kg/d increase in 3.5 % FCM and 1.65 kg/d increase in ECM ($P < 0.01$). The work of Desnoyers *et al.* (2009) identified that milk yield tended ($P < 0.10$) to increase linearly with yeast dose, and that the influence of supplementation on milk production increased with DMI, and the proportion of dietary concentrates, but was supressed with increased dietary levels of NDF, ADF and CP (Erasmus & Robinson, 2009). Whilst many individual studies have shown significant increases in milk production with yeast supplementation (Williams *et al.*, 1991; Piva *et al.*, 1993; Putnam *et al.*, 1997) and with supplementation of a DFM containing both yeast and bacterial strains (Nocek e*t al.*, 2003; Leicester e*t al.*, 2016), variation in results do exist. Some researchers have failed to observe a significant increase in milk production upon feeding yeast based DFM's (Erdman & Sharma, 1989; Swartz *et al.*, 1994; Hristov e*t al.*, 2009), a combination of yeast and *E. faecium* (AlZahal *et al.*, 2014) or a bacterial DFM comprised of *Lactobacillus* and *Propionibacteria* (Raeth-Knight *et al.*, 2007). Lactating dairy cows fed yeast cultures have been shown to reach peak milk yield earlier

(Wohlt *et al.*, 1991; Dann *et al.*, 2000), however this has not always translated into an improvement in overall milk production for the respective lactation (Dann *et al.*, 2000). An additional benefit of supplementation with DFM's may be the potential to reduce the decline in milk yield observed with cows experiencing SARA, as evidenced by Chiquette *et al.* (2015) who when supplementing cows with either *E. faecium* alone, *E. facecium* and *S. cerevisiae*, or *E. faecium a*nd *L. lactis*, observed a decrease in the decline of milk production by 0.9 kg/d, 0.8 kg/d and 0.9 kg/d versus the control (-7.5kg/d), respectively. Overall, results seem to indicate that feeding DFM's to lactating dairy cattle may lead to an improvement in milk production (Kalebich & Cardoso, 2017).

Supplemental DFM's may be beneficial to milk production, however, changes in milk composition are variable and typically minor (Krehbiel e*t al.*, 2003). The study of Desnoyers e*t al.* (2009) reported that yeast cultures tend ($P < 0.10$) to increase milk fat content but that milk protein content was unaffected, whist Poppy *et al.* (2012) was unable to detect any significant effects of supplementation on milk fat yield or milk protein yield. Despite these meta-analyses and the study of Swartz *et al.* (1994) which evaluated the effects of yeast cultures under various nutritional management programs of seven farms and found no response of milk components, some have observed alterations in milk composition. Upon feeding a DFM based on *S. cerevisiae*, *A. oryzae* and *Bacillus subtilis* fermentation extract to early-lactation dairy cows, Leicester *et al.* (2016) observed an increase in milk fat content, milk true protein content and lactose, whilst Nocek *et al.* (2003) upon feeding a combination of *E. faecium a*nd *S. cerevisiae* both pre- and post-partum found supplementation to increase the concentration of milk protein. Higginbotham *et al.* (1993) fed *A. oryzae* extracts to mid-lactation dairy cows and observed an increase in milk protein content and the concentration of SNF, which was confirmed in a later study utilising early-lactation cows in which milk protein content and yield were increased (Higginbotham *et al.*, 2004). From this information one can postulate that DFM's have the potential to enhance milk protein content, whilst having no effect on other milk components (Kalebich & Cardoso, 2017).

Direct-fed microbials are proposed to increase production efficiency of dairy cows and this is likely brought about through a number of interacting factors (McAllister *et al.*, 2011). The efficiency of milk production in response to yeasts, is likely enhanced by the reported increase in milk production or the decrease in DMI observed in late lactation (Poppy *et al.*, 2012). Another means by which the efficiency of production is increased is through the stabilising effect of DFM's, particularly *A. oryzae* based, on the ruminal milieu, which increases fibre digestion and ultimately the energy available from the diet for productive purposes (Williams & Newbold, 1990). Although limited there is evidence to suggest that both yeast products (McAllister e*t al.*, 2011) and bacterial based DFM's (Seo *et al.*, 2010) are capable of improving production efficiency.

In addition to production benefits, DFM supplementation has also been shown to give rise to various health benefits. Dietary inclusion of DFM's may improve general health, reduce the severity of nutritional disorders, such as SARA (Chiquette *et al.*, 2015) and ketosis (Nocek *et al.*, 2003), and enhance the immune system (Kalebich & Cardoso, 2017). The study of Nocek e*t al.* (2003) evaluated the effects of supplementation with a combination of *E. facecium* and *S. cerevisiae* and found increased concentrations of blood glucose and insulin and decreased levels of NEFA, these results are indicative of a reduction in fatty acid mobilisation and an increase in energy derived from dietary sources, and may lead to improvements in the health status of transition cows, in particular.

### 2.9.8 Bottom-line

Despite the positive responses to DFM supplementation observed in lactating dairy cattle, the basic mechanisms are ill-defined and more importantly positive production responses are neither predictable nor consistent. Variation in responses across studies may be due to factors such as method of administration, species of DFM, concentration of the microbial inoculant within the DFM, physiological status, degree of nutritional stress, and diet composition (Kalebich & Cardoso, 2017). Development of DFM's which are efficacious over a wide range of ruminant production systems is currently a challenge, owing to the lack of knowledge of the microbial communities inherent to the gastrointestinal system. Advancements in the fields of metagenomics and transcriptomics will potentially provide new insights into how these additives modify the ecology of the microbiome found within the entirety of the gastrointestinal tract. Thereby leading to

enhancements in our understanding of the mode of action, and ultimately allowing for the selection and development of DFM's which are more effective at optimising productive performance and improving the health status of production animals (Krehbiel *et al.*, 2003; McAllister e*t al.*, 2011).

## 2.10 Conclusion

From this discussion it is clear that energy and protein metabolism in lactating dairy cows is not a simple concept, with the microbial community playing a crucial role in the nutrition of these animals. For this reason, it is vital that research focuses on the effects of the various rumen modulators (i.e. feed additives) which are used widely within the industry, on parameters associated with N and energy metabolism. Particularly research needs to focus on the effects these additives have on the ruminal synthesis and flow of MCP, the AA composition of this protein source, differential flow of microbial fractions and the effect on microbial populations, as currently there is a gap in knowledge in this area of research, even for the extensively researched ionophores. Such research will allow for the integration of new knowledge into nutritional models allowing for improvements in the accuracy and precision with which nutrient supplies and requirements are predicted. This review also highlights the lack of consistency in the outcomes of feeding dietary additives on ruminal fermentation, digestibility and production, particularly for DFM products owing to the highly variable composition these products, thus illustrating the need for further investigation and validation of results *in vivo* to determine their efficacy in practical farm situations.

# CHAPTER 3

# METHODOLOGICAL REVIEW OF BACTERIAL ISOLATION

## 3.1 Introduction

As discussed, the rumen is a complex microbiome, consisting of bacteria, protozoa and fungi, and knowledge of the extent of ruminal microbial flow is imperative to our understanding of microbial digestion in the rumen and the role of these microbes in ruminant nutrition. The N- feed evaluation systems for ruminants rely on the ability to estimate ruminal MPS, the ruminal degradability of dietary protein and the relative contribution of these protein sources to the duodenal digesta. Ruminal MPS is primarily estimated by microbial markers which may be naturally occurring constituents of the microbes, i.e. DAPA or nucleic acids, or radioisotopes which are incorporated into the microbes during growth, i.e. $^{15}N$ or $^{35}S$ (Stern & Hoover, 1979). To determine the ruminal MCP flowing to the lower gastrointestinal tract, bacteria must be harvested and analysed for the marker to NAN ratio. Typically, free-floating microbes are isolated and across literature there is a wide variation in the methods used for the isolation of ruminal microorganisms from whole rumen contents (WRC). The following serves as a brief review of the literature, in order to justify the methodology behind the bacterial isolation procedure followed in this research trial.

## 3.2 Isolation of mixed rumen bacteria versus fluid- and particle-associated bacteria

Common across research is the use of either mixed rumen bacterial isolates or fluid-associated bacterial isolates to represent the total bacterial population both found in and leaving the rumen. The reason for this is due to the ease of rumen fluid sampling and the subsequent isolation of FAB or mixed bacteria from the liquid digesta, as compared to the multifarious methods required to dislodge and recover good preparations of PAB (Sok *et al.*, 2017). A common assumption also exists that the chemical composition of bacteria associated with the fluid phase and that of the solid phase are identical, this is used as a justification for merely isolating the bacteria from the liquid phase. However, it is recognised that the chemical composition of the bacteria associated to each phase does in fact differ (Merry & McAllan, 1983; Legay-Carmier & Bauchart, 1989). Additionally, research has shown that bacteria are clearly associated with two phases in the rumen, being the liquid and solid phase, and that the bacteria associated with the solid (i.e. particulate) phase represents the most prevalent fraction of bacteria in ruminal contents, up to 70 % to 80 %, (Forsberg & Lam, 1977; Craig *et al.*, 1987a) and subsequently the duodenal digesta (Faichney, 1975; Reynal & Broderick, 2005; Brito *et al.*, 2007ab). For these reasons the current assumption that FAB can be used to represent the entire microbial population flowing from the rumen to the duodenum is invalidated (Martín – Orúe *et al.*, 1998), thus future research should be focused on the isolation of rumen microorganisms from both the liquid and solid phases of the rumen, in order to obtain a true representation of the total bacterial population.

## 3.3 Sample collection, filtration and fraction separation

Approaches to obtaining rumen samples are known to be limited, methods used in literature include ruminocentesis, orogastric collection (i.e. oesophageal tubing) and sampling directly via a rumen cannula. The limitation of the first two methods being 1) only rumen fluid samples can be obtained and 2) the samples obtained are typically not representative of the entire rumen, not to mention that each of these methods requires a certain level of skill in order to be carried out without harming the animal. The simplest way of obtaining a rumen content sample is by sampling via a rumen cannula, here two options are available; the first being the use of a vacuum pump and the second manual sampling by placing one's hand directly into the rumen. The limitation of the vacuum pump method is that one mainly collects rumen fluid, and not solid digesta, giving rise to an unrepresentative sample (Taljaard, 1972). To ensure that a truly representative sample of whole rumen contents will be obtained the sampling method of Schwab *et al.* (2006) should be followed, in which samples of rumen contents are collected from nine locations within the rumen, including three samples from each the dorsal, medial, and ventral area. These samples are obtained by forcing a plastic beaker into the designated area, mixing the contents in the particular area, filling the beaker and removing the sample from the rumen, where after it is placed into a common collection container.

Primary filtering to separate the WRC sample into liquid and solid fractions is primarily done by straining the sample through fabric, this allows the particulate matter to be retained on the cloth whilst allowing the liquid fraction to filter through. Typically, cheesecloth is used to strain WRC, although nylon and dacron mesh (Schwab *et al.*, 2006) are also commonly used, the layers of cheesecloth used vary from two layers (Reynal & Broderick, 2005), four layers (Shabi *et al.*, 2000; Fessenden *et al.*, 2017) and eight layers (Whitehouse *et al.*, 1994). Some researchers also include additional layers of fabric with a smaller aperture to serve as a trap for smaller particulate matter which could easily filter through conventional cheesecloth, nylon with an aperture of 50 µm or 59 µm is often used (Martín – Orúe *et al.*, 1998). After this initial filtering one is left with the liquid fraction, which is ready for the isolation of the FAB, and a solid "digesta" fraction which must still undergo further treatment before the PAB can be successfully isolated.

### 3.4 Isolation from fresh versus previously frozen samples

Typically, rumen bacteria are isolated from fresh rumen samples, although some researchers have successfully isolated rumen bacteria from previously frozen rumen fluid samples (Erasmus *et al.*, 1992). Research into the effects of isolating from fresh versus previously frozen rumen samples has been done but the results have been conflicting. Cecava *et al.* (1990) reported that pre-isolation freezing had no effect on the composition of the isolated rumen bacteria nor did it have an effect on the estimation of bacterial N flows to the small intestine. However, Merry and McAllan (1983) reported that pre-isolation freezing lead to significant losses of RNA, this was confirmed by Hsu & Fahey (1990) who also reported that pre-isolation freezing lead to differences in all measured parameters when compared to fresh samples. Pre-isolation freezing can also result in a non-representative bacterial sample being isolated, as freezing can alter the physical structure of membrane lipid components (Strange, 1976). The swelling of water volume upon freezing can induce an immense turgor force which would be destructive, especially to gram-negative bacteria which are known to be more susceptible to changes in osmolality due to their cell wall structure (Mackie & Theron., 1984). For this reason harvesting rumen bacteria from fresh ruminal contents appears to be the most suitable option for obtaining a representative bacterial sample.

### 3.5 Isolation of the ruminal bacteria

Differential centrifugation is the simplest form of separation by centrifugation, and is typically used as the primary method for the isolation of bacteria. In brief, when a suspension containing a variety of cells is subjected to a series of increasing centrifugal force cycles a series of pellets will be obtained. This is due to particles of different densities and/or sizes, within a suspension, having different sedimentation rates. Larger, denser particles (i.e. protozoa and small feed particles) will sediment faster than smaller, less dense particles (i.e. bacteria) which require higher centrifugal forces in order to be pelleted. The main issues observed with differential centrifugation are contamination and poor recoveries, due to the heterogeneity of biological particles. The foremost approach to addressing contamination by different particles types is through the re-suspension of the pellet in a suitable rinse solution and repeated centrifugation cycles, which is referred to as washing the pellet (Graham, 2002).

Across literature wide-ranging centrifugal forces and times are applied to pellet bacteria, with no specific reason being given. At present it is clear that there is no consensus as to the ideal centrifugal force which suspensions should be subjected to in order to pellet bacteria.

Following the principle of differential centrifugation, suspensions (i.e. ruminal fluid and "wash solution") are subjected to an initial slow spin so as to pellet the protozoa and contaminating small feed particles remaining in the sample. A range of centrifugal forces have been applied to samples in order to achieve this, with forces as low as 150 x g, for 10 minutes (Hsu & Fahey, 1990) to those as high as 3,000 x g, for 25 minutes (Yáñez-Ruis *et al.*, 2006) having been used. Most researchers have utilised intermediate speeds, centrifuging at lower speeds of 500 x g, for 5 (Martín-Orúe *et al.*, 1998), 10 (Cecava *et al.*, 1990; Whitehouse *et al.*, 1994) or 15 minutes (Shabi *et al.*, 2000), and at higher speeds of 1,000 x g for 5 (Reynal & Broderick 2005; Fessenden *et al.*, 2017) or 10 minutes (Erasmus *et al.*, 1992; Martin *et al.*, 1994), with various other intermediate speeds and times being reported. Some researchers have repeated this initial spin to ensure that the clarified rumen fluid was free from any contaminating protozoa or small feed particles (Smith & McAllen,

1974; Cecava *et al.*, 1990; Boguhn *et al.*, 2006), however, most found a single initial, slow speed spin to be sufficient.

Following the initial spin, samples typically undergo two consecutive centrifugation cycles, the first being that of the clarified supernatant in order to pellet the bacteria, and the second of the bacterial pellet re-suspended in rinse solution, in order to remove any contaminants. Again, large variation is observed across studies in the centrifugal force and time applied to obtain the bacterial pellet. In some studies bacteria was pelleted at forces as low as 10,300 x g, for 20 minutes (Yang & Russel, 1993b) and others as high as 30, 000 x g for 15 (Smith & McAllan, 1974; Miller-Webster *et al.*, 2002; Vlaeminck *et al.*, 2006), and 20 minutes (Shabi *et al.*, 2000). Typically, intermediate forces of 15,000 x g, 20 minutes (Martin *et al.*, 1994; Fessenden *et al.*, 2017) and 20,000 x g, for 10 (Whitehouse *et al.*, 1991; Schwab *et al.*, 2006) or 20 minutes (Erasmus *et al.*, 1992; Martín-Orúe *et al.*, 1998) are used to pellet bacteria.

The reasoning behind the wide variation in centrifugal forces applied to rumen samples for the isolation of bacteria is unclear. Ruminal bacterial sizes approximate those of the eukaryote ranging from 0.2 µm to 5 µm, however, the range of centrifugal forces used for pelleting mitochondria is much narrower, 10,000 x g (Kimball, 1984) and 20,000 x g (Sheeler & Bianchi, 1980), for 15 minutes, than that used for ruminal bacteria (Hsu & Fahey, 1990). The concern is that the use of unnecessarily high centrifugal forces, increases the risk of obtaining an unrepresentative sample, i.e. intact bacterial samples contaminated by bacterial membranes or cell wall debris with the loss of cell contents of broken cells into the supernatant (Hsu & Fahey, 1990). Research into the effects of centrifugation speeds on the composition of ruminal bacterial samples was carried out by Hsu & Fahey (1990), with speeds of 4,640 x g, 30 min; 9,520 x g, 20 min, 25, 900 x g, 20 min being tested. This study reported that centrifugation speed did not affect either the quantity or chemical composition of the ruminal bacteria harvested, but upon microscopic examination it was observed that the bacterial samples subjected to higher centrifugal forces had greater contamination with cell debris. For this reason, Hsu & Fahey (1990) recommended pelleting bacteria using lower centrifugation speeds for a longer period of time, and suggested the conservative 4,640 x g for 30 minutes, to reduce the risk of cellular damage whilst ensuring sufficient bacteria is pelleted. This recommendation does not align with the typical centrifugation speeds utilised across research in animal science, with most researchers continuing to isolate bacterial fractions at the higher speeds of 15,000 x g and/or 20,000 x g, for 20 minutes.

Often an additional centrifugation cycle with distilled water is reported, with the aim of removing any residual salts which may be present and could affect ash analysis (Smith & McAllan, 1974; Putnam *et al.*, 1997; Boguhn *et al.*, 2006). Thereafter the bacterial pellet is stored frozen as is, or it is re-suspended in distilled water (Miller-Webster *et al.*, 2002; Fessenden *et al.*, 2017) and stored frozen at – 20 ºC.

Before the second (i.e. “rinse” step) centrifugation cycle, the bacterial pellet is re-suspended in a rinse solution. The most common solution used is 0.85 % (Whitehouse *et al.*, 1991; Martín - Orúe *et al.*, 1998; Fessenden *et al.*, 2017) or 0.9 % (Smith & McAllan, 1974; Shabi *et al.*, 2000; Boguhn *et al.*, 2006) saline solution. But other solutions have been used such as McDougall’s buffer (Reynal & Broderick, 2005) and Coleman’s buffer solution (Martin *et al.*, 1994).

## 3.6 Detachment procedures to obtain particle-associated bacteria

As previously mentioned, additional steps are required to dislodge and recover bacteria associated with the solid digesta of the rumen. A variety of both chemical and physical methods have been experimented with and are discussed below.

### 3.6.1 Chemical treatments

A variety of chemical treatments have been investigated, either alone or in combination with other chemical treatments in solution. Chemical treatments include the use of formaldehyde, surfactants, alcohols, low pH and cellulose powders. Although all have shown to be efficacious in dislodging adherent bacteria some have been shown to be more suitable than others, due to lack of interference with the chemical composition of the bacteria.

Several published methods have employed the use of formaldehyde (HCHO) in either the isolation or storage of microbial samples (Martin *et al.*, 1994; Rossi *et al.*, 2013; Fessenden *et al.*, 2017), however, it has been shown that the use of formaldehyde has adverse effects on microbial samples, specifically it alters the protein fraction and reacts with the AA's present (Barry 1976), regardless of bacterial type (Sok *et al.*, 2017). Volden & Harstad (1998) reported that the addition of formaldehyde to ruminal samples significantly decreased the ratio of true protein relative to CP, averaging 71.0 % *versus* 82.4 % ± 3.7 % (SEM) with and without formaldehyde, respectively. More specifically this work noted that the use of formaldehyde altered the concentrations of AA's, with the later work of Volden *et al.* (1999) reporting lower recoveries of the AA's; Lys; Met and Tyr in both FAB and PAB when formaldehyde was included in the treatment. This confirms the earlier works of Gruber & Mellon (1968), Stern *et al.* (1983) and Whitehouse *et al.* (1994) who reported that the addition of formaldehyde lead to a 100 % loss of Tyr, with one third of both Cys and His, and smaller amounts of Glu and Lys not being detected under hydrolysis conditions; that storage of WRC in 7.4 % (wt/vol) formaldehyde lead to lower concentrations of Lys and Tyr; and that when included in detachment solutions even at levels as low as 0.5 % (wt/vol) formaldehyde reduced the concentrations of Lys and Tyr in PAB by 8.9 % and 19.8 %, respectively. In addition, formaldehyde is known to reduce DM, OM, and N digestibility of isolated microbial cells (Fessenden *et al.*, 2017). Barry (1976) stated that formaldehyde readily reacts with the proteins present thus rendering the sample resistant to digestion. It is clear that Lys and Tyr are the two most severely affected AA's; it has been suggested that the phenol group of Tyr, and the epsilon group of Lys may react with formaldehyde, forming cross-links within the protein, thus rendering these AA's resistant to standard acid hydrolysis (Barry, 1976). For these reasons the use of formaldehyde in the isolation of microbial cells has been discouraged.

Alcohols, namely methanol and tertiary butanol, have been investigated as detaching agents due to their reported ability to remove the bacterial polysaccharide capsule (Barr *et al.*, 1975). Whitehouse *et al.* (1994) investigated various chemical methods for dissociating PAB from digesta residue, and found that the addition of 1.0 % methanol to the treatment solution increased the removal of PAB, from 50 % to 70 % and 60 % to 72 % in two experiments and was necessary for the complete dissociation of large coccal chains and motile spirochetes from digesta. Whitehouse *et al.* (1994) also investigated the efficacy of the inclusion of 1.0 % methanol in treatment solution with a pH less than 2.0, and shaking of the suspension with marbles (to mimic pummelling), and concluded that although the inclusion of 1.0% methanol enhanced the removal of PAB there was no appreciable benefit of shaking the suspension or the use of low pH. Tertiary butanol has been suggested as an alternate to methanol due to its decreased toxicity and detergent- like properties. Although Martín-Orúe *et al.* (1998) observed no effect of the addition of tertiary butanol to the basal treatment (i.e. cooling plus homogenisation) others have reported decreases in bacterial attachment (Fletcher, 1983) and increased removals of PAB, from 72 % to 82 %, with the addition of tertiary butanol to a treatment solution of pH 2.0 containing 0.1 % Tween 80 and 1.0 % methanol (Whitehouse *et al.*, 1994). A major concern with the use of alcohols is the ability of the alcohol to react with the lipid structure of cellular membranes, breaking non-covalent bonds in the hydrophobic regions of lipids, leading to the disorganisation of the lipid structure (Harold, 1970). This is likely to result in cell leakage or cell lysis of both adherent and free- floating bacteria, if the loss of cellular contents is significant the chemical composition of the bacterial pellet could be altered due to the disproportionality between cell wall and cytoplasm content.

Tween (Polysorbate) 80, is a surfactant commonly used in microbial isolations (Whitehouse *et al.*, 1994; Reynal & Broderick, 2005; Schwab *et al.*, 2006) as it is known to reduce bacterial attachment to a variety of surfaces (Brandl & Huynh, 2014). The inclusion of Tween 80 in treatment solutions has been shown to increase total bacterial counts to the same extent as chilling (Dehority & Grubb, 1980) thus increasing the quantity of PAB isolated (Whitehouse *et al.*, 1994). Tween 80 acts by increasing wettability (Dehority & Grubb, 1980; Merry & McAllan, 1983) and breaking surface tension, thereby allowing aqueous solutions to more effectively remove the adherent, colonising bacteria from the particulate matter (Brandl & Huynh, 2014).

Methylcellulose, has more recently become a common choice as a detachment agent (Fessenden *et al.*, 2017) owing to its ability to inhibit adhesion leading to an increased detachment of PAB (Rasmussen *et al.*, 1989) without altering cell integrity and composition (Martín-Orúe *et al.*, 1998). The dilution of solid residues with treatment solutions containing as little as 0.1 % methylcellulose have been shown to be most effective at removing adherent bacteria. Martín-Orúe *et al.* (1998) reported that solid residues diluted in a treatment

solution comprised of 0.85 % saline solution and 0.1 % methylcellulose, showed the highest recovery of PAB and increased PAB removal over the basal treatment (cooling and homogenisation) from 60 % to 66 %, as determined by both purine bases and $^{15}N$ content. Whitehouse *et al.* (1994) observed that by subjecting digesta residue to an initial incubation in 0.1 % methylcellulose before further treatment with microbial free extraction solution (MFES), which is 1.0 % methanol and 0.1 % Tween 80 in pH 2.0 saline, lead to the complete removal of tetrads and cocci, and ultimately increased PAB removal from 62 % to 83 %, using purine bases as a microbial marker. These two studies validated earlier works which reported either increased or complete removals of bacteria with the inclusion of 0.1 % methylcellulose in the treatment solutions (Minato & Suto, 1978, 1981), with gram-negative rods appearing to be the most dominant bacterial group removed (Minato & Suto, 1981). It is not yet known if methylcellulose brings about its detachment effect via binding inhibition, competitive binding (Kudo *et al.*, 1987) or its surfactant qualities (Pell & Schofield, 1993).

Lowering the pH of the treatment solution has also been shown to aid in the detachment of adherent bacteria. Whitehouse e*t al.* (1994) experimented with the reduction of the pH of saline solution to below 2.0, with the addition of hydrochloric acid (HCl), prior to the addition of the treatment solution to the ruminal digesta contents and observed that the treatments with lower pH resulted in the greatest removals of PAB, 70 %, however, it was concluded that this result was most likely brought about by the inclusion of 1.0 % methanol in the treatment solution, and that no appreciable benefit came from the use of low pH.

### 3.6.2 Physical treatments

Various physical treatments have been investigated, either alone or in combination with other chemical or physical treatments. Physical treatments include cooling and storage, homogenisation, agitation, and changes in temperature, however, these treatments can be difficult to delineate from each other, and often overlap or are used in conjunction.

Cooling, storage, homogenisation, and agitation can be discussed as one, as these treatments are typically applied collectively as the basis of most isolation procedures. Cooling and homogenisation have been shown to increase the removal of PAB from ruminal digesta (Dehority & Grubb, 1980; Merry & McAllan, 1983) either by disrupting adherence or aiding the release of microbes trapped in the particles (Warner, 1962). Dehority & Grubb (1980) concluded that the chilling of ruminal contents significantly increased the removal of PAB, as shown by increased colony counts when ruminal digesta were cooled from 0 to 8 hours, owing to the breakdown of the material responsible for cell-to-cell and cell-to-digesta attachments. Homogenisation is applied either before (Cecava *et al.*, 1990; Schwab *et al.*, 2006; Fessenden *et al.*, 2017) or after chilling (Whitehouse *et al.*, 1994; Martín – Orúe *et al.*, 1998) and Merry and McAllan (1983) demonstrated that the inclusion of an initial homogenisation treatment, regardless of subsequent treatment, lead to enhanced removals of PAB from ruminal digesta without affecting the physical form or chemical composition of the bacteria. This particular study concluded that a combination of homogenisation and mechanical pummelling of the ruminal digesta was overall the most effective at removing adherent PAB. Mechanical pummelling, serves to agitate the particles with the aim of disrupting attachments so as to release adherent microbes, this agitation can be achieved in a variety of ways such as stomaching (Merry & McAllan, 1983; Vlaemink *et al.*, 2006), the use of shaking water baths (Pérez *et al.*, 1998; Fessenden *et al.*, 2017), or by shaking the sample with marbles (Whitehouse *et al.*, 1994), although the latter has not been shown to have a significant benefit. By making use of microbial markers, researchers have established that a combination of cooling and homogenisation leads to PAB removals of approximately 60 % (Martín-Orúe *et al.*, 1998) to 65 % (Merry & McAllan, 1983).

Changes in temperature, have been little researched as a means to dislodge adherent PAB from digesta although it has been suggested that adhesion can be significantly reduced by either the lowering of temperature to 4 ºC (Minato & Suto, 1978), or increasing it above 38 ºC (Pell & Schofield, 1993). Martín – Orúe *et al.* (1998) investigated this by subjecting the basal treatment (cooling and homogenisation) to additional heating in a 60 ºC water bath for 10 minutes, followed by cooling in an ice bath for 10 minutes. This procedure was repeated twice, while never allowing the temperature inside the bottle to drop below 37 ºC. Although this treatment increased removal of PAB to the same extent as the methylcellulose treatment, it also showed the lowest recovery of detached purine bases, thus suggesting an adverse effect of this treatment on cell integrity. Investigation of the microbial pellet reflected a disproportionality between cell wall and cytoplasm content,

thus the use of wide changes in temperature is advised against if one aims to isolate a representative particle-associated bacterial pellet.

It is important to note that there is a high variability in the recovery of PAB, with recoveries ranging from 20% (Martín-Orúe *et al.*, 1998) to 80% (Whitehouse *et al.*, 1994). This variation may to an extent be attributable to the detachment procedure, but regardless we do not succeed in isolating most of the particle adherent bacteria, with the main fraction of detached bacteria being lost throughout experimental procedures (Martín – Orúe *et al.*, 1998). Whitehouse *et al.* (1994) clearly demonstrated that the effects of the detachment procedures are additive, and that a combination of both physical and chemical methods will prove most effective at dissociating the majority of the adherent microbial fraction, without compromising microbial integrity. In this work removal efficiencies of up to 80 % were realised by two treatment procedures, in brief, the first procedure comprised of chilling, saline washed digesta residue at 4 ºC for 24 hours in a solution of pH 2.0 saline, 0.1 % Tween 80, 1 % tertiary butanol and 1 % methanol, followed by homogenisation. While the second procedure comprised of a 30 minute incubation of saline washed digesta residue in a 0.1 % methylcellulose solution, after which the residue was subjected to chilling at 4 ºC for 24 hours in a solution of pH 2.0 saline, 0.1 % Tween 80 and 1 % methanol, followed by homogenisation.

## 3.7 Conclusion

After reviewing the literature it is clear that there is no one correct way to isolate bacteria associated with the fluid- and solid – phases of the rumen. While isolation of the FAB is straightforward, a combination of both physical and chemical treatments is required in order to effectively detach the PAB. Based on the research presented one could safely recommend the incubation, with agitation, of saline washed digesta residue, in a 0.1 % methylcellulose solution, followed by chilling for 24 hours and subsequent homogenisation, as a detachment method which would provide sufficient recovery of PAB without the risk of compromising the structural integrity of the isolated bacteria. For the isolation of bacteria by differential centrifugation, moderate centrifugal forces should be applied, such as 500 x g, for 10 minutes, to remove contaminating protozoa and small particulate matter, and 15,000 or 20,000 x g, for 20 minutes to pellet the bacteria. Owing to the significant variation in the ability of detachment procedures to recover PAB, the ability of the recovered bacteria to truly represent the total adherent microbial population is called into question. One can assume that the isolation process would have varying effects on the bacterial species, and as such these techniques may lead to the selective enrichment of certain bacterial species, giving a PAB extract not fully representative of the adherent population. For this reason, strict standardisation of sampling and isolation techniques are required in the future to allow for the better comparison of data across studies.

# CHAPTER 4

# MATERIALS AND METHODS

## 4.1 Farm and management

The research trial was conducted at the University of Pretoria's Hatfield Experimental Farm, which is situated on the LC de Villiers Campus, Hatfield, Pretoria East (S 25° 45'10'' and E 28° 14'46''). Four ruminally cannulated lactating Holstein cows, 223 ± 6.19 (SEM) DIM and producing 28.8 ± 2.79 (SEM) kg milk/d, were used in the trial. Cows were selected for the trial from the dairy herd according to DIM, milk production and body weight.

Cows were housed individually and remained in their originally assigned pen for the duration of the trial. Pens were semi-open, measured 4.6 m x 11.6 m, and were bedded with dried manure solids. Pens were cleaned regularly, by removing excess manure build-up, whilst the cows were being milked so as to prevent any stress or disturbance to normal eating behaviour. Access to fresh, cool water was available *ad libitum.* As per normal farm practise, the cows were milked three times daily starting at 06:00, 12:00 and 19:00.

**Figure 4.1:** Photograph illustrating the partially covered pens cows were kept in for the trial

The study was reviewed and approved by the University of Pretoria's Animal Ethics Committee (AEC) before commencement of the trial (Project number: EC016-16 (Amend 1)). The cows involved in this study were cared for according to the guidelines for "The Care and Use of Animals for Scientific Purposes" as per the specifications set by the South African National Standard (SANS 10386-2008).

**Table 4.1** Information on experimental cows as of the start of the trial

| Cow Number | Cow I.D. | Body weight (kg) | Body condition score | Lactation number | Days in milk | Milk Production (kg/day) |
|---|---|---|---|---|---|---|
| 1 | 1404 | 579 | 3.25 | 1 | 238 | 33.5 |
| 2 | 1401 | 584 | 3.25 | 1 | 214 | 33.0 |
| 3 | 1303 | 586 | 2.75 | 2 | 232 | 29.0 |
| 4 | 1406 | 638 | 3.5 | 1 | 208 | 19.6 |

## 4.2 Experimental design and treatments

Cows were randomly allocated to treatments according to a 4 x 4 Latin Square design comprised of four dietary treatments and four experimental periods. This experimental design ensures that by the completion of the trial all cows have received all four treatments. The total duration of the trial was 100 days, which consisted of 4 experimental periods of 25 days each. Each 25 day period consisted of a 14 day adaptation period followed by an 11 day data collection period. According to Dr T.G. Nagaraja, a rumen microbiologist at Kansas State University (tnagaraj@vet.k-state.edu) and Dr Xandra Smith a microbiologist (xandra.smith@agro-biosciences.com) a two week adaptation period is sufficient to prevent any carry-over effects from the previous dietary treatment.

**Table 4.2** Assignment of treatments to cows, according to Latin Square design

| Period | C[1] | AB10[2] | DFM[3] | MON[4] |
|---|---|---|---|---|
| 1 | Cow 1 | Cow 2 | Cow 3 | Cow 4 |
| 2 | Cow 4 | Cow 1 | Cow 2 | Cow 3 |
| 3 | Cow 3 | Cow 4 | Cow 1 | Cow 2 |
| 4 | Cow 2 | Cow 3 | Cow 4 | Cow 1 |

[1] C = Control diet
[2] AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d
[3] DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d
[4] MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.

The four dietary treatments were comprised of the same basal diet, with the treatment differences being brought about by the inclusion of a feed additive. The dietary treatments were as follows:

Treatment 1: Control diet.

Treatment 2: Control diet plus the buffer Acid Buf 10 (Acid Buf plus Marine MgO), included as 3.33 g/kg DM of Acid Buf and 0.417 g/kg DM of MgO, or as a percentage of dietary DM as 0.33 % of Acid Buf and 0.042 % of MgO. Henceforth this product will be referred to as Acid Buf 10, which at a combined inclusion level of 0.375 % of dietary DM provided an intake of 90 g/d (Control + AB10).

Treatment 3: Control diet plus Achieve$^{FE}$ a DFM, this product was not included in the diet but rather placed directly into the rumen daily at 10 g/d (Control + DFM).

Treatment 4: Control diet plus Rumensin® 200, an ionophore product, included at 54.2 mg/kg DM (i.e. 54.2 ppm) to provide an intake of 1.3 g/d. Which equates to an intake of 260 mg/d of the active ingredient monensin sodium, as Rumensin® 200 contains only 20 % of the active ingredient (Control + MON).

The basal diet supplied was a TMR, typical of those fed in industry. The diet was formulated to be highly degradable, so as to promote the flow of microbial protein to the duodenum. The four dietary treatments were formulated, according to the CPM Dairy software database – version 3.0 (Boston *et al.*, 2000), to be both iso-calorific and iso-nitrogenous. It is of importance to note that in order for the dietary treatments to be formulated on an iso-basis for minerals, that treatment 2 (i.e. Control + Acid Buf 10) had no magnesium sulphate or limestone included as did the other dietary treatments.

The feed additives used in this study, were supplied to the animals according to the manufacturer's specifications and a predicted feed intake of 24 kg DM/d.

Acid Buf 10 was supplied by Celtic Sea Minerals (Strand Farm, Currabunny, Carrigaline, Cork, Ireland). As described earlier Acid Buf is a CMA, which comes from the skeletal remains of the seaweed, *Lithothamnium calcareum*. However, the Acid Buf 10 used in this study was comprised of both Acid Buf and marine MgO. The additive was fed according to specification, 80 g/day Acid Buf and 10 g/d marine MgO, summing to a total of 90 g/d of product.

Achieve$^{FE}$ was supplied by MicroBasics Inc. (1186 East 990 South Eden, Idaho, 83325, USA). This DFM is comprised of yeast culture (*S. cerevisiae* yeast grown in a medium of barley and cane molasses); dried *A. niger* and *A. oryzae* fermentation solubles; dried *B. subtilis*, *L. acidophilus*, and *E. faecium* fermentation products; and *Yucca schidigera* extract. The manufacturers specified that the product should not be included in the TMR, and should instead be given in a manner to ensure that the entire 10 g recommended dose was received daily. Various options were considered such as top dressing the product, dosing or placing directly into the rumen via the cannula. It was decided that the best option would be to place the product directly into the rumen daily, by pre-weighing the product and packaging it in small tissue paper parcels. A parcel was placed into the rumen via the cannula daily, directly after the morning milking, and the animal was fed the basal diet (i.e. Control diet).

The ionophore product used in this trial was Rumensin® 200, active ingredient monensin sodium, supplied by Elanco Animal Health, a division of Eli Lilly and Company (Greenfield, Indianapolis, IN 46285, USA). The manufacturer specifies that for optimum efficiency the active ingredient should be fed continuously to lactating dairy cows at a rate of 11 – 22 g/t TMR DM/d, this is equivalent to 250 mg/cow/d to 300 mg/cow/d of monensin sodium. The final dose decided upon for this trial was 260 mg/d of monensin sodium.

Experimental diets were blended by Pennville (Pty) Ltd. (Pretoria West, Gauteng). The two feed additives included in the feed (i.e. Acid Buf 10 and Rumensin$^{TM}$) were mixed into the vitamin-mineral premix, which was then blended into the TMR.

**Table 4.3** Ingredient composition of the control diet fed to experimental animals (g/kg DM)

| **Ingredient** (g/kg DM) | |
|---|---|
| Lucerne Hay (A grade) | 403 |
| Yellow Maize (Finely ground) | 394 |
| Soybean oilcake meal | 56.5 |
| Sunflower oilcake meal | 76.2 |
| Molasses | 59.6 |
| Urea | 2.7 |
| Mono calcium phosphate | 1.8 |
| Salt | 4.5 |
| Vitamin – mineral premix[1, 2, 3] | 1.9 |

[1] Rizocore Dairy Premix/ Protein Feeds Dairy Premix V1 (Wisium SA, Fourways, Johannesburg).

[2] Contains per kg of premix: 6,000,000 I.U. of Vitamin A; 1,000,000 I.U. of Vitamin D3; 25,000 mg of Vitamin E; 400,000 mg of magnesium (Mg); 100,000 mg of manganese (Mn); 100,000 mg of zinc (Zn); 15,000 mg of copper (Cu); 1,500 mg of cobalt (Co); 1,700 mg of iodine (I); and 350 mg of selenium (Se).

[3] The Acid Buf 10 and monensin dietary treatments were created by the addition of these feed additives into the respective vitamin-mineral premix. For the Acid Buf 10 treatment the additive was included at a level to provide 3.75 g/kg DM, and for the monensin treatment Rumensin® 200 was included at a level to provide 10.84 mg/kg DM of monensin sodium. Note for the DFM treatment, Achieve$^{FE}$ was not included in the vitamin-mineral premix as this feed additive was administered directly into the rumen at 10 g/d.

## 4.3 Feeding and management

Experimental animals were individually fed *ad libitum*, and received their assigned experimental diets twice daily, directly after milking, at 08:00 and 14:00. Feed troughs were cleaned daily, by sweeping up the feed refusals from the previous day. These feed refusals were collected and weighed back daily thus allowing the amount of feed offered daily to be adjusted to achieve 5 % to 10 % feed residue. Feed intakes were monitored and recorded for the duration of the trial.

In order to simplify feeding each treatment was assigned a different colour:

Control (C) – Green

Control + Acid Buf 10 (AB10) – Blue

Control + Achieve$^{FE}$ (DFM) – Orange

Control + Rumensin® 200 (MON) – Yellow

Each dietary treatment was packaged according to the colour assignment. Pens were clearly marked with colour-coordinated information boards, which displayed both the cow's identification number and the current treatment. Information boards were updated at the start of each period.

## 4.4 Monitoring of body weight and body condition

Cows were weighed at the beginning of the trial, thereafter BW was monitored on a weekly basis, and recorded on the first and last day of each experimental period, with the final BW being recorded on the last day of the trial. Body weight was recorded three times daily, i.e. after each milking, as the cows exited the milking parlour. These weights were automatically saved to the milk recording software utilised by the farm (AfiFarm Dairy Farm Management Software, V 3.076AT2 – Test version – Limited edition), these three weights were averaged to give the final BW recorded. By calculating the BW in this manner, possible variation in BW due to events such as time since last urination, defecation, or water consumption were reduced.

Body condition was scored on the first and last day of the trial, additional scoring was carried out on the final day of each experimental period. Cows were scored according to the 5-point scoring system developed by Wildman *et al.* (1982), which is used as a tool to measure the relative amount of subcutaneous body fat. To assist in assigning a score the BCS system of Ferguson *et al.* (1994a) was used, which utilises a flow chart to direct the scorer to view and assess several anatomical characteristics of cows, allowing one to assign a quarter point score. Body condition scoring was always carried out directly after the morning milking.

## 4.5 Sample collection, preparation and analytical methods

### 4.5.1 Total mixed ration

Total mixed ration samples were taken each time feed was blended for the trial. Each experimental diet was blended separately, and in total four separate batches of feed were mixed for the duration of the trial. During the blending of each experimental diet, a certain amount of TMR was diverted into a bucket – this sample was considered as a representative sample of the particular TMR. This sample then underwent the cone and quartering method (Campos & Campos, 2017) for obtaining a representative sample. In brief, on a hard, clean, dry surface the sample was mixed by turning over the entire sample at least three times with a shovel. A conical pile of feed material was then formed, this pile was then flattened to an even thickness, and the sample was divided into quarters upon which two opposite quarters were discarded. The remaining quarters were then recombined and once again poured into a conical pile, flattened and divided, this process continued until left with the desired sample size. A representative sample (500 g) of each experimental diet was retained from each of the four batches, giving a total of 16 TMR samples for the trial. These samples were stored refrigerated until analysis.

Before proximate analysis could be carried out on the TMR samples, the four feed samples per treatment were pooled, giving one sample per treatment. Each of the four samples underwent the cone and quartering method for obtaining a representative sample, as described earlier. The samples were dried at 55ºC, in a forced-air oven, for 48 hours, thereafter the dried samples were ground to pass through a 1 mm screen on a Retsch SM 100 cutting mill (Retsch, Haan, Germany).

Proximate analysis of the experimental diets, was performed at the UP-Nutrilab (Department of Animal and Wildlife Sciences, University of Pretoria). Initial dry matter (iDM) determination was performed on the original "as-is" TMR samples, by sub-sampling 2 g of sample for drying in a forced-air oven at 105 ºC for 24 hours, after which it was weighed back. Dry matter was determined as the gravimetric loss when dried in a forced-air oven at 105 ºC for 24 hours, and was performed according to method 934.01 of AOAC (2000). Ash determination was performed, according to method 942.05 of AOAC (2000), allowing for the calculation of OM content. Ether extract (EE) was determined by Soxhlet extraction, method 920.39 of AOAC (2000). Crude protein was determined by the Leco (Leco TruMac Nitrogen determinator, Model FP 428, Leco Corporation, St Joseph, MI, USA) following method 990.03 of AOAC, 2000, whereby the percentage of N multiplied by the factor 6.25 is used to calculate percentage CP. Starch was determined with the Megazyme (Megazyme International, County Wicklow, Ireland) total starch assay kit, adopted by the AOAC, method 996.11 and

AACC International (2010), method 76-13.01. Before mineral analysis could be performed, samples had to undergo an acid digestion (Method 935.13, AOAC 2000), thereafter calcium (Ca) and phosphorous (P) were analysed for according to methods 965.09 and 965.17 respectively, of the AOAC (2000). Fibre analyses, namely NDF, NDF organic matter ($NDF_{OM}$), ADF and acid detergent lignin (ADL) were determined according to manufacturer's specifications, using the $ANKOM^{200}$ Fibre analyser with filter bag technology (ANKOM Technology, Macadon, NY, USA). Neutral detergent fibre, ADF, and ADL were determined following ANKOM methods 6, 5, and 8 (Goering & Van Soest, 1970), respectively. It is important to note that the NDF method followed, included the use of a heat-stable amylase which served to remove starch and inactivate enzymes present which could degrade the fibre, thus NDF is more accurately denoted as aNDF, $aNDF_{OM}$, was determined by ashing the filter bags from a completed aNDF run, at 600 ºC for 2 hours (ANKOM service procedure 034), thus allowing for aNDF to be expressed on a ash-free basis. Gross Energy was determined using the CAL2K equipment according to the manufacturer's specifications (Digital data systems, Randburg, Gauteng). Lastly in vitro organic matter digestibility (IVOMD) was determined according to Tilley & Terry (1963) as modified by Engels & van der Merwe (1967). The determination of GE and IVOMD allowed for the calculation of ME for each experimental diet according to Robinson *et al.* (2004).

### 4.5.2 TMR refusals

During the 11 day sampling period, TMR refusals were collected daily, on days 15; 16; 18; 19; 21; 22; 24 and 25, and samples were retained. Total mixed ration refusal samples were taken as grab samples, from the total refusals swept up from the feed trough prior to the morning feeding. These samples were kept refrigerated until the end of the study. All samples collected per treatment during an experimental period were composited. Thus, giving a total of 16 TMR refusal samples for the duration of the trial (i.e. one sample for each animal for each experimental period). Each of these samples underwent the cone and quartering method for obtaining a representative sample as described earlier. Each representative sample was then dried and milled as previously described. These feed refusal samples were analysed for DM, CP and aNDF, according to the previously mentioned procedures, in order to determine if dietary selection occurred.

### 4.5.3 Ruminal pH profile

Rumen pH was recorded at 03:00 and 15:00 on day 15; 06:00 and 18:00 on day 18; 09:00 and 21:00 on day 21; and 12:00 and 00:00 on day 24, when obtaining ruminal samples. This sampling design allowed one to obtain a sample every three hours over a 24 hour period. The pH of the rumen fluid was measured immediately upon collection, so as to reduce the change in pH which can occur from the exposure of the rumen digesta to oxygen, using a handheld pH meter (EcoSense pH100A, YSI Environmental, Yellow Springs, OH, USA). Although monitoring ruminal pH in this manner is not as ideal as continuous rumen pH recording over a 24 hour period using indwelling rumen pH probes, it does provide one with sufficient information to allow for conclusions to be drawn as to the effects of the various dietary treatments on shifts in ruminal pH and the time ruminal pH is below 5.6.

### 4.5.4 Ruminal fermentation

Rumen liquor samples, for the determination of rumen fermentation parameters, were collected according to the schedule described above, for rumen pH monitoring. Ruminal liquor samples were obtained directly from the rumen, via the rumen cannula, by inserting one's hand into the rumen, and using a cup to collect rumen contents. In order to obtain a final ruminal liquor sample which was representative of the entire rumen, samples were taken from various locations in the rumen, in brief samples were taken from 9 locations in the rumen, three samples were taken moving cranially to caudally, from the dorsal, medial and ventral areas. These smaller "sub – samples" formed the final sample obtained (Schwab *et al.*, 2006). Rumen content samples were upon collection immediately strained through two layers of cheesecloth in order to remove particulate matter. Three ruminal liquor samples were collected per cow per sampling time, and preserved according to Erasmus *et al.* (1992), for the determination of ammonia-N, VFA's and lactic acid.

For determination of ammonia-N ($NH_3$-N), 45 ml of filtered ruminal liquor was added to a sample bottle containing 5 ml of 50 % sulphuric acid ($H_2SO_4$), shaken, and stored frozen at - 20 ºC until analysed. Analysis was done according to the adapted method of Broderick & Kang (1980) this colorimetric method is generally

referred to as the Berthelot colour reaction or the Indophenol blue reaction. In brief, in the presence of a strong oxidising agent (i.e. Hypochlorite) the ammonia present in the sample will react with the Phenol reagent to produce an intense blue coloured solution of Indophenol, with the intensity of the blue colour being proportional to the concentration of ammonia. The colour is read by means of spectrophotometry at a wavelength of 630 nm, with results being measured against ammonia standards. Duplicate standard curves were included at both the start and end of each run from which the concentration of ammonia in the samples was calculated by making use of the equation y = mx + c

For determination of VFA's, 45 ml of filtered ruminal liquor was added to a sample bottle containing 5 ml of 10 % Sodium Hydroxide (NaOH), shaken, and stored frozen at - 20 ºC until analysed. Samples were analysed by Mr Sarel Marais (Department of microbiology, biochemical and food biotechnology, University of Free State, Bloemfontein, Free State) by means of high-performance liquid chromatography (HPLC) according to the method of Tabaru *et al.* (1988). Prior to being analysed each individual sample was subjected to a "clean-up" procedure (Siegfried *et al.*, 1984) during which the ruminal liquor was deproteinised and all sugars removed, resulting in a reasonably clean solution of fermentation products which could then be analysed for VFA's via HPLC.

For determination of lactic acid, 20 ml of unpreserved, filtered ruminal liquor was stored frozen at - 20 ºC until analysed. Samples were analysed by Mr Sarel Marais (Department of microbiology, biochemical and food biotechnology, University of Free State, Bloemfontein, Free State) according to the HPLC method of Tabaru *et al.* (1988).

### 4.5.5 Bacterial isolation from ruminal contents

Samples of mixed ruminal contents, for the isolation of rumen bacteria, were collected according to the schedule described above, for ruminal pH and fermentation parameters. Mixed ruminal content samples were obtained directly from the rumen, via the rumen cannula, by inserting one's hand into the rumen, and using a cup to collect rumen contents. In order to obtain a final sample which was representative of the entire rumen, samples were taken from various locations in the rumen, in brief samples were taken from nine locations in the rumen, three samples were taken moving cranially to caudally, from the dorsal, medial and ventral areas. These smaller "sub – samples" were composited giving a final representative sample of whole ruminal contents (500 ml).

These samples were fractionated into liquid and solid fractions, from which FAB and PAB could respectively be isolated, as follows (Figure 1): each entire mixed ruminal content sample was squeezed through 2 layers cheesecloth and nylon mesh (60 µm), with the nylon mesh serving to trap particulate matter. Solid residue obtained was re-suspended in three times its weight of saline solution (0.85 %). After gentle agitation, the suspension was filtered again, as before, to obtain the solid residue ("Washed" solid residue). This saline "wash" solution was combined with the strained rumen fluid (Filtrate + saline "wash"), and from this liquid phase FAB was isolated by differential centrifugation according to Whitehouse *et al.* (1994) with modifications. Briefly, the liquid phase was centrifuged at 500 x g for 5 minutes at 4 ºC (Rotor JS 5.3, Avanti J-E, Beckman Coulter (Pty) Ltd, Brea, CA, USA) to remove small feed particles and protozoa. The supernatant was then carefully decanted, collected and centrifuged at 20,000 x g for 20 minutes at 4 ºC (Rotor JA 20, Avanti J-E, Beckman Coulter (Pty) Ltd, Brea, CA, USA) in order to isolate the bacteria.

These supernatants were discarded, the bacterial pellets were then transferred into a single tube, using saline solution (0.85 %), and re-centrifuged at 20,000 x g for 20 minutes at 4 ºC. The bacterial pellet obtained was re-suspended in distilled water and underwent a final centrifugation at 20,000 x g for 10 minutes at 4 ºC in order to remove any residual salt which could interfere with the ash analysis.

In order to isolate the PAB the "washed" solid residue retained underwent a detachment procedure, during which the residue was weighed and transferred into a 1 L sampling bottle, re-suspended in three times its weight of 0.1 % Methylcellulose solution, agitated for one hour in a shaking water bath (39 ºC, moderate speed), and then placed in a cooler at 4 ºC for 24 hours.

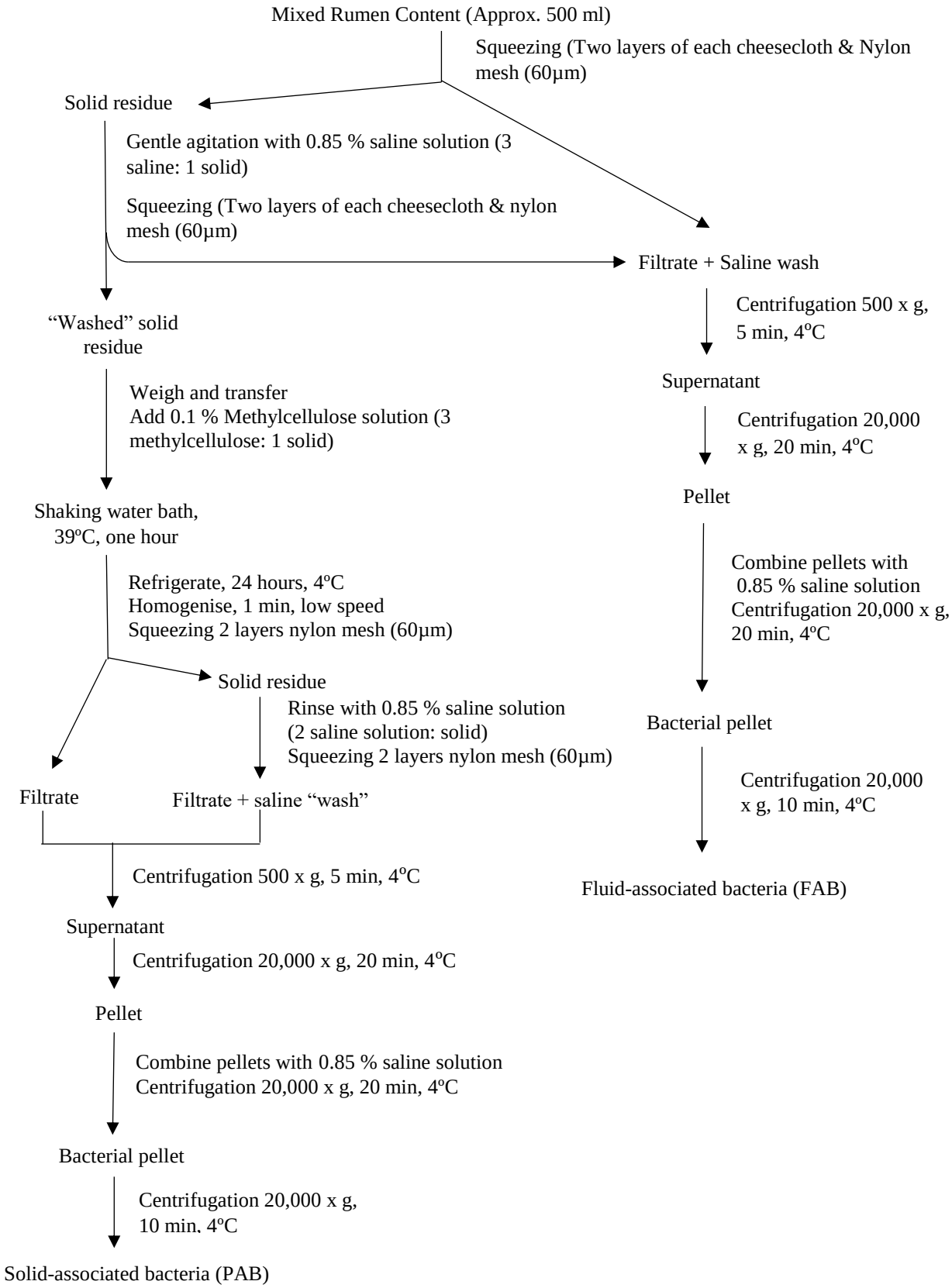


**Figure 4.2:** Diagram illustrating the procedure used in this study to isolate fluid-associated bacteria (FAB) and particle-associated bacteria (PAB) from ruminal contents (Modification of Whitehouse *et al.* (1994)).

After 24 hours, the contents of each PAB bottle were homogenised for one minute at low speed (Hamilton Beach Commercial blender, Hamilton Beach/Proctor-Silex Inc., Glen Allen, VA, USA), the contents were then strained through two layers of nylon mesh (60 µm), to remove any particulate matter, with the filtrate being collected in a common container. The solid residue remaining on the nylon mesh was re-suspended in two times its weight of saline solution (0.85 %), gently agitated and was again squeezed through two layers of nylon mesh (60 µm).

The particulate matter remaining on nylon mesh was discarded, and the two filtrates combined, this filtrate then underwent differential centrifugation, as previously detailed, in order to obtain the particle associated bacterial pellet for each treatment.

Once isolated both the FAB and PAB pellets, for each treatment, were transferred into pre-labelled sample bottles, re-suspended in distilled water and stored frozen at -20 ºC until freeze-dried to constant DM, thereafter the individual pellets were ground using a mortar and pestle. Before analysis the samples were composited to give one FAB and PAB sample per treatment and per period, i.e. all time periods were composited per treatment per period for each FAB and PAB. The resulting 32 samples were sent to the ARC, Irene (Animal production unit, ARC, Irene, Gauteng) for analysis of the AA composition, following a method which involves the acid hydrolysis of samples, followed by pre-column derivatisation and ultimately separation by HPLC with detection using a fluorescence detector (Einarsson *et al.*, 1983). The microbial samples were also analysed for DM content, ash content for the determination of OM content, and N content, according to methods 934.01, 942.05, and 990.03 of the AOAC (2000), respectively.

### 4.5.6 Urine

Spot urine samples were collected on sampling days 15, 18, 21 and 24, when the animals were being handled (i.e. the two sampling times). Manual stimulation was attempted to ensure a urine sample would be obtained from each cow at each sampling time, however little success was had, instead urine samples were collected from voluntarily urinating cows only. Immediately upon collection, the SG of each untreated urine sample was measured using a digital handheld pen refractometer (Pen-urine SG, Atago Inc, Tokyo, Japan), directly after the measurement was taken the samples were placed on ice. This data was later used to calculate the volume of urine passed per cow per day.

Aliquots of urine (7 ml) were transferred into sample bottles containing 2 ml of 10 % Sulphuric acid ($H_2SO_4$), the addition of acid being essential, as the final pH of the urine samples needed to be reduced to below 2.0 so as to prevent the bacterial destruction of AL. The samples were then diluted with deionised water, to prevent precipitation of uric acid, to a final volume of 35 ml, and stored frozen at - 20 ºC until analysed (Swanepoel *et al.*, 2014).

Note that duplicate samples were prepared per sample collected. One set of duplicate samples were chemically analysed for CR at the Clinical pathology laboratory (Faculty of Veterinary Science, University of Pretoria, Onderstepoort, Pretoria, Gauteng) according to the Jaffé method, using a urine CR kit (Roche Diagnostics Corporation, Indianapolis, USA).

The other set of samples were chemically analysed for AL according to Chen & Gomes (1992) based on the method of Young & Conway (1942). In brief, during this colorimetric method, AL is hydrolysed to allantoic acid, which in weak acidic conditions is further degraded to urea and glyoxylic acid. The glyoxylic acid reacts with phenylhydrazine hydrochloride to produce a phenylhydrazone of the acid, this product then forms an unstable chromophore with potassium ferricyanide leading to the development of colour. The colour was read at a wavelength of 522 nm with the results being measured against AL standards. Standards were prepared to create working concentrations of 20; 40; 60; 80 and 100 mg/L of AL. Duplicate standard curves were included at both the start and end of each run from which the concentration of AL in the samples was calculated by making use of the equation $y = mx + c$. Note that samples had to be diluted 60 times to fit the standard curve. Additionally, samples were thawed and centrifuged, at 1200 x g for 5 minutes at room temperature, prior to analysis in order to remove a precipitate which could interfere with the colorimetric readings.

### 4.5.7 Milk recording and composition

Cows were milked three times daily at 06:00, 12:00 and 19:00 in a 10-point milking parlour equipped with weigh all milk meters (Waikato S.A. Milking Systems, 20 Dobson Street, North End, Port Elizabeth, South Africa). Data from each milking was automatically updated on the AfiFarm Dairy Farm Management Software, allowing the milk production for the entire duration of the trial to be recorded.

Milk samples from each experimental cow were taken daily during the 11 day sampling period (days 15 -25) at each milking. Sampling was done, as per national herd recording scheme guidelines, by attaching a sample bottle to the milk line via suction, into which milk was diverted drip by drip during milking, this ensured that the sample obtained was representative of the entire milking. This sample bottle was then removed from the milk line, gently mixed by inverting the bottle a few times, and sufficient milk sample was decanted into a sampling bottle. Milk samples for the day were stored refrigerated at 4 ºC. At the end of the day composite milk samples were prepared by taking a sub-sample (1 ml to represent each hour since the last milking) of the milk sample taken at each milking and combining these to form one composite milk sample per cow per day. In other words, an 11 ml sample was taken from the 06:00 milking, 6 ml sample from the 12:00 milking, and a 7 ml sample from the 19:00 milking.

Duplicate samples were taken on days 15, 18, 21 and 24 and were preserved with Broad Spectrum Microtabs II (Weber Scientific, Hamilton, New Jersey, USA), which is a preservative containing 8 mg Bronopol and 0.30 mg Natamycin to prevent spoilage of the milk sample. Samples were stored refrigerated at 4 ºC until the end of the sampling period at which point the samples were sent to Mériux NutriSciences (Swift Micro Laboratories (Pty) Ltd, Midrand, Gauteng) for analysis. Here samples were analysed for the compositional parameters; milk fat percentage, milk protein percentage, milk lactose percentage and milk urea nitrogen (MUN) using the MilkoScan/ Bentley FTS FT+/ Bentley FTS fully automated Fourier transformation infrared spectrophotometer (FT-IR), which utilises infrared technology to determine the composition of milk samples (Foss Analytical, Hillerød, Denmark/ Bentley instruments, Chaska, MN, USA). The SCC of the milk samples was determined by flow cytometry using the Bentley flow cytometer (Bentley instruments, Chaska, MN, USA).

In addition to these standard milk analyses, milk samples were also taken for the analysis of the milk nitrogen fractions (i.e. whey protein, casein and NPN). These samples were taken on days 16, 19, 22, and 25, in the same manner as previously described, the only differences being firstly that 2.5 ml of milk was taken to represent each hour since milking instead of 1 ml, this was to ensure that there would be enough sample for the analysis. And secondly that the composite sample did not receive any preservative, the raw milk sample was frozen as is at -20 ºC until analysed. Prior to shipment of the samples for analysis, the samples were thawed in order to allow for the samples to be pooled. Milk samples from day 16 and 19, and 22 and 25 were pooled together, thus giving 2 samples per animal per period to be analysed. Samples were analysed by Penny Barnes at the Animal production institute – ARC (General Chemistry laboratory, ARC, Irene, Gauteng) following the methods of Rowland (1938a), for the determination of the various nitrogen fractions by laboratory analyses and calculations.

### 4.5.8 Calculations

Organic matter content of the experimental diets was calculated as 100 minus the ash (% basis) content of the experimental diets.

Metabolizable energy (MJ/kg DM) of the experimental diets was calculated as the GE of the diet multiplied by the IVOMD, multiplied by a factor of 0.82 (Robinson *et al.*, 2004).

Non-fibrous carbohydrate (NFC) content of the experimental diets was calculated as 100 minus the sum of the NDF, CP, EE and ash contents of each experimental diet, according to the NRC (2001).

Milk efficiency was calculated as the quotient of daily milk yield (kg/d) and daily DMI (kg/d).

Energy-corrected milk (ECM) (kg/d) was calculated according to the equation found in Robinson & Erasmus (2010), where each milk component is multiplied by its respective energetic value, the sum of which

is then divided by the energetic value of one kg of 'standard' milk (i.e. 0.721 Mcal/kg). With 'standard' milk being defined as 3.75 % milk fat, 3.0 % true protein and 4.9 % lactose. The formula is as follows:

[(((((milk fat % *41.65) + (milk true protein % *24.13) + (milk lactose % *21.6)) – 11.72) / 1000) *2.204)) * milk yield (kg/d)] / (0.721)

Fat – corrected milk (i.e. 4 % FCM) (kg/d) was calculated according to Gains (1928) and NRC (2001) as:

(0.4 x milk yield (kg)) + (15 x milk fat yield (kg))

The total AA-Nitrogen (AA-N) content for each bacterial fraction was calculated according to the advice of Dr Jeff. Firkins (Personal communication), at the Ohio State University (firkins.1@osu.edu), as follows;

Firstly, each individual AA was converted from being expressed as grams (g) of AA per 100 g of AA, to g of AA per 100 g of DM which was done by multiplying each individual AA by the total AA percentage.

Thereafter each individual AA (g/100 g of DM) was converted to AA- Nitrogen, i.e. g of N/100 g of DM) by multiplication of the individual AA by the molar percentage of N per mol of AA, as follows:

AA (g/100 g of DM) *((# N*14.0067 (g/mol)) / MW of AA (g/mol)

where # N is the number of nitrogen atoms in each AA; 14.0067 (g/mol) is the molecular mass of N; and MW is the molecular weight of each AA.

Total AA-N (g AA-N/100 g of DM) for each sample is simply the sum of AA-N for each individual AA. To express total AA-N as a percentage of total N, as determined by Kjeldahl analysis, total AA-N (g of TAA-N/ 100 g of DM) was divided by total N (g of N/100 g of DM), and multiplied by 100 to obtain a percentage.

Urine volume (L/d) was calculated by means of an equation derived from the published data of Burgos *et al.* (2005) as:

$$332.66 * (((SG - 1) * 1000)^{-0.884})$$

where SG is the specific gravity of the urine sample.

Allantoin output (mmol/d) was calculated as the concentration of AL (mmol/L) multiplied by the urine volume (L/day).

Total daily PD excretion (mmol/d) is the sum of the daily PD excretion in urine and the daily PD excretion in milk of lactating dairy cows (Chen & Gomes, 1992). Purine derivative excretion in urine was calculated as the quotient of AL output (mmol/d) by the coefficient 0.906, which expresses the concentration of AL as a proportion of total urinary PD excretion, obtained from values reported by Vagnoni & Broderick (1997), Valadares *et al.* (1999), Gonzalez-Ronquillo *et al.* (2003) and Moorby *et al.* (2006). Daily PD excretion in milk is taken to be a constant 0.05 of urine PD excretion (Chen & Gomes, 1992).

Daily absorption of microbial PD from the intestine (X, mmol/d) was calculated according to the equation of Chen & Gomes (1992) as:

$$(\text{Total daily PD} - 0.385 * (BW^{0.75})) / 0.85$$

assuming that the net endogenous contribution of PD to total excretion is 0.385mmol/kg$BW^{0.75}$, and that the recovery of absorbed purines as PD in the urine is 0.85.

Microbial CP production (g CP/d) was then estimated as:

$$[(X \text{ (mmol/d)} * 70 / (0.116 * 0.83 * 1000)] * 6.25$$

assuming an N content of 70 mgN/mmol for purines and a ratio of purine N: total N in mixed rumen microbes as 11.6: 100. The coefficient for microbial purine digestibility was taken to be 0.83, and the factor 6.25 was used to convert microbial N to microbial CP (Swanepoel *et al.*, 2016).

Purine derivative to creatinine index is calculated as the quotient of total purine derivatives and the concentration of creatinine (Chen & Ørskov, 2004). However, as urinary AL is the predominant PD in cattle and constitutes an almost constant molar proportion of PD, uric acid was not measured in this study. Instead total PD was calculated by correcting the AL concentration (mg/L) by a factor of 0.906 (Previously described). The calculated PDC values obtained were then corrected for metabolic BW ($BW^{0.75}$) in kg to allow for comparison amongst cows (Swanepoel, 2014).

PDC Index = $(AL_{adj}: CR)^* (BW)^{0.75}$

### 4.5.9 Statistical analysis

Data were analysed statistically as a 4 x 4 Latin Square design using the general liner model (GLM) analysis of variance (Statistical Analysis System, 2018) for the average effects over time.

The linear model used is described by the following equation:

$$Y_{ijk} = \mu + T_i + C_j + P_k + e_{ijk}$$

where $Y_{ijk}$ is the response variable studied; $\mu$ is the overall mean; $T_i$ is the fixed effect of the $i^{th}$ treatment ($i$ = C, AB10, DFM, MON); $C_j$ is the random effect of the $j^{th}$ cow ($j$ = 1, 2, 3, 4); $P_k$ is the fixed effect of the $k^{th}$ period ($k$ = 1, 2, 3, 4); and $e_{ijk}$ is the random residual error associated with the related observation.

For the statistical analysis of repeated period measures, i.e. the ruminal fermentation characteristics (pH, NH3-N, and VFA's), sampling time, and sampling time x treatment were added to the model and analysed as Repeated Measures Analysis of Variance using the GLM model. For the ruminal bacteria data, i.e. chemical composition and amino acid profile, the contrast procedure in SAS was applied to compare the control *versus* AB10, control *versus* DFM, and control *versus* MON. These factorial contrasts allowed for one to test and identify the main effects of feed additive supplementation on the composition of the ruminal bacteria. In addition, the two bacterial fractions (i.e. FAB and PAB) were included in the model as levels so as to test the difference between the levels (i.e. bacterial fractions) in chemical composition and amino acid profile.

Results are reported as least square means ± standard error of the means (SEM). For the different statistical tests, significance of difference between means was declared at $P < 0.05$ and a tendency of difference at $0.05 < P \leq 0.10$, as determined by Fisher's test (Samuels, 1989).

# CHAPTER 5

# RESULTS AND DISCUSSION I:

# EFFECT OF FEED ADDITIVES ON PRODUCTIVE PERFROMANCE AND RUMEN FERMENTATION PARAMETERS IN DAIRY COWS

## 5.1 Experimental ration evaluation

The experimental diets were formulated to supply sufficient nutrients for a 600 kg Holstein dairy cow producing 35 kg/d of 4 % FCM (NRC, 1989). All diets were formulated to be identical in terms of both the ingredients and chemical composition, with the exception of the respective feed additives, and to be similar to dairy rations fed in industry. Proximate analysis of the dietary experimental treatments verified the conformity of the four dietary treatments (See Table 5.1 below), thus iso-proximate nutrient rations were achieved. Chemical analysis of the feed residue for DM, CP and aNDF content indicated that little, if any, selective feeding occurred (Data not shown) as the chemical composition of the diets consumed differed little from the mean chemical composition of the ration offered.

**Table 5.1** The chemical composition of each of the experimental diets fed (g/kg DM)

| | Treatment[1] | | | |
|---|---|---|---|---|
| Nutrient | C | AB10 | DFM | MON |
| DM (g/kg) | 951 | 954 | 953 | 950 |
| Ash (g/kg DM) | 72.9 | 80.6 | 70.9 | 78.9 |
| OM (g/kg DM)[2] | 927 | 919 | 929 | 921 |
| GE (MJ/kg DM) | 17.9 | 17.5 | 18.0 | 17.4 |
| ME (MJ/kg DM)[3] | 12.2 | 12.0 | 12.1 | 11.7 |
| Starch (g/kg DM) | 272 | 272 | 272 | 264 |
| CP (g/kg DM) | 188 | 190 | 195 | 194 |
| aNDF (g/kg DM)[4] | 230 | 232 | 229 | 225 |
| $aNDF_{OM}$ (g/kg DM)[5] | 218 | 208 | 216 | 211 |
| ADF (g/kg DM) | 179 | 181 | 178 | 180 |
| ADL (g/kg DM) | 50.4 | 53.3 | 47.9 | 51.5 |
| NFC (g/kg DM)[6] | 486 | 475 | 479 | 477 |
| EE (g/kg DM) | 23.6 | 22.8 | 26.6 | 25.2 |
| IVOMD (%) | 82.8 | 83.5 | 82.0 | 82.4 |
| Ca (g/kg DM) | 8.15 | 9.04 | 8.71 | 9.37 |
| P (g/kg DM) | 3.91 | 3.97 | 4.03 | 3.88 |
| Ca:P | 2.08 | 2.28 | 2.16 | 2.41 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] OM (g/kg DM) calculated as OM = 100 – Ash.
[3] ME (MJ/kg DM) calculated as ME = 0.82 x (GE x IVOMD) (Robinson *et al.*, 2004).
[4] aNDF, NDF assayed with heat-stable amylase, expressed inclusive of residual ash.
[5] $aNDF_{OM}$, aNDF expressed free of residual ash.
[6] NFC = 100 – (NDF, % + CP, % + EE, % + Ash, %) (NRC, 2001).

The chemical composition of the actual dietary treatments fed although comparable do deviate slightly from the nutritional requirements set out by the NRC (2001) for large breed dairy cows. Much of the variation can be attributed to the superior quality of lucerne hay included in the ration as evidenced by the lower than anticipated fibre content and improved *in vitro* digestibility. Mean dietary aNDF and ADF content for the experimental rations are 229 g/kg DM and 179 g/kg DM, respectively, which is less than the NRC (2001) recommendations of a minimum of 250 g/kg DM to 330 g/kg DM of dietary NDF and 170 g/kg DM to 210 g/kg DM dietary ADF. This decreased level of dietary fibre, means that there is less dilution of nutrients by fibre and explains the slightly higher than expected ME content of the rations, as dietary NDF is negatively

correlated to energy concentration (NRC, 2001). Owing to the lower fibre content of the experimental diets, the mean non- fibrous carbohydrate (NFC) content was in slight excess of the recommended maximum NFC of 360 g/kg DM to 440 g /kg DM (NRC, 2001), thus the experimental diets have the potential to induce acidosis and other metabolic problems (Nocek, 1997). The observed lower fibre content of the experimental rations is accompanied by higher mean dietary CP (μ = 192 g/kg DM), which is in excess of the 160 g/kg DM recommended by the NRC (2001), and as such is in support of a superior quality of lucerne hay. This superior quality of lucerne hay, resulted in a higher IVOMD (μ = 82.7 %), with this improvement in diet digestibility resulting in greater dietary ME, as predicted by the equation of Robinson *et al.* (2004), than one would typically expect for large breed dairy cow rations. The ash content of the diets is slightly higher than expected, this could be due to soil contamination, as South African lucerne hay is known to be contaminated (Scholtz, *et al.*, 2009) and lucerne contributes almost 40 % of the experimental diets. Ash content of the dietary treatments are comparable; however, the AB 10 and MON treatments show elevated ash contents, which is likely due to the inorganic content of the feed additive itself.

## 5.2 Feed intake, body weight and body condition

Throughout the research trial feed intake, BW and body condition, by means of body condition scoring, were monitored, the results of this data can be found below in Table 5.2. Please note that no published studies were available on the DFM product Achieve$^{FE}$, and owing to its unique composition comparisons of the results to those of others was not possible. Nevertheless, results obtained have been compared to various other studies on live yeasts, yeast cultures, bacterial strains, *A. oryzae* fermentation extracts, and various combinations of these.

**Table 5.2** Feed intake, body weight and body condition score of lactating dairy cows, supplemented with Acid Buf 10, a direct-fed microbial and monensin, when fed a total mixed ration (n = 16)

| | Treatments[1] | | | | SEM[2] |
|---|---|---|---|---|---|
| | C | AB10 | DFM | MON | |
| Dry matter intake (kg/d) | 20.8 | 19.8 | 19.8 | 20.0 | 0.50 |
| Body weight | | | | | |
| At start (kg) | 602 | 600 | 597 | 590 | 5.9 |
| At end (kg) | 615 | 596 | 609 | 600 | 8.1 |
| Mean (kg) | 611 | 599 | 603 | 595 | 5.9 |
| Change (kg/ 25-day period) | 17.5 | -1.11 | 16.8 | 11.7 | 11.43 |
| Body condition score | | | | | |
| At start (units) | 3.31 | 3.25 | 3.25 | 3.25 | 0.048 |
| At end (units) | 3.31 | 3.25 | 3.38 | 3.31 | 0.063 |
| Mean (units) | 3.31 | 3.25 | 3.31 | 3.28 | 0.050 |
| Change (units/ 25-day period) | 0.02$^{cd}$ | 0.00$^{d}$ | 0.12$^{c}$ | 0.06$^{cd}$ | 0.041 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[c, d] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

### 5.2.1 Feed intake

The results of this study show no DM intake response to supplementation with dietary additives, with an average DMI of 20.1 kg/d across treatments, at a mean DM intake of 3.32 % of BW, which is in accordance with the acceptable range of 2 % to 4 % of BW as specified by the NRC (2001). The observed lack of DM intake response in this study is not unusual as across feed additive based studies effects on DMI are variable and often inconsistent as discussed below.

When feeding CMA (i.e. Acid Buf) one would expect outcomes similar to those observed when feeding sodium bicarbonate or buffers alike, as buffers serve to stabilise the pH of the ruminal milieu, and by doing so enhance DMI (Erdman, 1988; Staples & Lough, 1989). However, when fed to lactating dairy cows, at doses comparable to that used in this study, on potentially acidotic diets, CMA is yet to be shown to increase DMI (Beya, 2007; Bernard *et al.*, 2014; Cruywagen *et al.*, 2015; Wu e*t al.*, 2015). Lack of response in this trial may be due to the intake of AB10 being slightly less than the manufacturers recommendation of 80 g/cow/d to 90 g/cow/d, when planning this trial buffer intake was based on an anticipated DMI of 24 kg/d, however actual intakes averaged 20.1 kg/d thus only approximately 75.4 g/cow/d of AB10 was ingested. However, a more likely reason for lack of DMI response is that although this diet had the potential of being acidotic, owing to the higher NFC (μ = 479 g/kg DM) and lower NDF (μ = 229 g/kg DM) content than recommended by the NRC (2001), the diet was composed of approximately 400 g/kg DM of lucerne hay which is known to have high buffering capacity. The meta-analyses of Erdman (1988), Staples & Lough (1989) and Hsu & Murphy (2005), determined that sodium bicarbonate has little effect on DMI in non-maize silage-based diets, or when added to diets with a forage content higher than 30 % and with hay or pasture as the predominant forage source. The reason for this is that such diets are well buffered owing to the inherent buffering capacity of forages, and this might explain why in this study and those of Beya (2007), Calitz (2009) and Cruywagen *et al.* (2015) no DM intake response was observed.

Dry matter intake response to ionophores, specifically monensin, has been highly variable. The meta-analysis of Duffield *et al.* (2008b) determined that monensin consistently decreases DMI by 0.3 kg, this is in agreement with Ipharraguerre & Clark (2003) who evaluated 14 experiments, in which lactating dairy cows were administered ionophores within the effective dose range of 240 mg/cow/d (Wilkinson *et al.*, 1997) and not exceeding 350 mg/cow/d, and found a mean decrease in DMI of 0.3 kg/d. In the present study although there was no significant decrease in DMI when supplemented with MON, this treatment was numerically decreased compared to the C treatment (MON = 20.0 kg/d *versus* C = 20.8 kg/d), although slightly higher this 0.77 kg decrease in DMI is in agreement with Duffield *et al.* (2008b) and Ipharraguerre & Clark (2003). This numerically lower but non-significant decrease in DMI has also been observed by Phipps e*t al.* (2000) and Erasmus *et al.* (2005). Duffield *et al.* (2008b) stated that failure to report consistent, significant effects is reflective of inadequate sample size and lack of statistical power to detect small changes, the present trial was a 4 x 4 Latin Square design, and this could be the reason that the 3.7 % decrease in DMI as compared to the control diet was not detected.

Direct-fed microbials have been reported to improve DMI in dairy cows (Seo e*t al.*, 2010; Krehbiel *et al.*, 2003). The meta-analysis of Poppy *et al.* (2012) examined the influence of *S. cerevisiae-based* yeast cultures and found a 0.62 kg/d increase ($P$ =0.003) in DMI in early-lactation cows, however a decline in DMI of 0.78 kg/d was identified for mid-late lactation cows ($P$ = 0.001). This parallels the results of the present study in which a numerical 1.0 kg/d decline in DMI is observed for the DFM treatment (DFM = 19.8 kg/d *versus* C = 20.8 kg/d), this lack of change in DMI in response to yeast based DFM supplementation has also been reported by (Erdman & Sharma, 1989; Piva *et al.*, 1993). Fungal DFM's have the potential to improve DMI but most studies have not detected an effect of DMI (Sievert & Shaver, 1993; Takiya *et al.*, 2017), whilst the effects of bacterial based DFM's on DMI remain unclear, with most researchers reporting no effect (Raeth-Knight e*t al.*, 2007; AlZahal *et al.*, 2014; Chiquette *et al.*, 2015).

### 5.2.2 Body weight & body condition

Body weight and body condition were monitored throughout the duration of the trial with no treatment effect expected, and as predicted body weight was unaffected by supplementation. Body weight alone is not a good measure of cow size, with changes in BW often not reflective of true changes in tissue stores. In early lactation as feed intake increases so does the weight of the gastrointestinal contents, because the average gut fill in dairy cows is approximately 15 % of BW, decreases in body tissue weight during early lactation, due to tissue mobilisation, are masked by the increased gut fill. Whereas, mid- to late- lactation, feed intake and the associated gut fill declines, such that increases in BW undervalue true changes in body tissue weight (NRC, 2001).

For this reason, body condition monitoring, although subjective by nature, is a more effective tool for evaluating body tissue (i.e. energy) stores of lactating dairy cows, as BCS is correlated with body fat and energy contents, whilst being independent of BW or frame size (NRC, 2001). Body condition scoring involves a combination of visual appraisal and manual palpation to assign a condition score to individual cows (Wildman *et al.*, 1982), with an ideal BCS for each stage of lactation, which will optimise productive performance, whilst minimising health and reproductive disorders, thus maximising economic returns (Wildman *et al.*, 1982; Gearhart *et al.*, 1990). In this research trial there was no effect of treatment on body condition, as determined by body condition scoring, at the start or end of the trial, with no differences in mean BCS which averaged 3.29. Since the experimental cows utilised in this trial were in late-lactation, approximately 200 to 300 DIM, the mean BCS of 3.29 was within the ideal BCS of 3.00 to 3.50 (Ferguson *et al.*, 1994b) for this stage of lactation. At this stage of lactation cows are in a positive energy balance, with the nutritional objective at this physiological stage being to maintain milk production whilst allowing the cow to restore body tissue reserves in preparation for the subsequent lactation (Gearhart *et al.*, 1990). Body condition changes throughout the lactation cycle, with these changes being related to both live weight change and change in body composition (Wright & Russell, 1984), however, typically after approximately 239 DIM no further changes in BCS are observed (Wildman *et al.*, 1982). As such no significant change in BCS was expected across dietary treatments, although there was a tendency ($P$ = 0.08) for BCS change per period to differ between the AB 10 (0.00) and DFM (0.12) treatments, this was not biologically significant.

Across research supplementation with dietary buffers, specifically Acid Buf, and DFM products typically have no effect on BW, body condition or change when fed to mid- to late-lactation dairy cows (Bernard *et al.*, 2014; Wu e*t al.*, 2015; Leicester e*t al.*, 2016) which is in accordance with the observed results of this trial. Lactating dairy cows supplemented with monensin typically maintain higher body condition throughout lactation (McGuffey *et al.*, 2001; Erasmus e*t al.*, 2005), but this is not always accompanied by an increase in BW (Erasmus *et al.*, 2005). In this study no beneficial effect of MON supplementation on either BW, body condition, or change was observed, this is in accordance with the study of Gandra e*t al.* (2010) and likely due to the fact that these cows were in late lactation, and so in positive energy status unlike their early-lactation counterparts who typically lose less body condition when supplemented with monensin (McGuffey *et al.*, 2001).

As a side-note, this research trial consisted of only 4 dairy cows and involved only a portion of the lactation cycle, the tail - end, for this reason the lack of effect of these feed additives on BW, BCS and change cannot be accepted as a general statement. Lactation studies encompassing the entire lactation with a greater number of animals are required to obtain conclusive results on such effects of these feed additives.

## 5.3 Milk production, composition and milk efficiency

The results for daily milk yield, milk composition, MUN and efficiency of milk production are presented in Table 5.3. It must be said that these results should be interpreted with caution owing to the 4 x 4 Latin Square design of the trial, and the use of cows which are later in lactation and as such are typically less responsive to the mode of action of feed additives. Conventionally herd-based trials involving a full lactation cycle are more suited to evaluating the effects of additives on milk production and composition.

### 5.3.1 Milk yield

In the present study, mean milk production, 4 % FCM and ECM (kg/d) were not affected by dietary treatments, with a mean production across treatments of 24.7 kg/d ± 0.82 kg/d, 25.7 kg/d ± 1.01 kg/d and 26.1 kg/d ± 0.96 kg/d for milk, 4% FCM and ECM, respectively. The milk yields observed in this study were less than anticipated, although not uncharacteristic for cows in late- lactation (Vijayakumar *et al.*, 2017) as typically one can expect a 10 % to 15 % decline in milk per month after peak milk production has been reached, with milk production declining by 12 % to 20 % from peak milk yield for cows in late-lactation (Ishler *et al.*, 2017). Dietary NFC in the present study averaged 479 g/kg DM, which may have influenced milk production as Hoover & Stokes (1991) found that when dietary NFC was either greater than 450 g/kg DM to 500 g/kg DM or less than 250 g/kg DM to 300 g/kg DM that milk production declined. The lack of treatment effect on milk production observed in this trial is supported by the inability of the dietary treatments to favourably modify ruminal fermentation in this study, as shown in Table 5.7, as none of the feed additives exhibited a positive

effect on the concentration of total VFA's, nor was fermentation shifted to support higher concentrations or relative ratios (i.e. molar percentages) of the glucogenic precursor, propionate. It is these two alterations of ruminal fermentation which typically enhance the energy available to the dairy cow (Russell & Strobel, 1989) for milk synthesis.

Acid Buf when fed at levels of 0.35 % DM to 0.4 % DM (i.e. 80 g/cow/d to 90 g/cow/d) to lactating dairy cows fed concentrate rich, potentially acidotic TMR's, has been reported to increase milk production (Cruywagen *et al.*, 2004; Beya, 2007; Cruywagen *et al.*, 2015). Acid Buf has been shown to increase milk production by 4 kg/d ($P = 0.01$) compared to a non-significant increase of 1.5 kg/d with the traditional buffer, sodium bicarbonate (Beya, 2007). In accordance with the present study the research trials of (Calitz, 2009; Bernard *et al.*, 2014; Wu *et al.*, 2015) did not observe an increase in milk production when supplementing diets with Acid Buf. The lack of response in these studies and the present study is likely due to the diets being sufficiently buffered owing to the significant inclusion of dietary forages (> 30 %), thus preventing significant alteration of ruminal pH and fermentation to the detriment of milk production, thus diminishing the extent to which a positive response to supplementation would be observed.

The comprehensive studies of Desnoyers *et al.* (2009), Erasmus & Robinson (2009) and Poppy *et al.* (2012) evaluated various commercial preparations of yeast cultures and found supplementation to increase milk yield by 0.8 kg/d, 0.9 kg/d and 1.18 kg/d, respectively. Additionally, Poppy e*t al.* (2012) found yeast-based supplementation to increase 3.5 % FCM by 1.61 kg/d and ECM by 1.65 kg/d. Research on the supplementation of DFM products containing a combination of both yeast and bacterial strains have too shown significant enhancements in milk production (Nocek *et al.*, 2003; Leicester *et al.*, 2016). Contrary to this the present study showed no effect of supplementation with a DFM comprised of yeast culture (*S. cerevisiae*); *A. oryzae* fermentation solubles; dried *B. subtilis*, *L. acidophilus*, and *E. faecium* fermentation product, on milk production. This absence of response is in agreement with studies in which various DFM products were evaluated and found to have no effect on milk production (Raeth-Knight *et al.*, 2007; AlZahal *et al.*, 2014).

Following an extensive review of literature, it is clear that the effects of monensin supplementation on lactation performance are inconsistent. Despite the observed variability in response, the extensive reviews of McGuffey e*t al.* (2001), Ipharraguerre & Clark (2003) and Duffield *et al.* (2008b), have shown supplementation with monensin to increase milk yield by 1.3 kg/d, 1.5 kg/d and 0.7 kg/d, respectively. Contrary to this and in agreement with the present study, many researchers have reported no effect of monensin on milk production, when fed at doses within the range of 250 mg/cow/d to 350 mg/cow/d, to lactating dairy cows past peak milk production and fed a TMR comprised of approximately 60 % forage to 40 % concentrate (Broderick, 2004; Yang *et al.*, 2007; do Prado *et al.*, 2015). As discussed in *section 2.8.8* various factors modify the response to ionophore supplementation, the most notable of these being level of supplementation and diet composition. Level of supplementation, i.e. dose, is an important factor to consider when evaluating the effects of ionophores on production, Gandra *et al.* (2010) found that when supplementing mid- and late-lactation cows better productive performance was observed at doses of 24 mg/kg DM to 35 mg/kg DM, with no favourable responses being observed at levels below this, 12 mg/kg DM to 20 mg/kg DM, or in excess of this. This could explain why no favourable effects on milk production were observed in the present trial, as sodium monensin was only fed at approximately 10.84 mg/kg DM (i.e. 260 mg/cow/d), however, the values suggested by Gandra *et al.* (2010) are in excess of the typical level of supplementation used in industry, and the present study did meet manufacturer guidelines of 250 mg/cow/day to 350 mg/cow/d. The dietary composition of experimental diets is likely the chief factor influencing the response to monensin, with the meta-analysis of Duffield e*t al.* (2008b) determining that milk production response to monensin supplementation was greater in studies based on pasture-based herds than TMR fed herds. This finding was in agreement with Ipharraguerre & Clark (2003) who upon grouping of experiments according to the proportion of dietary DM supplied as forage into high (≥700 g/kg of dietary DM) and low (≤500 g/kg of dietary DM) found ionophore supplementation to have a greater positive effect on milk production in pasture based herds (+ 1.5 kg/d, i.e. + 9.4 %) as opposed to herds on low forage or concentrate diets (+ 0.7 kg/d, i.e. + 1.5 %). In their discussion Erasmus e*t al.* (2005) also found that upon examination of published studies as either pasture-based or TMR that significant increases in milk production due to monensin supplementation occurred in all pasture-based studies but not in all TMR studies. These findings support the results of the present TMR study. This observation may be explained through the effects of ionophores on ruminal fermentation. When grazing high quality pastures, which supply

abundant CP but limited energy, the productive performance of the cows will likely be restricted by an energy deficit. By increasing the supply of glucogenic precursors, i.e. propionate and AA, through enhanced ruminal propionate production and protein sparing (Bergen & Bates, 1984) ionophores increase the supply of precursors for milk synthesis, allowing for increased milk production. In contrast, when feeding a high energy, starchy TMR to dairy cows, a milk production response would be less likely (Erasmus *et al.*, 2005; Duffield *et al.*, 2008b).

**Table 5.3** Milk yield, milk components, milk urea nitrogen and milk efficiency of lactating dairy cows supplemented with Acid Buf 10, a direct-fed microbial and monensin when fed a total mixed ration (n =16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| | C | AB10 | DFM | MON | SEM[2] |
| Production (kg/d) | | | | | |
| Milk | 25.1 | 24.2 | 25.4 | 24.1 | 0.82 |
| 4 % FCM[3] | 26.1 | 25.1 | 26.1 | 25.4 | 1.01 |
| ECM[4] | 26.7 | 25.5 | 26.5 | 25.8 | 0.96 |
| Fat | 1.07 | 1.03 | 1.06 | 1.05 | 0.048 |
| Protein | 0.83 | 0.78 | 0.81 | 0.76 | 0.026 |
| Lactose | 1.24 | 1.20 | 1.26 | 1.21 | 0.040 |
| Composition (%) | | | | | |
| Fat | 4.35 | 4.27 | 4.24 | 4.36 | 0.113 |
| Protein | 3.35$^{a}$ | 3.27$^{ab}$ | 3.23$^{ab}$ | 3.19$^{b}$ | 0.046 |
| Casein | 2.64 | 2.56 | 2.50 | 2.48 | 0.062 |
| Whey protein | 0.47 | 0.46 | 0.53 | 0.55 | 0.040 |
| Non- casein nitrogen (NCN) | 0.11 | 0.11 | 0.12 | 0.12 | 0.007 |
| Non-protein nitrogen (NPN) | 0.04 | 0.04 | 0.03 | 0.04 | 0.002 |
| Lactose | 4.97 | 4.97 | 4.95 | 4.98 | 0.023 |
| MUN (mg/dL) | 15.9 | 16.9 | 15.7 | 15.6 | 1.13 |
| Milk efficiency[5] | 1.20$^{d}$ | 1.22$^{cd}$ | 1.28$^{c}$ | 1.20$^{d}$ | 0.027 |
| 4 % FCM efficiency[6] | 1.25 | 1.27 | 1.32 | 1.27 | 0.033 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[3] 4 % FCM = 4 % fat corrected milk, calculated as (0.4 x milk yield (kg)) + (15 x milk fat yield (kg)), according to Gains (1928) and NRC (2001)
[4] ECM = Energy corrected milk, calculated as [(((((milk fat % *41.65) + (milk true protein % *24.13) + (milk lactose % *21.6)) – 11.72) / 1000) *2.204)) * milk yield (kg/d)] / (0.721), according to Robinson & Erasmus (2010)
[5] Milk efficiency calculated as the quotient of daily milk yield (kg/d) and daily dry matter intake (kg/d).
[6] 4% FCM efficiency calculated as the quotient of daily 4 % FCM yield (kg/d) and daily dry matter intake (kg/d).
[a, b] Means within a row with different superscripts differ ($P < 0.05$)
[c, d] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

## 5.3.2 Milk fat

Of the three principal constituents of milk, milk fat is the most amendable to dietary manipulation, in terms of concentration and composition (Sutton, 1989; Palmquist *et al.*, 1993). Milk fat concentration can be considerably altered, over a range of approximately three percentage units, by the manipulation of nutritional factors such as level of concentrate intake, carbohydrate composition of concentrates, energy intake, forage to concentrate ratio, and dietary fat (Sutton, 1989). Milk fat depression (MFD) is often observed when feeding

concentrate-based, high-energy diets but the physiological mechanisms behind this are not fully understood. Depression in milk fat has typically been explained by two theories, the first being that when feeding high energy, low fibre diets there is a decline in the relative proportion of and ruminal production of the lipogenic precursors acetate and butyrate to support d*e novo* fatty acid synthesis in the mammary gland (Jenkins & McGuire, 2006). The second theory is known as the glucogenic-insulin theory and attributes MFD to a shortage in supply of lipogenic precursors to the mammary gland. Microbial fermentation of diets rich in energy from grain sources results in increased concentrations of the glucogenic VFA, propionate, which undergoes gluconeogenesis in the liver to give rise to glucose. Increased blood glucose levels stimulate the release of insulin, which inhibits the mobilisation of lipids, and stimulates lipid synthesis in adipose tissue, thus diverting lipid precursors towards adipose tissue and away from the mammary gland, ultimately reducing milk fat synthesis (Sutton, 1989; Griinari e*t al.*, 1998). Recently the biohydrogenation theory has come to light, which attributes MFD to inhibitors of milk fat synthesis that form during altered ruminal biohydrogenation (Griinari *et al.*, 1998), specifically when feeding high grain diets low in effective fibre which enhance the production of *trans* – 10, c*is* – 12 conjugated linoleic acid (CLA) a potent inhibitor of milk fat synthesis (Griinari e*t al.*, 1998; Jenkins & McGuire, 2006).

The results for the present study can be found in Table 5.3. Neither milk fat percentage nor milk fat yield (kg/d) were altered by the dietary treatments. No MFD was observed in this study, with an average milk fat content across treatments of 4.31 % ± 0.113 %. Although somewhat high for Holsteins, this milk fat value falls within the range of 3.4 % to 4.6 % suggested by Cerbulis & Farrell (1974) for the breed and similar values have been observed in the studies of Benchaar *et al.* (2008), Aikman *et al.* (2011) and Cruywagen *et al.* (2015). The lack of MFD in this study suggests that there was no shortage of lipogenic precursors for milk fat synthesis in the mammary gland, this is supported by the ruminal fermentation data (Refer to Table 5.7) in which none of the dietary treatments reduced the ruminal concentrations of acetate or butyrate, and the acetate to propionate ratio was on average 3.55 which far exceeds that given in the review of Erdman (1988) who reported the greatest reductions in milk fat when the ratio was below 2.0.

Buffer supplementation has been shown to improve nutrient digestibility, ruminal fermentation and passage rate (Rogers *et al.*, 1982; Erdman & Sharma, 1989) which optimises the metabolic functions associated with milk production, thus alleviating MFD (Rogers *et al.*, 1982) and leading to increased milk production and milk fat content (Erdman, 1988). Acid Buf when included in concentrate rich, potentially acidotic TMR's at levels of 0.35 % DM to 0.4 % DM (i.e. 80 g/cow/d to 90 g/cow/d) has been reported to increase milk fat content with the concomitant increase in milk fat yield, due to increased milk production, with these increases exceeding those of experimental animals fed diets buffered with traditional buffers (Cruywagen *et al.*, 2004; Beya, 2007; Cruywagen *et al.*, 2015). The study of Beya (2007) found Acid Buf to have a strong tendency ($P = 0.06$) to increase milk fat content by 25 % compared to the control. In contrast, the studies of Calitz (2009), Bernard *et al.* (2014) and Wu *et al.* (2015) found no effect of Acid Buf on milk fat content or yield, this was likely due to the inability of the diets to induce MFD as in the present study.

Ionophore supplementation is widely known to reduce milk fat content. The study of Ipharraguerre & Clark (2003) found that when fed at a maximum dose of 350 mg/d, ionophores caused a 4.5 % reduction in milk fat concentration as compared to untreated cows. The exhaustive study of Duffield *et al.* (2008b) found monensin to decrease milk fat content by 3.0 % whilst having no effect on milk fat yield, in agreement McGuffey *et al.* (2001) summarised the finding from 11 studies, feeding monensin at an average of 300 mg/d and found milk fat percentage to decrease ($P < 0.01$) from 3.98 % to 3.78 %, whilst milk fat yield was unchanged. Other studies in which monensin was supplemented support these observations (Phipps e*t al.*, 2000; Benchaar *et al.*, 2006b; Odongo e*t al.*, 2007). Frequently the milk depressing effect of ionophores has been attributed to reduced ruminal acetate and butyrate, potentially resulting in a shortage of lipogenic precursors (Ipharraguerre & Clark, 2003; Van der Werf *et al.*, 1998), as well as to an inhibition of biohydrogenation of long-chain fatty acids (Fellner *et al.*, 1997). In the present study monensin intake averaged 237.6 mg/d, this low level of supplementation probably explains the lack of influence on milk fat. The papers of Kennelly & Lien (1997) and Duffield e*t al.* (2008b) highlight that stage of lactation, breed, diet composition, level of supplementation and delivery method are all factors which should be considered when assessing the likelihood of a response.

Supplementation with DFM's gives rise to variable and typically minor changes in milk composition (Krehbiel e*t al.*, 2003). The DFM supplemented in the present study contained a significant level of the yeast *S. cerevisiae*, the meta-analysis of Desnoyers e*t al.* (2009) found yeast cultures to have a tendency ($P = 0.099$) towards increased milk fat content (+ 0.05 %), however, Poppy *et al.* (2012) was unable to detect any significant effects of supplementation on milk fat yield (+ 0.06 kg/d). More often than not DFM supplemented diets do not have an effect on milk fat content, as observed in the present study. Others to report this lack of response upon feeding various DFM's, include Dann *et al.* (2000), Erasmus *et al.* (2005), Nocek *et al.* (2003), Raeth-Knight *et al.* (2007) and AlZahal *et al.* (2014) to name a few. In contrast, Leicester e*t al.* (2016) observed a tendency ($P = 0.07$) towards increase in milk fat yield, when feeding a DFM based on *S. cerevisiae*, *A. oryzae* and *B. subtilis* fermentation extract to early-lactation dairy cows, however this was not due to an increase in milk fat content but rather to an increase in milk production. Supplemental DFM's are proposed to improve nutrient, specifically fibre, digestibility (Kalebich & Cardoso, 2017) and thus potentially could enhance milk fat synthesis, although not often observed. The absence of a milk fat response in the present study may be as a result of the already high milk fat content, thus diminishing the ability of supplementation to elicit a positive treatment response, and the use of cows late in lactation which are typically less responsive to feed additives.

### 5.3.3 Milk protein and nitrogen fractions

Milk protein can be altered by dietary manipulation, however, when compared to the alteration possible in milk fat, the scope is much smaller, the reasons for this being that biologically the natural variation is smaller and that the factors relating to milk protein synthesis are poorly understood (Sutton, 1989). The nitrogenous fractions of milk can be divided into three categories; casein, whey protein, and non-protein N (NPN) (Rowland, 1983b; DePeters & Ferguson, 1992). Casein accounts for the majority of N in milk, with lesser amounts of whey protein and NPN, for the Holstein breed each fraction comprises on average 78.2 %, 16.9 % and 4.9 %, respectively, of total milk N (Cerbulis & Farrell, 1974). In the present study mean milk protein concentration was 3.26 % ± 0.046 %, with an observed 4.78 % relative decrease ($P = 0.04$) in milk protein concentration with monensin supplementation as compared to the control (MON = 3.19 % *versus* C = 3.35 %). Supplementation with either AB10 or DFM did not alter the milk protein concentration as compared to the other treatments, and no treatment differences were observed in milk protein yields across treatments.

Regarding the nitrogenous fractions of milk, supplementation with dietary additives had no influence on concentration or yield (Refer to Table 5.3). The results were not unexpected owing to the difficulty in manipulating the protein fraction of milk. The mean concentration across treatments of casein, whey protein, and NPN in the present study were 2.55 %, 0.50 % and 0.04 %, respectively and are in accordance with the breed values reported by Cerbulis & Farrell (1974) with the exception of NPN. The study of Gandra *et al.* (2010) in which milk protein fractions were measured in mid- to late-lactation cows, reported a mean value of 0.23 % for NPN.

**Table 5.4** Milk nitrogenous fractions, expressed as a percentage of total milk CP, of lactating dairy cows supplemented with Acid Buf 10, a direct-fed microbial and monensin when fed a total mixed ration (n =16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| | C | AB10 | DFM | MON | SEM[2] |
| Casein, % CP[3] | 78.7 | 78.6 | 77.4 | 77.9 | 1.22 |
| Whey protein, % CP | 14.0 | 13.9 | 16.3 | 17.3 | 1.25 |
| Non-casein nitrogen, % CP | 3.26 | 3.33 | 3.63 | 3.80 | 0.214 |
| Non-protein nitrogen, % CP | 1.12 | 1.17 | 1.05 | 1.10 | 0.061 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[3] CP = crude protein

When expressed as a percentage of total milk CP (Refer to Table 5.4), casein and whey protein are within the range reported by Cerbulis & Farrell (1974) but NPN in the present study is on average 1.11 % of

milk CP, which is far below the accepted value of 5 to 6 % reported by Cerbulis & Farrell (1974) and DePeters & Ferguson (1992). The reason for the extremely low values observed in the present trial is not known and is unexpected considering that the mean MUN (μ = 16.0 mg/dL) and ruminal ammonia-N concentrations (μ = 22.6 mg/dL) were on the higher end of the scale.

Generally, it appears that traditional dietary buffers do not consistently alter milk protein concentration and yield (Erdman *et al.*, 1982; Harrison *et al.*, 1989; Solorzano *et al.*, 1989), the same can be said for Acid Buf which failed to exert a milk protein response in the present study and the studies of Beya (2007), Calitz (2009), Bernard *et al.* (2014) and Cruywagen *et al.* (2015).

The decrease in milk protein content observed with monensin supplementation in the present study is not unusual and is corroborated by the findings of McGuffey *et al.* (2001) and Duffield *et al.* (2008b), who reported significant 0.05 and 0.03 percentage unit declines in milk protein percentage, respectively. Individual studies to observe this decline include the long – term studies of Phipps e*t al.* (2000) and Odongo *et al.* (2007). It has been proposed that the observed decline in milk protein percentage is due to dilution from the increased milk production observed in these studies (Phipps *et al.*, 2000), however this theory does not explain the reduction observed in the present study. The observed reduction in the present study may be explained by the reduced MCP observed with MON supplementation, although not measured directly in this study, this may be indicative of reduced microbial N flow to the small intestine (Refer to section *6.1 Microbial crude protein synthesis*). An increase in milk protein yield is often observed with monensin supplementation, with McGuffey e*t al.* (2001) reporting a 26 g/d increase and Duffield e*t al.* (2008b) a 0.016 kg/d increase, but this was not observed in the present study, and was likely a result of the inability of the MON treatment to alter milk yield relative to the control. There are few published studies detailing the effects of ionophore supplementation on the nitrogenous fractions of milk, however, one such study was found (Gandra e*t al.*, 2010) and supported the present observation that supplementation with monensin did not exact any changes on the milk N fractions.

Although not observed in the present study research points to DFM's having the potential to enhance milk protein content (Kalebich & Cardoso, 2017), as evidenced by the studies of Higginbotham *et al.* (1993; 2004), Nocek *et al.* (2003) and Leicester *et al.* (2016) who found that upon feeding DFM's based on *A. oryzae* extracts, a combination of *E. faecium* and *S. cerevisiae*, and *S. cerevisiae*, *A. oryzae* and *B. subtilis* fermentation extract, respectively, that milk protein content and yield could be increased. Our results are more in agreement with those of Desnoyers e*t al.* (2009) who reported that yeast cultures, a primary component of the DFM examined in this study, do not affect milk protein content.

When evaluating studies for the effect of additives on milk protein it is imperative to distinguish between responses which affect milk protein content and those affecting milk protein yield, as often positive responses in milk and protein yields are accompanied by decreases in milk protein content (Jenkins & McGuire, 2006).

### 5.3.4 Milk lactose

Lactose is the principal carbohydrate found in milk, and is a disaccharide composed of the monosaccharides glucose and galactose (McDonald *et al.*, 2011). Lactose is the primary osmole (i.e. osmotically active constituent) in milk (Sutton, 1989) and its synthesis is responsible for drawing water into milk during milk synthesis, owing to this relationship lactose content is the least variable milk component (NRC, 1988). Occasionally minute changes in lactose concentration are observed in response to dietary changes, however, these changes are inconsistent, insignificant and are not of practical value (Sutton, 1989). Such changes are typically only observed under extreme and unusual feeding situations and not when cows are fed according to normal standards (Jenkins & McGuire, 2006). Thus, is it generally accepted that milk lactose concentrations cannot be altered by dietary manipulation. This validates the results of the present study in which no difference in milk lactose content or yield was observed across dietary treatments. When fed sodium bicarbonate, a long-established dietary buffer, no change in the milk lactose content of dairy cows was observed (Erdman & Sharma, 1989; Rogers *et al.*, 1982), this is in agreement with the lack of treatment effect observed upon the inclusion of Acid Buf (Beya, 2007; Calitz, 2009; Bernard *et al.*, 2014; Cruywagen *et al.*, 2015). Although milk lactose content was not altered in these studies, some researchers did report increased milk lactose yields with Acid Buf supplementation as a result of increased milk yields (Beya, 2007; Cruywagen *et al.*, 2015). Most published research on ionophores (Phipps *et al.*, 2000; Erasmus e*t al.*, 2005; do Prado e*t*

*al.*, 2015) and DFM's (Kalebich & Cardoso, 2017) have found that milk lactose content and yields are unaffected by supplementation. The mean lactose content of this study was 4.97 % ± 0.023 % which is in agreement with the work of Cerbulis & Farrell (1974), who determined milk lactose content for Holsteins to be 4.93 % ± 0.61 % SD.

### 5.3.5 Milk urea nitrogen

Milk urea nitrogen is a management tool used to monitor the nutritional status of lactating dairy cows, particularly protein status (Jonker *et al.*, 1999). Research has established that the concentration of MUN is representative of surplus ruminal nitrogen not captured by the ruminal microbes, and that there is a significant relationship between MUN and the efficiency of protein utilisation by the dairy cow (Hof e*t al.*, 1997). Ruminal ammonia-N in excess of the microbial requirement and surplus N from the deamination of excess AA's and peptides are rapidly converted to urea (Swenson & Reece, 1993) in the liver, this urea then enters the circulatory system to become part of the blood urea N pool (Jonker *et al.*, 1998). The quantity of urea excreted in the urine is directly proportional to the concentration of urea in the blood (Ciszuk & Gebregziabher, 1994), with this being proportional to the concentration of urea present in milk (Roseler *et al.*, 1993). For this reason, MUN is a suitable non-invasive alternate to blood urea N (BUN). Milk urea nitrogen values can range from 8 mg/dL to 25 mg/dL (Drudik *et al.*, 2007) for individual cows, and can be difficult to interpret without a good, reliable baseline value, as values vary by factors such as season, month, parity and stage of lactation (Biswajit e*t al.*, 2011). When lactating dairy cows are fed well - balanced diets developed according to the NRC recommendations average MUN concentrations of 10 mg/dL to 16 mg/dL can be expected (Baker *et al.*, 1995; Jonker e*t al.*, 1998). This is in accordance with the mean MUN concentration of 16.0 mg/dL ± 1.13 mg/dL observed in this trial. No treatment differences were observed in this trial (Refer to Table 5.3) this was to be expected as the dietary treatments did not differ in ingredient or chemical composition. There are very few published studies detailing the effect of dietary feed additives on MUN from which comparisons can be drawn. Only two studies have examined the effects of Acid Buf on MUN (Bernard *et al.*, 2014; Wu e*t al.*, 2015), the work of Bernard e*t al.* (2014) found that the Acid Buf treatment had higher a MUN concentration than the unbuffered control diet ($P = 0.03$), but this was not observed in the present study. When feeding ionophores one would typically expect a reduction in the concentration of MUN owing to the inhibitive effect of ionophores on the microbial species involved in AA deamination (Russell & Strobel, 1989), however most published studies report no significant effect of ionophores on MUN concentrations when administered to lactating dairy cows fed rations similar in CP level and dose of monensin as used in this study (Benchaar *et al.*, 2006b; Martinuae *et al.*, 2007; do Prado e*t al.*, 2015). In agreement with this study, Dann *et al*. (2000), Raeth-Knight *et al*. (2007), Chiquette *et al*. (2015) and Malekkahi *et al*. (2016) reported no effect of supplementation with various DFM's on MUN concentrations.

### 5.3.6 Efficiency of milk production

The expression of feed efficiency in dairy cows is not standardised, with measures utilised across research including ratios such as milk yield (kg/d) to DMI (kg/d), FCM (kg/d) to DMI (kg/d), ECM (kg/d) to DMI (kg/d), and NE milk to NE intake. No matter the definition used, improvement of the efficiency of production is crucial to increasing the profitability of dairy production. In the present study, when expressed as either the quotient of milk yield (kg/d) and DMI (kg/d) or 4 % FCM (kg/d) and DMI (kg/d), there was no significant improvement in the efficiency if milk production in response to feed additive supplementation. However, there was an observed tendency for the efficiency of production to be higher with DFM supplementation (DFM = 1.28) as compared to non- supplemented cows (C = 1.20, $P = 0.07$) or cows supplemented with monensin (MON = 1.20, $P = 0.08$). This tendency towards an improved efficiency of production for DFM supplemented cows was not observed when efficiency was corrected for fat at a 4 % level.

This lack of improvement in efficiency was expected owing to the similar DMI and milk yields observed across treatments. Although not observed in the present study, Acid Buf has been shown to significantly ($P < 0.001$) improve the efficiency of feed conversion into milk by Cruywagen e*t al.* (2015) who found Acid Buf to produce more milk per kilogram of DMI than unbuffered diets or diets buffered with sodium bicarbonate. When supplemented with sodium monensin, lactating dairy cows typically display a numerical or significant improvement in milk production efficiency (Ipharraguerre & Clark, 2003). The meta-analysis of Duffield *et*

*al.* (2008b) reported an improvement in efficiency of approximately 2 % owing to the increase of DMI and milk yields, whilst the review of Ipharraguerre & Clark (2003) evaluated studies with large numbers of animals, and doses not exceeding 350 mg/cow/d and found efficiency to increase relatively by 11.4 % and 3.6 %, on high forage and high concentrate diets, respectively. These improvements in efficiency are due to the enhancement of the energetic value of feed through alteration of ruminal fermentation leading to increased propionate production, decreased methanogenesis, and increased DM digestibility (Erasmus e*t al.*, 1999). The lack of observed improvement in production efficiency upon ionophore supplementation observed in this trial is in agreement with the study of Yang *et al.* (2007) who when feeding monensin at 330 mg/cow/d to lactating dairy cows, fed a TMR comprised of 60 % concentrates and 40 % forage, found no improvement in efficiency. Very few published studies detail the effects of DFM's on milk efficiency, but reviews have suggested that both yeast-based DFM's (McAllister *et al.*, 2011) and bacterial based DFM's (Seo e*t al.*, 2010) are capable of improving production efficiency. With the improvements likely brought about through various interacting factors, such as the effect of these products on the stabilisation of ruminal fermentation and enhanced fibre digestibility. The tendency towards higher efficiency with DFM supplementation in the present trial, may be explained by the decrease in DMI, of approximately 1 kg as compared to the control, without a substantial increase in milk yield. This is in agreement with Poppy *et al.* (2012) who postulated that the improvement in efficiency with yeast culture supplementation in late-lactation cows was likely due to the decreased intakes.

## 5.4 Ruminal pH

Ruminal pH is one of the most variable factors influencing both microbial populations and VFA production, and reflects the dynamics of ruminal acid production, absorption, utilisation as well as the inherent buffering capacity of the ruminant (Erdman, 1988). For the maintenance of a well-balanced ruminal population ruminal pH should fall within the range of 5.8 to 6.4 (Ishler *et al.*, 1996), however, with high-yielding dairy cows fed high-concentrate rations rich in starch rumen pH may decrease well below 5.8 (Cruywagen e*t al.*, 2015). In the present study mean ruminal pH across treatments averaged 5.58 ± 0.029, which indicates that the highly fermentable basal diet was capable of reducing ruminal pH to values well below the ideal for optimal fermentation, but not unusual for TMR fed dairy cows. Although not directly examined in this study one could postulate that at this pH level the numbers and activity of both fibrolytic bacteria and protozoa dwindled, as these microbes are known to function optimally within a pH range of 6.2 to 6.8 and have shown declines when pH drops below 6.0, particularly for an extended time period (Hungate *et al.*, 1966; Ishler e*t al.*, 1996). As a result of the decline in the fibrolytic populations, a reduction in ruminal fibre-digestion is typically observed when ruminal pH falls below 5.9 (Russell & Dombrowski, 1980), although not investigated in this study it is unlikely that any substantial decrease in ruminal fibre digestion occurred as ruminal acetate concentrations were maintained at acceptable levels across dietary treatments (Refer to section *5.5.1.2 Acetate*). Accompanying the decline in fibrolytic populations, a shift towards amylolytic ruminal populations would be expected as these species thrive in a pH range of 5.2 to 6.0 (Ishler *et al.*, 1996). At low ruminal pH populations of acid tolerant LAB, e.g. *S. bovis*, typically increase leading to an accumulation of lactic acid (Russell & Hespell, 1981) however, this was not observed in the present study with only negligible quantities of lactic acid having been detected and therefore not reported.

**Table 5.5** Mean, maximum, and minimum ruminal pH and hours spent below pH 5.5 of lactating dairy cows, supplemented with Acid Buf 10, a direct-fed microbial and monensin when fed a total mixed ration (n = 16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| | C | AB10 | DFM | MON | SEM[2] |
| Mean pH | 5.55$^{d}$ | 5.64$^{c}$ | 5.55$^{d}$ | 5.59$^{cd}$ | 0.029 |
| Maximum pH | 5.86 | 6.02 | 5.84 | 5.91 | 0.067 |
| Minimum pH | 5.33 | 5.34 | 5.33 | 5.33 | 0.039 |
| Hours below pH 5.5 | 10.13$^{c}$ | 6.13$^{d}$ | 9.25$^{cd}$ | 7.13$^{cd}$ | 1.430 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[c, d] Means within a row with different subscripts tend to differ ($0.05 < P \leq 0.10$)

In the present study no significant differences were observed amongst dietary treatment for mean, minimum or maximum ruminal pH (Refer to Table 5.5). However, mean pH over time tended to be greater for the AB10 treatment (μ = 5.64) as compared to the C (μ = 5.55, *P* = 0.07) and DFM (μ = 5.55, *P* = 0.07) treatments, but did not differ from the MON treatment (μ = 5.59).

Research on sodium bicarbonate has established that this buffer effectively increases ruminal pH postprandially (Erdman *et al.*, 1982; Erdman, 1988) however, the buffering capacity of Acid Buf has not been as well researched. Upon feeding Acid Buf to lactating dairy cows fed a ration capable of inducing SARA, Beya (2007) reported no effect of this buffer on mean, minimum or maximum ruminal pH but did observe that the control tended (*P* = 0.11) to have lower ruminal pH than the buffered diets. In a similar study Cruywagen e*t al.* (2015) found no difference in mean daily pH and maximum pH between the unbuffered and Acid Buf treatments, but did observe the minimum pH to be higher (*P* = 0.04) for the Acid Buf treatment as compared to the unbuffered treatment. Caltiz (2009) studied the effect of Acid Buf alone and in combination with sodium bicarbonate both *in vivo* and *in vitro* and found Acid Buf to have a higher buffering capacity than sodium bicarbonate (*P* < 0.01). Whilst Cruywagen e*t al.* (2004) found ruminal pH to increase with increasing Acid Buf dosages, with the optimal level of supplementation being 80 g/d. Ionophores have the potential to improve ruminal pH when potentially acidotic rations are fed, by reducing the populations of the ionophore-sensitive LAB, particularly *S. bovis*, and in doing so reducing ruminal lactic acid concentration (Ipharraguerre & Clark, 2003). However, in most studies ruminal pH is unchanged in response to monensin supplementation for both dairy cows (Yang & Russell, 1993b; Ali-Haïmoud *et al.*, 1995; Erasmus *et al.*, 2005) and feedlot cattle (Guan e*t al.*, 2006), with responses only being pronounced in trials based on high concentrate diets or with animals undergoing adaptation. The response of ruminal pH to yeast culture supplementation has been variable (Chaucheyras-Durand *et al.*, 2008). Whist the meta-analysis of Desnoyers e*t al.* (2009) found supplementation with yeast-based products to be capable of increasing ruminal pH, on average by 0.03 units, it stated that effects were more pronounced in dairy cattle fed diets rich in concentrates, lower in NDF and having a higher level of DMI. This may explain the lack of response observed in the current study, as DMI's were lower than anticipated and the basal diet contained sufficient forage. Although several other studies have too failed to observe an effect of yeast cultures on ruminal pH both *in vitro* (Sullivan & Martin, 1999; Miller-Webster e*t al.*, 2002) and *in vivo* (Erasmus *et al.*, 1992; Erasmus *et al.*, 2005; Hristov e*t al.*, 2010). The variable effects of yeasts on ruminal pH stabilisation are likely related to the particular yeast strain, stage of lactation and the basal diet fed (McAllister *et al.*, 2011). The effect of bacterial DFM's and *A. oryzae* fed alone or in combination with yeasts on ruminal pH has also been variable, with many studies reporting no effect (Yoon & Stern, 1996; Higginbotham *et al.*, 2004; Raeth-Knight *et al.*, 2007). However, Nocek e*t al.* (2002) did observe an increase in minimum pH when feeding a combination of *L. plantarum* and *E. faecium*. Whilst Chiquette *et al.* (2015) found a combination of either *E. faecium* and *S. cerevisiae* or *L. lactis* to maintain greater ruminal pH during both adaptation and a SARA challenge.

Ruminal pH is influenced by feeding time and eating pattern, and is subject to diurnal variation. In the present study ruminal pH was measured at three hour intervals over a 24 hour period (Refer to Table 5.6 and Figure 5.1) but wasn't found to differ over time or with dietary treatments (*P* > 0.05). Only at 18:00 was ruminal pH found to be greater for the AB10 treatment as compared to the C (*P* = 0.047), DFM (*P* = 0.04) and MON (*P* = 0.003) treatments. Whilst the MON treatment resulted in lower ruminal pH than the C (*P* = 0.047) treatment and tended to have lower ruminal pH than the DFM treatment (*P* = 0.06). The highest pH values were observed at either 03:00 or 06:00 which is in agreement with Bargo *et al.* (2002) and Guedes *et al.* (2008) who observed maximal ruminal pH to be reached in the pre-feeding period. Cruywagen e*t al.* (2015) also observed an increase in ruminal pH during the early morning hours across treatments and attributed this rise to the type and quantity of substrate remaining in the rumen, suggesting that the rate of fermentation should be lower at this time and that salivary buffers assist in the recovery of ruminal pH. One hour post-feeding the ruminal pH started to decline for the AB10, DFM and MON treatments, with nadir pH values being reached at 12:00 for the DFM treatment, 15:00 for AB10 and at 18:00 for both MON and C treatments. This correlates to 4 hours after the morning feed and 1, and 4 hours post afternoon feed, when the rate of ruminal fermentation was high, and is in agreement with Enjalbert *et al.* (1999) and Guedes *et al.* (2008) who reported nadir pH values between two to five hours post-feed.

**Table 5.6** The effect of supplementation with Acid Buf 10, a direct-fed microbial and monensin on the ruminal pH profile, measured at three hour intervals over a 24 hour period, for lactating dairy cows fed a total mixed ration (n = 16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| Time | C | AB10 | DFM | MON | SEM[2] |
| 09:00 | 5.63 | 5.69 | 5.60 | 5.62 | 0.049 |
| 12:00 | 5.49 | 5.59 | 5.41 | 5.51 | 0.151 |
| 15:00 | 5.47 | 5.42 | 5.48 | 5.50 | 0.088 |
| 18:00 | 5.46[b] | 5.50[a] | 5.46 [bcd] | 5.41[ce] | 0.013 |
| 21:00 | 5.59 | 5.54 | 5.54 | 5.51 | 0.046 |
| 24:00 | 5.54 | 5.72 | 5.55 | 5.62 | 0.081 |
| 03:00 | 5.64 | 5.76 | 5.65 | 5.71 | 0.093 |
| 06:00 | 5.58 | 5.88 | 5.70 | 5.81 | 0.174 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve[FE], administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[a, b, c] Means within a row with different superscripts differ ($P < 0.05$)
[d, e] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

This post-prandial decline in ruminal pH after the morning feed is characteristic and unavoidable, but dietary buffers have been shown to reduce this decline (Beya, 2007, Cruywagen e*t al.*, 2015). However, this was not observed in this study, although the ruminal pH remained higher for the AB10 treatment the decline was no less severe than for the other treatments. The effect of the second feeding at 14:00 on ruminal pH was less clear. After 18:00 ruminal pH gradually increased to pre-feed levels. Overall Acid Buf appeared to buffer ruminal pH over time as compared to the other treatments, and this is likely due to its slow release feature.

Perhaps of greater importance than maximum, mean and nadir ruminal pH is the length of time for which ruminal pH continuously remains below pH 5.5. Sub-acute ruminal acidosis has been described in literature as occurring when ruminal pH falls between 5.0 and 5.8 (Beauchemin & Yang, 2005; Krause & Oetzel, 2006; Nagaraja & Titgemeyer, 2007) with the upper threshold varying amongst researchers. De Veth & Kolver (2001) suggested that the length of time that ruminal pH is suboptimal is likely more critical to the aetiology of SARA than is mean ruminal pH or brief fluctuations in pH below the threshold for SARA. In the present study SARA was defined as a continuous period in which ruminal pH persisted at levels below 5.5 (Krause & Oetzel, 2006) and it was observed that the AB10 treatment ($\mu$ = 6.13 hours) reduced the time ruminal pH spent below this threshold compared to all other treatments. However, this reduction only approached significance as compared to the C treatment ($\mu$ = 10.13 hours, $P = 0.095$). This data can be found in Table 5.5 and is illustrated in Figure 5.1, were one can clearly observe the duration pH was below 5.5 for each of the dietary treatments. The ability of Acid Buf to reduce the time ruminal pH is below the threshold has been previously observed by both Beya (2007) and Cruywagen *et al.* (2015). Both studies supplemented Acid Buf at a level of 90 g/cow/d to lactating dairy cows fed acidotic diets and by means of continuous ruminal pH monitoring found Acid Buf to significantly ($P < 0.001$) reduce the time spent below pH 5.5. In these studies cows supplemented with Acid Buf experienced suboptimal pH levels for only 4 hours as opposed to the 13 hours and 13.8 hours respectively, which cows fed the unbuffered spent below the SARA threshold. Acid Buf also proved more efficacious than the conventionally used sodium bicarbonate buffer. Active dry yeasts have been shown to reduce the period of time ruminal pH remains below the threshold for SARA. Thrune *et al.* (2009) examined the effect of an ADY (*S. cerevisiae*, $10^{10}$ cfu/ d) fed to lactating dairy cows and found it to reduce ($P < 0.05$) the time ruminal pH spent below pH 5.6 (C = 0.69 hr/d *versus* ADY = 0.06 hr/d), pH 5.8 (C = 1.68 hr/d *versus* ADY = 0.37 hr/d), and pH 6.0 (C = 3.81 h/d *versus* ADY = 1.56 hr/d). Whilst more recently Malekkahi e*t al.* (2016) found that an ADY (*S. cerevisiae*, 20 x $10^9$ cfu/ d) fed at 10 g/d, tended to reduce the time spent below pH 5.8 and 5.6 ($P = 0.09$ and $P = 0.07$, respectively). Bacterial DFM's may too have the ability to reduce SARA, as by supplementing *M. elsdenii* directly into the rumen, Aikman e*t al.* (2011) observed that fluctuations in ruminal pH were lessened as was the time ruminal pH was below 5.6, although these reductions were only numerical.

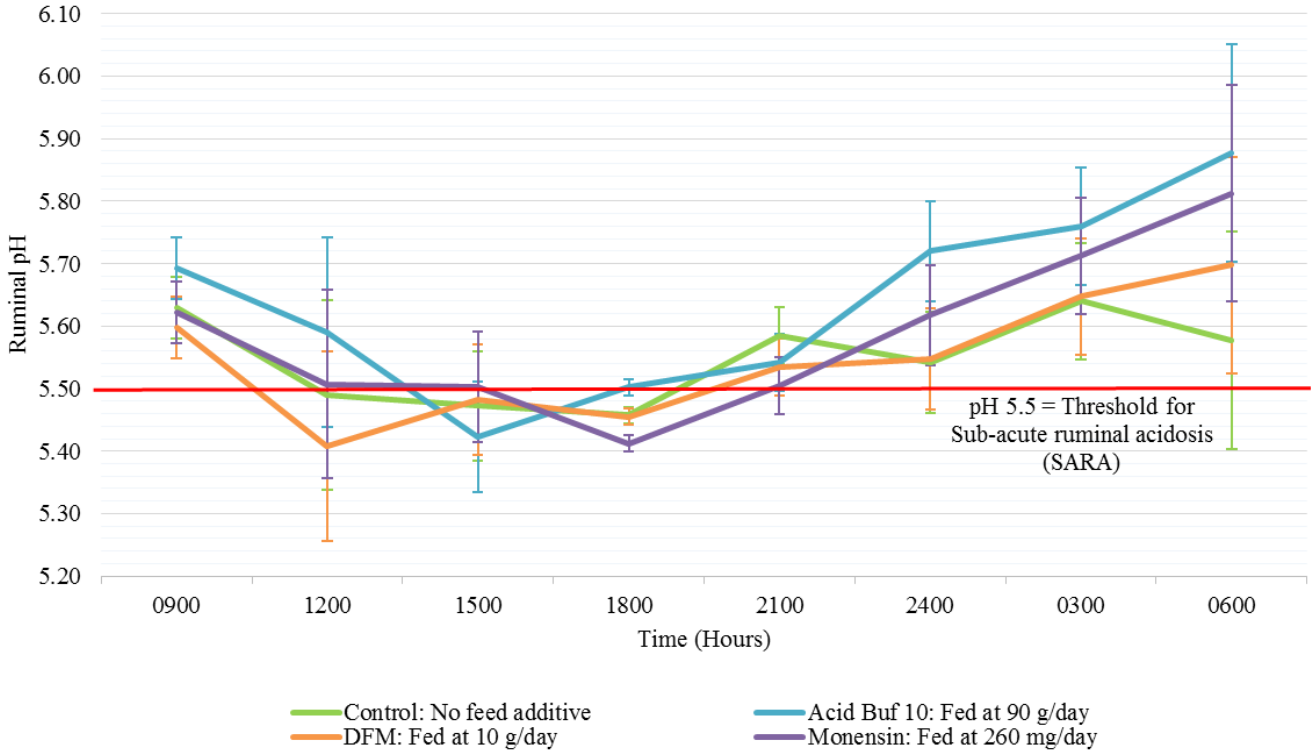


**Figure 5.1** The effect of Acid Buf 10, a direct-fed microbial and monensin on the ruminal pH profile, measured with a handheld pH meter at three hours intervals over a 24 hour period, of lactating dairy cows fed a total mixed ration *ad libitum*, as compared to the control (n = 16). Results are means ± SEM.

## 5.5 Ruminal fermentation

The results for the effect of dietary supplementation with feed additives on the various rumen parameters are presented in Table 5.7, these values are the mean values over all time periods. Data on the effects of supplementation at various time-points for each parameter will be presented and discussed in the respective sections.

### 5.5.1 Volatile fatty acid profile

The pattern of VFA's produced by the ruminal fermentation of OM have a major influence on the level of production and product composition, with the relative proportions in which VFA's are produced being influenced by factors such as substrate composition, substrate availability and the microbial species present (Dijkstra, 1994). These factors all interact complicating the interpretation of results at various time intervals over a 24 hour period. When interpreting results one should be mindful that the relative proportion of VFA's reported might not represent relative production rates, particularly at lower ruminal pH. Molar proportions of VFA's are generally assumed to represent the relative proportions in which they are present in the ruminal fluid, however, research has indicated that the absorption rates of the individual VFA's vary with ruminal pH and the concentration of ruminal VFA's, which itself is influenced by turnover rates (Dijkstra, 1994). As such this assumption is not necessarily valid across studies, with the molar proportions and relative ruminal concentrations diverging more when concentrate-rich rations are fed (Dijkstra, 1994).

**Table 5.7** Measurements of ruminal fermentation in lactating dairy cows, as affected by supplementation with Acid Buf 10, a direct-fed microbial and monensin, when fed a total mixed ration (n = 16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| | C | AB10 | DFM | MON | SEM[2] |
| Volatile Fatty Acids (mmol/L)[3] | | | | | |
| Acetate (A) | 67.0$^{d}$ | 70.1$^{cd}$ | 68.7$^{cd}$ | 71.2$^{c}$ | 1.21 |
| Propionate (P) | 19.6 | 20.4 | 20.2 | 19.1 | 0.66 |
| Butyrate | 9.35$^{b}$ | 10.11$^{ab}$ | 9.58$^{abd}$ | 10.37$^{ac}$ | 0.281 |
| Iso-butyrate | 0.76 | 0.73 | 0.74 | 0.76 | 0.054 |
| Valerate | 1.32 | 1.29 | 1.28 | 1.34 | 0.114 |
| Iso-valerate | 0.85$^{abd}$ | 0.92$^{ab}$ | 0.83$^{b}$ | 0.97$^{ac}$ | 0.044 |
| Total VFA | 99.0 | 103.5 | 101.3 | 103.8 | 1.83 |
| Volatile Fatty Acids (Molar %) | | | | | |
| Acetate | 67.8 | 67.7 | 67.9 | 68.6 | 0.44 |
| Propionate | 19.9$^{a}$ | 19.6$^{abc}$ | 19.9$^{a}$ | 18.4$^{bd}$ | 0.35 |
| Butyrate | 9.41 | 9.81 | 9.45 | 10.01 | 0.317 |
| Iso-butyrate | 0.76 | 0.71 | 0.72 | 0.72 | 0.056 |
| Valerate | 1.32 | 1.23 | 1.25 | 1.29 | 0.100 |
| Iso-valerate | 0.85 | 0.88 | 0.80 | 0.93 | 0.049 |
| A:P ratio | 3.45$^{b}$ | 3.52$^{abd}$ | 3.47$^{abd}$ | 3.77$^{ac}$ | 0.090 |
| Ammonia – N (mg/dL) | 20.7$^{b}$ | 21.8$^{abd}$ | 22.9$^{ab}$ | 25.2$^{ac}$ | 1.05 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[3] mmol/L is synonymous with mM
[a, b] Means within a row with different superscripts differ ($P < 0.05$)
[c, d] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

### 5.5.1.1 Total volatile fatty acids

In this study, mean ruminal total VFA's ranged from 101.3 mmol/L for cows supplemented with DFM, to 103.5 mmol/L and 103.8 mmol/L for cows supplemented with AB10 and MON, respectively, but these differences did not differ ($P > 0.05$) from the C treatment at 99.0 mmol/L (Refer to Table 5.7). These results were expected as most published studies report no effect of these additives on total VFA concentrations. To date the inclusion of Acid Buf to potentially acidotic dairy rations has not been shown to have an appreciable effect on total VFA production (Beya, 2007; Calitz, 2009), with both studies only observing a numerical increase in total VFA's with the inclusion of buffers, as in the present study. Cruywagen e*t al.* (2015) did however, report a substantial increase in total VFA concentrations ($P = 0.01$) when supplementing Acid Buf at 90 g/cow/d. The most universal response to monensin supplementation is an increase in propionate (As previously discussed in *Chapter 2.8 Ionophores*), however, this is a molar increase arising from a shift in ruminal VFA patterns and is typically not accompanied by an increase in total VFA's. Some *in vitro* studies have reported increased ruminal VFA concentrations (Richardson *et al.*, 1976; Chalupa *et al.*, 1980; Busquet *et al.*, 2005), however, *in vivo* most studies have found monensin to have no effect on total ruminal VFA's (Richardson e*t al.*, 1976; Yang & Russel, 1993b; Martineau e*t al.*, 2007; do Prado e*t al.*, 2015). The effect of supplementation of various DFM's on total ruminal VFA concentrations and VFA patterns has been highly variable. A meta- analysis study on yeast cultures reported increases in total VFA concentrations of 2.17 mmol/L (Desnoyers *et al.*, 2009) whilst Enjalbert e*t al.* (1999) reported a 20 % increase in the concentration of total VFA's in non-productive cows. However, the majority of studies report no significant effect of yeast

cultures on total VFA production in lactating dairy cows (Harrison *et al.*, 1988; Putnam *et al.*, 1997; Hristov *et al.*, 2009). Supplementation of lactating dairy cows with a combination of *A. oryzae* and yeast culture (Yoon & Stern, 1996), *A. oryzae* alone (Sievert & Shaver, 1993; Higginbotham *et al.*, 2004) or *M. elsdenii* (Aikman *et al.*, 2011; Weimer *et al.*, 2015) have too not been reported to alter total ruminal concentrations of VFA's.

**Table 5.8** The effect of supplementation with Acid Buf 10, a direct-fed microbial and monensin on the ruminal concentration of total volatile fatty acids (mmol/L), measured at three hour intervals over a 24 hour period, for lactating dairy cows fed a total mixed ration (n = 16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| Time | C | AB10 | DFM | MON | SEM[2] |
| 09:00 | 104.6 | 101.4 | 99.0 | 102.4 | 3.48 |
| 12:00 | 103.4 | 108.7 | 115.0 | 105.8 | 4.67 |
| 15:00 | 105.3[b] | 117.9[a] | 103.4[b] | 105.9[b] | 3.29 |
| 18:00 | 99.8 | 112.5 | 98.3 | 108.5 | 6.90 |
| 21:00 | 92.9 | 94.6 | 99.5 | 102.0 | 6.09 |
| 24:00 | 91.3[b] | 94.9[bc] | 102.1[ac] | 105.8[a] | 2.86 |
| 03:00 | 94.7 | 96.5 | 93.6 | 100.1 | 4.95 |
| 06:00 | 99.7 | 101.4 | 99.2 | 99.6 | 4.92 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve[FE], administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[a, b, c] Means within a row with different superscripts differ ($P < 0.05$)

Research has shown there to be an effect of diet and time on the ruminal concentration of total VFA's (Evans *et al.*, 1975). In the present study when measured at three hour intervals over a 24 hour period (See Table 5.8) treatment effects were observed at 15:00 and 24:00. At 15:00 the AB10 treatment had greater levels of total ruminal VFA's as compared to the C ($P = 0.03$), DFM ($P = 0.02$) and MON ($P = 0.04$) treatments. At 24:00 the C and AB10 treatments did not differ from each other but both differed from the MON treatment ($P = 0.01$ and $P = 0.04$, respectively), C also differed from the DFM (P = 0.04) treatment. Throughout the 24 hour period, the AB10 treatment displayed numerically higher total concentrations of VFA's, which is in agreement with Beya (2007) who found Acid Buf to numerically increase the concentration of the total VFA's. Peak VFA concentrations for all dietary treatments were observed at 12:00, which was 4 hours after the morning feed, and 15:00, which was 7 hours after the morning feed and approximately 1 hour after the afternoon feed. These increases persisted until about 18:00 after which they declined. These results are supported by Evans e*t al.* (1975) who found the concentration of total VFA's to increase post-prandially in both sheep and cows, with values obtained 1.5 hours to 5.5 hours post feeding to be greater than those observed before feeding and 7.5 hours post feeding. The lack of treatment by time interaction ($P = 0.54$) is possibly indicative of more regular ruminal fermentation and lessened post-prandial variation when supplementing diets with these additives, however, it could also be explained by meal pattern, as when fed a TMR *ad libitum* cows generally consume many small meals at shorter intervals which could prevent any large fluctuations in ruminal fermentation over time.

### 5.5.1.2 Acetate

Acetate is the principal VFA, predominating on high forage diets and has an important role in milk fat synthesis (Hsu & Fahey, 2005). In the present study, ruminal acetate concentrations did not differ across dietary treatments but concentrations tended ($P = 0.051$) to be greater for the MON treatment compared to C. No differences were observed in the molar percentages of acetate across dietary treatments with a mean value of 69.3 mmol/L ± 1.21 mmol/L (Refer to Table 5.7). The numerical increase in ruminal acetate concentrations with Acid Buf as compared to the control is in accordance with the study of Beya (2007). An increase in both acetate concentration ($P = 0.01$) and molar percentage ($P = 0.02$) was observed by Cruywagen *et al.* (2015), although higher acetate concentrations have been reported by Calitz (2009) when Acid Buf was fed in combination with sodium bicarbonate as opposed to Acid Buf alone ($P = 0.03$). Ionophore supplementation usually results in a decline in the concentration and molar proportions of acetate, this has been observed *in*

*vitro* (Richardson *et al.*, 1976; Busquet e*t al.*, 2005; Castillejos *et al.*, 2006) and *in vivo* (Richardson *et al.*, 1976; Yang & Russell, 1993b; Broderick, 2004; Wang e*t al.*, 2015) on both concentrate and forage rich diets. Contrary to this the present study found that monensin tended ($P = 0.05$) to increase acetate concentrations but that the molar percentage of acetate was unchanged, as has been previously reported by others (Ali-Haïmoud *et al.*, 1995; Martineau *et al.*, 2007; do Prado e*t al.*, 2015). The reason for the lack of response of monensin on ruminal acetate in this study could be due to the relatively low level of monensin fed which may have been incapable of influencing the ruminal microbiome (i.e. reducing the gram positive, fibrolytic population). The lack of effect of DFM's on ruminal acetate is commonly observed across literate, with studies on bacterial DFMs (Raeth-Knight *et al.*, 2007), *A. oryzae* (Higginbotham *et al.*, 1993; 2004), *M. elsdenii* (Aikman *et al.*, 2011), yeast cultures (Erasmus *et al.*, 1992; Putnam *et al.*, 1997; Enjalbert *et al.*, 1999) and ADY (Thrune *et al.*, 2009) failing to observe a response. The overall high levels of acetate observed in the present study are manifested in the high milk fat values observed (Refer to section *5.3.2 Milk fat*).

**Table 5.9** The effect of supplementation with Acid Buf 10, a direct-fed microbial and monensin on ruminal acetate concentration (mmol/L), measured at three hour intervals over a 24 hour period, for lactating dairy cows fed a total mixed ration (n = 16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| Time | C | AB10 | DFM | MON | SEM[2] |
| 09:00 | 71.0 | 68.6 | 66.7 | 69.6 | 2.21 |
| 12:00 | 69.7$^{e}$ | 72.9$^{de}$ | 77.8$^{d}$ | 73.6$^{de}$ | 2.84 |
| 15:00 | 71.0$^{b}$ | 79.4$^{a}$ | 71.0$^{b}$ | 71.9$^{b}$ | 1.91 |
| 18:00 | 68.7 | 77.0 | 66.2 | 74.5 | 4.48 |
| 21:00 | 63.1 | 65.3 | 67.4 | 70.3 | 4.61 |
| 24:00 | 61.9$^{b}$ | 64.1$^{bce}$ | 70.1$^{acd}$ | 73.0$^{a}$ | 2.13 |
| 03:00 | 63.6 | 65.8 | 63.5 | 68.3 | 3.17 |
| 06:00 | 67.4 | 67.6 | 67.0 | 68.4 | 2.70 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[a, b, c] Means within a row with different superscripts differ ($P < 0.05$)
[d, e] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

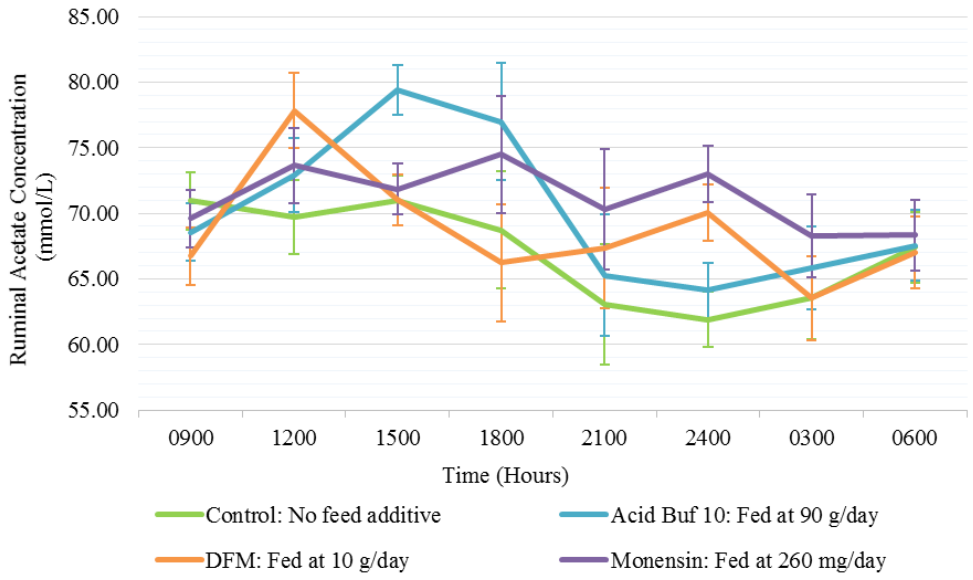


**Figure 5.2** The effect of Acid Buf 10, a direct-fed microbial and monensin on ruminal acetate concentrations (mmol/L), measured at three hour intervals over a 24 hour period, in lactating dairy cows fed a total mixed ration, *ad libitum*, as compared to the control (n = 16). Results are means ± SEM

As presented in Table 5.9 and illustrated in Figure 5.2 the concentration of acetate differed amongst dietary treatments over a 24 hour period. At 12:00 the DFM treatment tended ($P$ = 0.09) to have higher acetate concentrations than the C treatment. At 15:00 the AB10 treatment had greater ruminal acetate than the C ($P$ = 0.02), DFM ($P$ = 0.02) and MON ($P$ = 0.03) treatments. At 24:00 the C and AB10 treatments did not differ from each other, however, both DFM ($P$ = 0.04) and MON ($P$ = 0.01) treatments had greater ruminal acetate levels than C. The MON treatment also differed from the AB10 treatment ($P$ = 0.03) whilst the DFM treatment only tended to differ ($P$ = 0.095). In accordance with the total VFA concentrations, acetate concentrations appeared to be higher at 12:00, 15:00 and 18:00, which is to be expected as fermentation rates are most intense up to 5.5 hours after feeding (Evans *et al.*, 1975). Overall there appeared to be no interaction between sampling time and the dietary treatments ($P$ = 0.43).

### 5.5.1.3 Propionate

Propionate is the second most abundant VFA, produced from the fermentation of NSC (Hutjens, 2008) and is the principal precursor of glucose in ruminants (Ipharraguerre & Clark, 2003). The average propionate concentration and molar percentage for each dietary treatment over time in the present study is presented in Table 5.7. Dietary treatments had no effect on the ruminal concentrations of propionate, which averaged 19.82 mmol/L ± 0.66 mmol/L across treatments, however, the molar percentage of propionate was altered by dietary treatments. Contrary to the characteristic increase of the molar proportion of propionate with ionophore feeding, the present study found the MON treatment to have a lower propionate molar percentage (μ = 18.44 %) as compared to the C (μ = 19.88 %, $P$ = 0.03) and DFM (μ = 19.88 %, $P$ = 0.03) treatments, whilst tending to be lower than for the AB10 treatment (μ = 19.64 %, $P$ = 0.05). Typically, ionophores enhance propionate production through alteration of the ruminal microbiome, by enriching the ionophore resistant gram-negative populations (McGuffey *et al.*, 2001) and altering the metabolism of these populations (Bergen & Bates, 1984). Richardson e*t al.* (1976) investigated the effects of monensin *in vitro* across a range of doses (0.1 ppm to 25 ppm) and found this ionophore to increase propionate across all doses regardless of whether a concentrate or roughage substrate system was utilised, this was later confirmed by the *in vitro* studies of Chalupa *et al.* (1980), Busquet *et al.* (2005) and Castillejos e*t al.* (2006). The *in vivo* studies of Richardson e*t al.* (1976) corroborated the *in vitro* results, with increases in propionate being observed at all examined doses (25 mg/cow/d to 500 mg/cow/d) and persisting over a 148 day cattle response trial. Other more recent *in vivo* studies corroborating this observation in lactating dairy cows include Ali-Haïmoud *et al.* (1995), Erasmus *et al.* (2005) and do Prado *et al.* (2015). No studies were found which reported a decrease as observed in the present study, however, upon ionophore supplementation at 24 mg/kg DM to mid-lactation Holstein dairy cows fed a TMR, Martineau *et al.* (2007) reported no change in ruminal propionate proportions. Generally traditional dietary buffers reduce the molar percentage of propionate (Erdman *et al.*, 1982) in concentrate –rich diets and Acid Buf has been reported to have the same effect by Cruywagen e*t al.* (2015), who found decreases in the both the concentration and molar percentage of propionate. In agreement, with the present study Beya (2007) reported Acid Buf to have no effect on ruminal propionate. The effect of DFM's on ruminal propionate is still to be fully elucidated and is complicated by the highly variable composition of commercial DFM products. Yeast cultures have been reported to either increase propionate (Harrison *et al.*, 1988; Enjalbert *et al.*, 1999; Erasmus *et al.*, 2005) or to have no significant effect (Erasmus e*t al.*, 1992; Thrune e*t al.*, 2009; Hristov e*t al.*, 2010). Bacterial DFM's have been shown to increase ruminal propionate concentrations but responses differ based on the combination of species utilised (Krehbiel e*t al.*, 2003).

When measured at three hour intervals over a 24 hour period (Refer to Table 5.10 and Figure 5.3) a treatment effect was observed only at 15:00 where the AB 10 treatment tended to have a higher ruminal propionate levels than the MON treatment ($P$ = 0.08). Similar to ruminal total VFA's and acetate, peak ruminal propionate concentrations were observed at 12:00 to 15:00, which represent 4 hours after the morning feed and 1 hours after the afternoon feed and is in agreement with Wang e*t al.*, 2015, who found propionate to increase post-prandially.

**Table 5.10** The effect of supplementation with Acid Buf 10, a direct-fed microbial and monensin on ruminal propionate concentration (mmol/L), measured at three hour intervals over a 24 hour period, for lactating dairy cows fed a total mixed ration (n = 16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| Time | C | AB10 | DFM | MON | SEM[2] |
| 09:00 | 20.6 | 18.5 | 19.4 | 18.8 | 1.05 |
| 12:00 | 20.8 | 22.2 | 22.0 | 18.4 | 1.50 |
| 15:00 | 21.0[cd] | 24.3[c] | 20.4[cd] | 20.0[d] | 1.49 |
| 18:00 | 18.8 | 21.7 | 20.6 | 20.0 | 1.70 |
| 21:00 | 18.6 | 17.4 | 20.0 | 18.6 | 1.18 |
| 24:00 | 18.6 | 19.0 | 18.5 | 19.5 | 1.16 |
| 03:00 | 19.5 | 18.6 | 19.7 | 18.6 | 1.60 |
| 06:00 | 19.2 | 21.1 | 20.7 | 19.00 | 1.87 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve[FE], administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[c, d] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

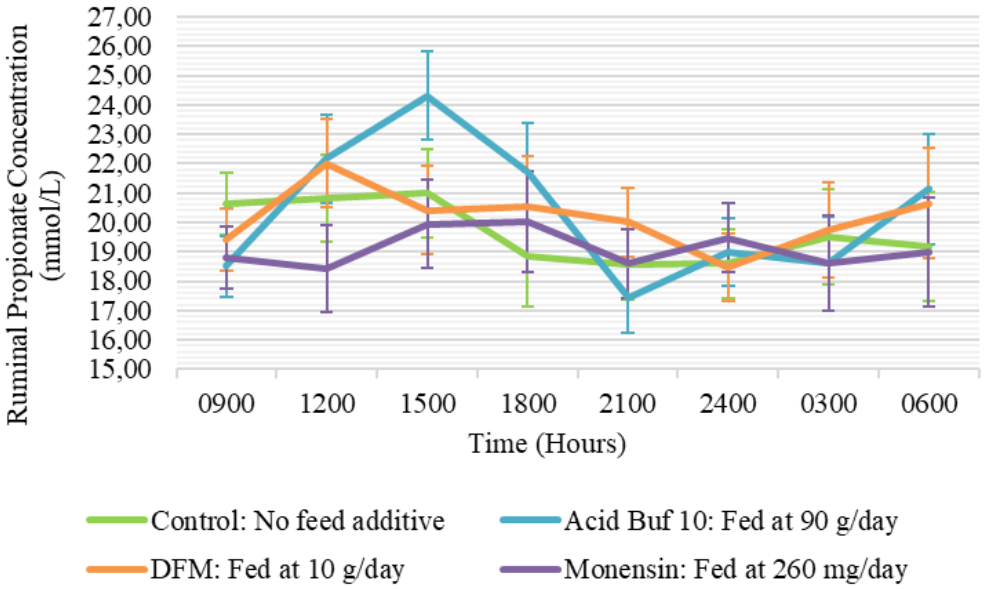


**Figure 5.3** The effect of Acid Buf 10, a direct-fed microbial and monensin on ruminal propionate concentrations (mmol/L), measured at three hour intervals over a 24 hour period, in lactating dairy cows fed a total mixed ration, *ad libitum*, as compared to the control (n = 16). Results are means ± SEM

### 5.5.1.4 Acetate to propionate ratio

In this study the acetate to propionate ratio for the MON treatment was higher (3.77) than compared to all other dietary treatments (C = 3.45, AB10 = 3.52, DFM = 3.47), it differed from the C ($P = 0.04$) and tended to differ from the AB10 ($P = 0.10$) and DFM ($P = 0.06$) treatments (Refer to Table 5.7). The observed increase in the acetate to propionate ratio with MON supplementation is atypical but is explained by the acetate and propionate data. Mediated by the alteration of the ruminal microbiome, monensin supplementation typically shifts ruminal VFA patterns towards a reduced lipogenic VFA (i.e. acetate and butyrate) to glucogenic VFA (i.e. propionate) ratio. This shift in VFA patterns is a hallmark of increased energy availability to the dairy cow (Russell & Strobel, 1989; Callaway *et al.*, 2003) as propionate is a highly efficient precursor for gluconeogenesis, and the shift in microbial populations reduces energy wasteful methanogenesis by diverting $H_2$ to more efficient pathways (McGuffey *et al.*, 2001). Reduced acetate to propionate ratios when feeding monensin to lactating dairy cows have been observed by Erasmus *et al.* (2005), Martineau *et al.* (2007) and do

Prado *et al.* (2015). In agreement with the results of the present study Broderick (2004) observed a small increase in the acetate to propionate ratio when supplementing monensin to lactating dairy cows at a level of 10 mg/kg/d, and attributed the small magnitude of response on ruminal fermentation to the relatively low level of monensin fed. The levels of monensin fed in this study were similar to those of the present study and may explain the observed lack of effect of monensin on the principal ruminal VFA's. In the present study Acid Buf did not influence the acetate to propionate ratio which is in agreement with Beya (2007) but Cruywagen *et al.* (2015) did observe an increased ratio with Acid Buf. The meta-analysis of Desnoyers *et al.* (2009) found yeast supplementation to have no influence on the acetate to propionate ratio, however some studies have found yeast cultures to reduce the ratio (Harrison *et al.*, 1988; Enjalbert *et al.*, 1999). The effect of bacterial strains and *A. oryzae* both of which are ingredients in the DFM utilised in this study have not yet been shown to consistently alter ruminal VFA patterns.

**Table 5.11** The effect of supplementation with Acid Buf 10, a direct-fed microbial and monensin on the ruminal acetate to propionate ratio, over a 24 hour period, for lactating dairy cows fed a total mixed ration (n = 16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| Time | C | AB10 | DFM | MON | SEM[2] |
| 09:00 | 3.44 | 3.82 | 3.45 | 3.73 | 0.150 |
| 12:00 | 3.34[b] | 3.38[b] | 3.61[abd] | 4.08[ac] | 0.169 |
| 15:00 | 3.42 | 3.28 | 3.59 | 3.65 | 0.227 |
| 18:00 | 3.73[a] | 3.65[abc] | 3.22[bd] | 3.77[a] | 0.141 |
| 21:00 | 3.44 | 3.90 | 3.38 | 3.80 | 0.209 |
| 24:00 | 3.37 | 3.38 | 3.88 | 3.79 | 0.221 |
| 03:00 | 3.30 | 3.54 | 3.28 | 3.73 | 0.211 |
| 06:00 | 3.53 | 3.21 | 3.34 | 3.62 | 0.181 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[a, b] Means within a row with different superscripts differ ($P < 0.05$)
[c, d] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

When measured at three hour intervals over a 24 hour period (Refer to Table 5.11) treatment differences were observed at 12:00 and 18:00. At 12:00 there were no differences in the acetate to propionate ratio amongst the C, AB10 and DFM treatments but MON differed from the C ($P = 0.02$) and AB10 treatments ($P = 0.03$) and tended to differ from the DFM treatment ($P = 0.096$). At 18:00 the acetate to propionate ratio for the DFM treatment was lower than for all other treatments, differing from the C ($P = 0.04$) and MON ($P = 0.03$) treatments and tending to differ from the AB10 ($P = 0.07$) treatment.

### 5.5.1.5 Butyrate

Butyrate is the third most abundant VFA, comprising 5 % to 15 % of the total VFA's produced (McDonald *et al.*, 2011). Butyrate is a lipogenic VFA involved in the synthesis of fatty acids in the mammary gland (Ishler *et al.*, 1996), thus maintaining adequate concentrations is important for the prevention of MFD. In the present study, butyrate concentrations were shown to be altered by the various dietary treatments. The mean butyrate concentration for each dietary treatment over time is presented in Table 5.7. The average butyrate concentration was higher for the MON treatment than the C treatment (MON = 10.37 *versus* C = 9.35, $P = 0.04$) and there was a tendency for the concentration of butyrate to be higher for the MON treatment compared to the DFM treatment ($P = 0.09$). No treatment differences were observed when butyrate was expressed as a molar percentage. Although all treatments increased the concentration of butyrate, only a significant effect of MON was observed. This outcome was unexpected as most studies have reported a decline in the concentration and molar proportions of butyrate with sodium monensin supplementation both *in vitro* (Richardson *et al.*, 1976; Busquet e*t al.*, 2005; Castillejos *et al.*, 2006) and *in vivo* (Broderick, 2004; do Prado *et al.*, 2015) when supplemented at doses similar to those used in the present study, 10 mg/kg DM to 16 mg/kg DM. The reason for this reduction being that ionophores selectively inhibit gram-positive bacterial species

which produce butyrate, in addition to acetate, as end products of fermentation (Russell & Strobel, 1989). One trial which supported the outcome of the present trial was that of Erasmus *et al.* (2005) who upon supplementing monensin, at a dose of 10 mg/kg DM, to early-lactating Holstein-Friesian cows fed a TMR twice daily, observed a numerical ($P = 0.11$) increase in the concentration of butyrate. The lack of a treatment response of ruminal butyrate to Acid Buf was in agreement with the study of Calitz (2009), however, Cruywagen *et al.* (2015) observed an increase in the concentration of butyrate ($P = 0.001$) and a tendency towards to increased molar proportions when supplementing lactating dairy cows with Acid Buf. The lack of effect of DFM supplementation on butyrate was expected with the vast majority of studies on bacterial species (Raeth-Knight *et al.*, 2007), *A. oryzae* (Higginbotham, 2004), *M. elsdenii* (Aikman e*t al.*, 2011) and yeast cultures (Putnam *et al.*, 1997; Erasmus *et al.*, 2005; Hristov *et al.*, 2010), all of which are included in many commercial preparations of DFM's, reporting no alteration of ruminal butyrate. Contrary to these studies, upon supplementation of an ADY to late-lactation Holsteins, Thrune e*t al.* (2009) observed an increase in the molar proportion of butyrate ($P < 0.05$).

**Table 5.12** The effect of supplementation with Acid Buf 10, a direct-fed microbial and monensin on ruminal butyrate concentration (mmol/L), measured at three hour intervals over a 24 hour period, for lactating dairy cows fed a total mixed ration (n = 16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| Time | C | AB10 | DFM | MON | SEM[2] |
| 09:00 | 10.1 | 11.1 | 9.93 | 10.7 | 0.491 |
| 12:00 | 9.89$^{d}$ | 10.6$^{cd}$ | 11.8$^{c}$ | 10.8$^{cd}$ | 0.680 |
| 15:00 | 10.2$^{cd}$ | 11.0$^{c}$ | 9.7$^{d}$ | 10.7$^{cd}$ | 0.476 |
| 18:00 | 9.19 | 10.8 | 9.16 | 11.0 | 0.896 |
| 21:00 | 8.70 | 9.65 | 9.19 | 10.2 | 0.560 |
| 24:00 | 8.31$^{b}$ | 9.21$^{abd}$ | 10.4$^{ac}$ | 10.4$^{ac}$ | 0.386 |
| 03:00 | 8.88$^{ab}$ | 8.94$^{ab}$ | 7.95$^{b}$ | 9.92$^{a}$ | 0.416 |
| 06:00 | 9.51 | 9.68 | 8.57 | 9.31 | 0.491 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[a, b] Means within a row with different superscripts differ ($P < 0.05$)
[c, d] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

As presented in Table 5.12 the concentration of butyrate differed amongst dietary treatments over a 24 hour period. At 12:00 there was a tendency for the DFM treatment to have higher ruminal butyrate concentration than the C treatment ($P = 0.09$), whilst at 15:00 there was a tendency for the AB10 treatment to have higher concentrations relative to the DFM treatment ($P = 0.08$). At 24:00 there was a marked difference in ruminal butyrate between the C treatment and both the DFM ($P = 0.01$) and MON ($P = 0.01$) treatments, and a tendency towards lower butyrate with AB10 supplementation as opposed to DFM ($P = 0.07$) or MON ($P = 0.08$) supplementation was observed. At 03:00 the DFM treatment had lower butyrate concentrations relative to the MON treatment ($P = 0.02$). Although DFM supplementation increased the concentration of butyrate relative to C at both 12:00 and 24:00 overall supplementation did not enhance butyrate concentrations.

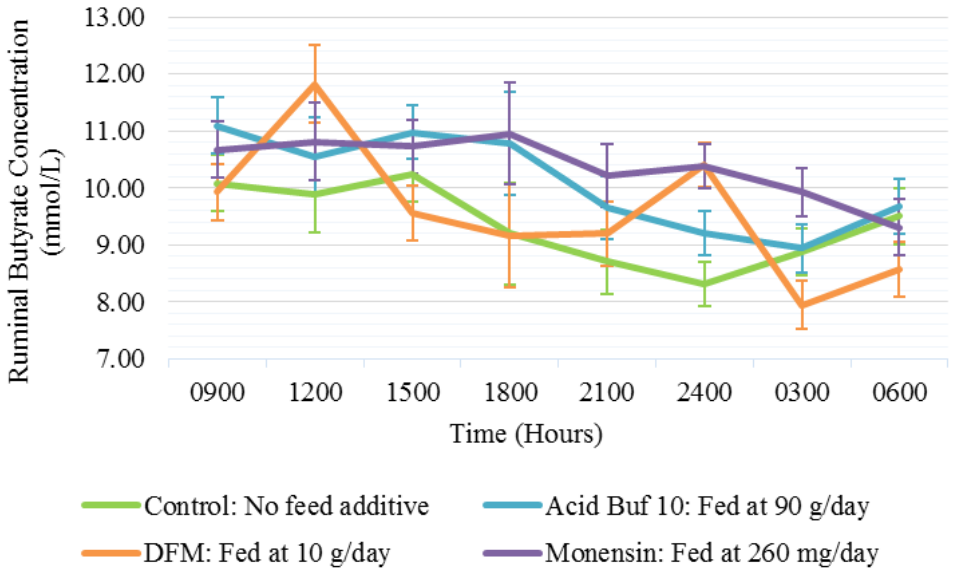


**Figure 5.4** The effect of Acid Buf 10, a direct-fed microbial and monensin on ruminal butyrate concentrations (mmol/L), measured at three hour intervals over a 24 hour period, in lactating dairy cows fed a total mixed ration, *ad libitum*, as compared to the control (n = 16). Results are means ± SEM

### 5.5.1.6 Isoacids

Valerate, iso-valerate and iso-butyrate are collectively known as isoacids, these VFA's although produced in small quantities are an important nutrient for the cellulolytic bacteria and are said to have an overall positive effect on ruminal fermentation (Andries *et al.*, 1987).

#### 5.5.1.6.1 Valerate

Valerate is the fifth most abundant VFA arising from the microbial fermentation of structural and non-structural carbohydrates and is of lesser importance than the previously discussed VFA's (Ishler e*t al.*, 1996). The average valerate concentration for each dietary treatment over time in the present study is presented in Table 5.7 as is the average valerate concentration expressed as a molar percentage. In this study dietary treatments had no effect on the concentration or molar proportion of valerate, with a mean concentration across treatments of 1.31 mmol/L ± 0.113 mmol/L. This response, or lack thereof, is in accordance with published literature, which reports no effects of Acid Buf on ruminal valerate concentrations (Calitz, 2009) and typically no effect of monensin or DFM's either. *In vitro* valerate production has been shown to be unaffected by monensin supplementation although the molar proportion was increased (Whetstone *et al.*, 1981). This lack of effect has been confirmed *in vivo* by the study of Ali-Haïmoud *et al.* (1995) in a 3 x 3 Latin Square design, supplementing MON at 33ppm to 60:40 TMR fed cows, and by Broderick (2004) and Wang *et al.* (2015) who found no change in the molar percentage and concentration of valerate, respectively. Contrary to this some researchers have found the concentrations of valerate to decline with monensin supplementation *in vitro* (Richardson *et al.*, 1976; Busquet *et al.*, 2005) while the *in vivo* study of Erasmus *et al.* (2005) found that monensin had the tendency ($P = 0.07$) to increase ruminal valerate concentrations. Typically supplementation with yeast cultures (Erasmus e*t al.*, 1992; Putnam e*t al.*, 1997; Malekkahi *et al.*, 2016), *A. oryzae* (Higginbotham *et al.*, 1993, 1994, 2004), *M. elsdenii* (Aikman *et al.*, 2011) and bacterial DFM's (Raeth-Knight *et al.*, 2007) have no effect on ruminal valerate concentrations or molar proportions, thus DFM products comprised of a combination of these should effect a similar lack of response, as was observed in the present study.

**Table 5.13** The effect of supplementation with Acid Buf 10, a direct-fed microbial and monensin on ruminal valerate concentration (mmol/L), measured at three hour intervals over a 24 hour period, for lactating dairy cows fed a total mixed ration (n = 16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| Time | C | AB10 | DFM | MON | SEM[2] |
| 09:00 | 1.36 | 1.32 | 1.36 | 1.51 | 0.188 |
| 12:00 | 1.35 | 1.34 | 1.58 | 1.24 | 0.128 |
| 15:00 | 1.50 | 1.48 | 1.14 | 1.53 | 0.191 |
| 18:00 | 1.32 | 1.43 | 1.21 | 1.43 | 0.146 |
| 21:00 | 1.20[ab] | 0.98[b] | 1.41[a] | 1.26[ab] | 0.119 |
| 24:00 | 1.04 | 1.15 | 1.24 | 1.21 | 0.135 |
| 03:00 | 1.28 | 1.30 | 1.06 | 1.38 | 0.225 |
| 06:00 | 1.48 | 1.29 | 1.28 | 1.17 | 0.230 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve[FE], administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[a, b] Means within a row with different superscripts differ ($P < 0.05$)

When measured at three hour intervals over a 24 hour period, a treatment effect was observed at 21:00 ($P = 0.04$), at this time the ruminal valerate concentration for AB10 was lower than that for the DFM treatment (AB10 = 0.98 mmol/L *versus* DFM = 1.41 mmol/L), but neither treatment differed ($P > 0.05$) from the control or MON treatments (Refer to Table 5.13).

### 5.5.1.6.2 The branched-chain volatile fatty acids

Iso-butyrate and iso-valerate are BCVFA's formed in the rumen as a result of the microbial deamination of the AA's Val and Ile, respectively (McDonald e*t al.*, 2011). These BCVFA's are typically produced in small quantities and are a minor proportion of the total VFA's, for this reason there is limited published information on the effects of dietary additives on these VFA's as compared to the predominant, previously discussed, VFA's. The average iso-butyrate and iso-valerate concentrations for each dietary treatment over time in the present study are presented in Table 5.7, as is the average concentration of both BCFA's expressed as a molar percentage. Dietary treatment had no effect on the concentration or molar proportion of iso-butyrate, with a mean concentration across treatments of 0.75 mmol/L ± 0.054 mmol/L which is typical of values observed across literature, whilst iso-valerate concentrations were shown to be altered by the various dietary treatments. The iso-valerate concentration for the MON treatment was higher than that for the DFM treatment ($P = 0.047$) and tended to be higher than that of C treatment ($P = 0.09$), with no treatment differences being observed when iso-valerate was expressed as a molar percentage.

The lack of response of the BCVFA's to supplementation with Acid Buf was expected, and in agreement with the study of Calitz (2009) who found that supplementation of lactating dairy cows fed a potentially acidotic diet did not influence the concentrations of the BCVFA's. The effect of monensin on the BCVFA's has been inconsistent, the study of Wang e*t al.* (2015) reported that when feeding Holstein heifers monensin iso-butyrate was not altered, as observed in the present study. In support is the *in vitro* study of Yang & Russell (1993b) who reported BCVFA's to be unchanged by monensin supplementation, however, others have reported a decrease in the concentration of BCFVA's *in vitro* (Busquet e*t al.*, 2005; Castillejos *et al.*, 2006). In the present study MON increased the concentration of iso-valerate as compared to both the control and DFM treatments, this rise in concentration is contrary to the majority of studies which found a decrease in the concentration of iso-valerate in response to monensin supplementation both *in vitro* (Richardson e*t al.*, 1976) and *in vivo* (Richardson *et al.*, 1976; Wang *et al.*, 2015). The response of the BCVFA's to DFM supplementation have been variable, with most studies concluding that yeast cultures (Erasmus *et al.*, 1992; Thrune *et al.*, 2009; Hristov *et al.*, 2010), bacterial (Raeth-Knight *et al.*, 2007) and fungal (Takiya *et al.*, 2017) based DFM's have no effect on the BCVFA's. Some studies have, however, reported decreases in the concentration and molar proportions of iso-valerate when supplemented with yeast cultures (Carro *et al.*, 1992; Harrison *et al.*, 1988) or *A. oryzae* based products (Yoon & Stern, 1996).

When measured at three hour intervals over a 24 hour period, no treatment by time interaction was observed for any of the dietary treatments on the ruminal concentration of iso-butyrate (Refer to Table 5.14).

**Table 5.14** The effect of supplementation with Acid Buf 10, a direct-fed microbial and monensin on ruminal iso-butyrate concentration (mmol/L), measured at three hour intervals over a 24 hour period, for lactating dairy cows fed a total mixed ration (n = 16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| Time | C | AB10 | DFM | MON | SEM[2] |
| 09:00 | 0.75 | 0.80 | 0.83 | 0.93 | 0.105 |
| 12:00 | 0.74 | 0.76 | 0.68 | 0.74 | 0.136 |
| 15:00 | 0.79 | 0.79 | 0.72 | 0.81 | 0.083 |
| 18:00 | 0.72 | 0.74 | 0.61 | 0.77 | 0.080 |
| 21:00 | 0.65 | 0.59 | 0.77 | 0.67 | 0.088 |
| 24:00 | 0.76 | 0.66 | 0.94 | 0.73 | 0.127 |
| 03:00 | 0.69 | 0.76 | 0.72 | 0.79 | 0.046 |
| 06:00 | 0.93 | 0.73 | 0.64 | 0.65 | 0.137 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean

However, the concentration of iso-valerate was shown to differ amongst dietary treatments over a 24 hour period (Refer to Table 5.15). At 15:00 the concentration of iso-valerate was lower for the DFM treatment relative to AB10 ($P$ = 0.04) and MON ($P$ = 0.02), this persisted at 18:00 with the DFM treatment differing from the C treatment ($P$ = 0.03) whilst tending to differ from the AB10 and MON treatments ($P$ = 0.08). At 21:00 the AB10 treatment tended to have lower ruminal iso-valerate than the MON treatment ($P$ = 0.08) but did not differ from the C and DFM treatments. At 24:00 it was observed that the C treatment tended to present lower iso-valerate concentrations relative to the DFM ($P$ = 0.08) and MON ($P$ = 0.09) treatments. At 03:00 there was no difference between the C and DFM treatments in iso-valerate concentrations, but both differed from the AB ($P$ = 0.03 and $P$ = 0.01, respectively) and MON ($P$ = 0.048 and $P$ = 0.02, respectively) treatments.

**Table 5.15** The effect of supplementation with Acid Buf 10, a direct-fed microbial and monensin on ruminal iso-valerate concentration (mmol/L), measured at three hour intervals over a 24 hour period, for lactating dairy cows fed a total mixed ration (n = 16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| Time | C | AB10 | DFM | MON | SEM[2] |
| 09:00 | 0.88 | 1.14 | 0.98 | 0.93 | 0.099 |
| 12:00 | 0.86 | 0.95 | 1.08 | 0.95 | 0.107 |
| 15:00 | $0.77^{ab}$ | $0.94^{a}$ | $0.56^{b}$ | $1.02^{a}$ | 0.106 |
| 18:00 | $0.97^{a}$ | $0.86^{abc}$ | $0.56^{bd}$ | $0.86^{abc}$ | 0.101 |
| 21:00 | $0.74^{cd}$ | $0.64^{d}$ | $0.77^{cd}$ | $0.90^{c}$ | 0.086 |
| 24:00 | $0.67^{d}$ | $0.76^{cd}$ | $0.98^{c}$ | $0.97^{c}$ | 0.105 |
| 03:00 | $0.73^{b}$ | $1.10^{a}$ | $0.64^{b}$ | $1.05^{a}$ | 0.092 |
| 06:00 | 1.18 | 0.96 | 1.05 | 1.11 | 0.146 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[a, b] Means within a row with different superscripts differ ($P < 0.05$)
[c, d] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

### 5.5.2 Ammonia-Nitrogen

Ruminal ammonia – N arises from the deamination of AA's obtained from the microbial fermentation of dietary protein. Various factors influence ruminal ammonia-N concentration such as ruminal pH, frequency of feeding, N recycling, as well as the fermentative capacity of the diets, which encompasses the type of dietary

carbohydrate and protein and the extent to which the dietary protein can be degraded (Satter & Roffler, 1975). The optimal ruminal ammonia concentration is one which results in either the maximum rate of ruminal fermentation or that which allows the maximum production of MCP per unit of substrate fermented (Mehrez e*t al.*, 1977). Ruminal ammonia is the principal N source utilised by the ruminal microbes for the synthesis of protein for growth and fermentation (Owens & Bergen, 1983; Erdman *et al.*, 1986) with the requirement being related to substrate availability, fermentation rate, microbial mass and yield (Hespell & Bryant, 1979; Russell *et al.*, 1983; NRC, 1985). Considerable controversy exits as to the optimum concentration of ruminal ammonia required for maximal microbial growth (Satter & Slyter, 1974; Satter & Roffler, 1975; Mehrez *et al.*, 1977). Mean ruminal ammonia-N values for the present study ranged from a low of 20.7 mg/dL (C) to a high of 25.2 mg/dL (MON) (See Table 5.7). These values far exceed the recommended value of 5 mg of $NH_3$-N/dL of rumen fluid to support maximal bacterial growth rates and protein production, as suggested by Satter & Slyter (1974) who investigated the effect of ammonia concentration on microbial protein production *in vitro* in continuous culture fermenters charged with the ruminal contents from steers fed different diets. Although this study identified that 5 mg of $NH_3$-N/dL could satisfy the needs of the bacterial population it did highlight that excessively high levels of ruminal ammonia, up to 80 mg $NH_3$-N/dL, were not inhibitory to microbial growth. Higher values have been suggested by the *in vivo* study of Mehrez *et al.* (1977) who upon feeding sheep with whole barley found that a concentration of 22.5 mg $NH_3$-N/dL of rumen fluid was required for maximal digestion. Erdman e*t al.* (1986) suggested that the minimum ruminal ammonia concentrations required for maximal digestion and microbial growth are not constant but rather a function of the ruminal digestibility (i.e. fermentability) of the feed, with the minimum requirement for ruminal ammonia-N increasing with increased diet fermentability. From this study the following equation was derived, minimum ammonia concentration (mg/dL) = 0.452 fermentability % - 15.71. For this reason, one would expect greater minimum ammonia-N concentrations in high-producing dairy cows which are fed TMR's rich is fermentable OM. This could explain the high ammonia-N values observed in the present study, as OM digestibility was shown to be high, with a mean IVOMD of 82.7 % across dietary treatments. Other possible explanations for the high ruminal ammonia values observed are that ruminal carbohydrate and protein digestion and supply of nutrients may have been asynchronous, resulting in the insufficient supply of energy from fermentation to stimulate the utilisation of all the dietary CP degraded in the rumen, thus limiting the efficiency of MPS. Alternatively, ruminal ammonia may have been supplied in excess of the microbial requirement as lucerne hay is known to be high in CP and highly degradable in the rumen (Cronje, 1983) and comprised 40 % of the total ration DM.

In the present study greater ammonia-N concentrations were observed with the MON treatment as compared to the C ($P$ = 0.02) treatment, MON values also tended to be higher than those of the AB10 ($P$ = 0.06) treatment. Ionophore supplementation characteristically reduces ruminal ammonia-N concentrations, by acting upon ionophore-sensitive proteolytic and obligate AA fermenting bacterial species, in particular the HAP species, and in doing so inhibit AA deamination (Yang & Russell, 1993ab) increasing the ruminal escape of dietary protein. Ruminal ammonia-N concentrations were shown to be decreased *in vitro* by 27 % and 63 %, respectively, on high concentrate and high forage diets (Fuller & Johnson, 1981), in response to ionophore supplementation, others to report a decrease *in vitro* include Yang & Russel (1993a) and Busquet e*t al.* (2005). Many *in vivo* studies feeding monensin to cattle at 330 mg/d to 350 mg/d have too observed a decrease in ruminal ammonia –N (Ali-Haïmoud *et al.*, 1995; Guan *et al.*, 2006; Wang *et al.*, 2015). However, this decrease is not always statistically significant as observed in the study of Erasmus *et al.* (2005) in which monensin supplemented at a level of 10 g/kg DM to early lactation dairy cows fed a TMR comprised of 383 g/kg of lucerne hay and 617 g/kg of concentrates, was found to only numerically decrease ruminal ammonia. Others to report this lack of response when feeding monensin at levels of 10 mg/kg DM to 24 mg/kg DM to lactating dairy cows include Broderick (2004), Martineau *et al.* (2007) and do Prado *et al.* (2015). At doses of 250 mg/d or less the lack of response it likely due to an insufficient dose unable to reduce AA or peptide catabolism in the rumen. The increase in ruminal ammonia- N observed in the present study, however, cannot be explained.

In agreement with the results of this study, both Beya (2007) and Cruywagen *et al.* (2015) failed to observe an effect of Acid Buf on ruminal ammonia-N, which is typical of buffers. Dietary supplementation with DFM's does not often alter ruminal ammonia-N concentrations, and when effects have been observed they have typically been numerical decreases not of statistical importance or biological value. Upon feeding a yeast culture to lactating Holstein dairy cows Erasmus *et al.* (1992) observed a 10 % decline in ruminal

ammonia-N, however, this decline was not significant ($P > 0.05$), others to observe numerical decreases include Harrison *et al*. (1988), Piva *et al.* (1993) and Enjalbert *et al.* (1999). Most commonly no ruminal ammonia-N response is observed when feeding yeast cultures to dairy cows (Carro *et al.*, 1992; Erasmus e*t al.*, 2005; Thrune *et al.*, 2009). Bacterial strains and *A. oryzae* are commonly included in commercial DFM's but have shown variability in their ability to alter ruminal ammonia-N, with Raeth-Knight e*t al.* (2007) failing to observe a treatment response when supplementing a combination of *L. acidophilus* and *P. freudenreichii* to Holstein cows, as did Sievert & Shaver (1993) and Higginbotham *et al.* (2004) when supplementing early-lactation Holsteins with *A. oryzae extracts*. Upon feeding a combination of yeast culture and *A. oryzae* both Higginbotham e*t al.* (1994) and Yoon & Stern (1996) failed to observe a significant reduction in ruminal ammonia-N.

**Table 5.16** The effect of supplementation with Acid Buf 10, a direct-fed microbial and monensin on ruminal ammonia- nitrogen concentrations (mg/dL), measured at three hour intervals over a 24 hour period, for lactating dairy cows fed a total mixed ration (n = 16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| Time | C | AB10 | DFM | MON | SEM[2] |
| 09:00 | 23.7 | 25.8 | 24.5 | 25.8 | 1.12 |
| 12:00 | 23.9$^{cd}$ | 23.1$^{d}$ | 26.8$^{cd}$ | 28.9$^{c}$ | 2.08 |
| 15:00 | 21.5$^{b}$ | 30.7$^{a}$ | 27.6$^{a}$ | 28.7$^{a}$ | 1.35 |
| 18:00 | 23.5$^{abd}$ | 21.6$^{b}$ | 24.8$^{ab}$ | 28.4$^{ac}$ | 1.66 |
| 21:00 | 19.8 | 19.2 | 21.2 | 24.4 | 2.05 |
| 24:00 | 19.0 | 19.9 | 22.8 | 23.8 | 1.81 |
| 03:00 | 17.0 | 16.6 | 16.9 | 20.8 | 2.33 |
| 06:00 | 17.2 | 17.2 | 18.7 | 20.5 | 2.68 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[a, b] Means within a row with different superscripts differ ($P < 0.05$)
[c, d] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

As presented in Table 5.16 the concentration of ammonia-N differed amongst dietary treatments over a 24 hour period. Sampling time has been reported to have a significant effect ($P < 0.05$) on ruminal ammonia-N concentrations (Bargo *et al.*, 2002) although this was not observed in the present study. This lack of significant variation in the pattern of ruminal ammonia-N concentrations over a 24 hour period is typical of the more constant pattern observed when cows are fed a TMR (Bargo *et al.*, 2002). Peak ammonia –N concentrations were observed at 12:00 and 15:00 which correlates to 4 and 7 hours after the morning feed, which is later than the one to two hours post feed reported by others (Enjalbert e*t al.*, 1999; Raeth-Knight *et al.*, 2007; Wang *et al.*, 2015) although Guedes e*t al.* (2008) did report increases at four hours post feed. At 12:00 there was a tendency for ruminal ammonia-N concentrations to differ between the AB10 and MON treatments ($P = 0.09$). At 15:00 the C treatment had markedly lower ammonia-N concentrations than the AB10 ($P = 0.002$), DFM ($P = 0.02$) and MON ($P = 0.01$) treatments which were all alike. At 18:00 both C ($P = 0.08$) and AB10 ($P = 0.03$) treatments appeared to differ from the MON treatment, from this time point onwards all treatments steadily reduced ruminal ammonia concentrations, reaching a nadir at 03:00.

### 5.5.3 Lactic Acid

Modern dairy diets are formulated to be highly digestible, and are composed primarily of readily fermentable carbohydrate sources, to meet the energy demands of lactation. A disadvantage of feeding such diets is that there is a rapid production of ruminal VFA's which accumulate and along with $H^+$ drive down the pH of the ruminal milieu (Slyter & Rumsey, 1976), increasing the risk of the common metabolic disorder, SARA. Although accumulation of ruminal VFA's alone have been shown to depress ruminal pH of dairy cattle at risk or suffering from SARA (Oetzel e*t al.*, 1999; Krause & Oetzel, 2006), lactic acid has historically been implicated. Passive absorption of VFA's is facilitated at low pH levels, however, the gains in VFA absorption may be offset by the production of lactic acid (Krause & Oetzel, 2006). Typically, lactate is utilised at the same

rate as produced, however, when dietary levels of readily fermentable starches and sugars are high and ruminal pH low there is a shift towards the lactic acid producer, *S. bovis*, which ferments glucose to lactate instead of VFA's and in doing so drives ruminal pH down even further (Krause & Oetzel, 2006). In the present study, no lactic acid data was obtained as when the samples were analysed lactic acid was detected at levels below 10 mg/L, which is outside the reliable range of quantitation. This was unexpected as the pH values observed in this trial ranged from 5.55 to 5.64 for the dietary treatments, which suggests that the cows were at risk of experiencing SARA. Lactate is supposedly removed fairly quickly preventing its accumulation in the ruminal fluid (Hibbard *et al.*, 1995; Enemark 2008) under normal or SARA conditions. Small, transient increases in ruminal lactate are also virtually impossible to measure *in vivo* (McAllister *et al.*, 2011), these two factors may explain why lactic acid was not detected in this study.

# CHAPTER 6

# RESULTS AND DISCUSSION II:

# EFFECT OF FEED ADDITIVES ON MICROBIAL PROTEIN PRODUCTION AND THE AMINO ACID PROFILE OF THE RUMINAL BACTERIA

## 6.1 Microbial protein synthesis

In the present study various urine parameters were investigated so as to allow for the prediction of MCP flow using spot urine samples. By determining MCP flow in this manner the need for duodenally cannulated animals, duodenal flow marker techniques, and total urine collection were avoided. The results for the study are presented in Table 6.1.

**Table 6.1** The effect of supplementation with Acid Buf 10, a direct-fed microbial and monensin on urine parameters for the prediction of microbial crude protein yield, for lactating dairy cows fed a total mixed ration (n = 16)

| | Treatments[1] | | | | |
|---|---|---|---|---|---|
| | C | AB10 | DFM | MON | SEM[2] |
| Creatinine (CR) (mg/L) | 751 | 749 | 772 | 641 | 48.1 |
| Allantoin (AL) | | | | | |
| mg/L | 2692 | 2641 | 2692 | 2245 | 171.7 |
| mmol/L | 17.0 | 16.7 | 17.0 | 14.2 | 1.09 |
| AL:CR | 3.81 | 3.57 | 3.64 | 3.54 | 0.204 |
| PDC Index[3] | 522 | 466 | 488 | 469 | 33.0 |
| Specific Gravity | 1.03 | 1.02 | 1.03 | 1.02 | 0.001 |
| Urine volume (L/day) | 18.9 | 19.9 | 18.9 | 20.4 | 0.80 |
| Total PD excretion[4] (mmol/d) | $368^{cd}$ | $382^{c}$ | $362^{cd}$ | $320^{d}$ | 19.8 |
| Intestinal flow of microbial N (g N/d) | $275^{cd}$ | $286^{c}$ | $269^{cd}$ | $235^{d}$ | 16.6 |
| MCP[5] yield (g/d) | $1716^{cd}$ | $1788^{c}$ | $1683^{cd}$ | $1468^{d}$ | 103.7 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[3] PDC Index = $(AL_{adjusted}: CR) \times (\text{Body weight (kg)})^{0.75}$
[4] Total PD excretion = Total purine derivative excretion
[5] MCP = Microbial crude protein yield.
[c, d] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

Originally proposed by Topps & Elliot (1965) as an indirect, non-invasive method for the quantitative estimation of the intestinal flow of ruminal MCP, researchers have since demonstrated and confirmed the use of urinary PD excretion as an effective alternate method by which to estimate intestinal MCP flow (Gonzalez-Ronquillo *et al.*, 2003; Chen & Ørskov e*t al.*, 2004). Urinary CR excretion may be used as a marker for the determination of total urine output (Valadares *et al.*, 1999) when total collection is not practical, and the ratio of AL to CR in spot urine samples can be used to estimate relative changes in ruminal microbial growth and MCP supply. In the present study no treatment differences were observed for the AL to CR ratio, with a mean ratio across treatments of 3.64 ± 0.204, which is slightly higher than those reported by Vagnoni & Broderick (1997) and Swanepoel *et al.* (2014) who reported mean values of 3.15 and 3.35, respectively. This may be due to the slightly lower CR concentrations observed in this study which could be related to differences in the proportion of lean body tissue of animals used in these studies, as CR excretion is known to be related to body protein mass turnover (Chen & Ørskov, 2004). Chen *et al.* (1995) found that in the absence of total urine collection, the PD to CR ratio in spot urine samples correlated well with both feed intake and the intestinal

flow of microbial purines, therefore allowing for the use of this index as a qualitative indicator of ruminal MCP supply. However, urinary CR is known to be a function of BW (Susmel e*t al.*, 1995), with daily CR excretion being related to body protein mass turnover, and for this reason varies amongst individual animals and studies. To account for this variation, Chen & Ørskov (2004) developed the purine derivative to creatinine (PDC) index which by accounting for metabolic BW in the calculation allows for the comparison of PD: CR ratios across animals within the same breed. To determine the PD: CR ratio in the present study, urinary AL was corrected to total PD as described by Swanepoel *et al.* (2014). In brief, urinary AL is known to be the predominant PD in cattle and constitutes and almost constant molar proportion of PD, thus in the absence of uric acid measurement, total PD can be calculated by correcting AL concentrations by a factor of 0.906 obtained from the reported values of Vagnoni & Broderick (1997), Valadares *et al.* (1999), Gonzalez-Ronquillo *et al.* (2003) and Moorby *et al.* (2006). As with the AL: CR ratio the PDC index was not altered by dietary treatments (µ = 486 ± 33.0) in the present study, unfortunately for comparative purposes not many studies report PDC index. In the present study MCP flow was determined through a series of calculations, detailed in section *4.5.8 Calculations*, using only the direct measurements of the specific gravity and the concentrations of AL present in the spot urine samples. Amongst dietary treatments there was no difference in urine specific gravity (µ = 1.03 ± 0.001), volume of urine excreted (µ = 19.5 L/d ± 0.80 L/d), or the concentrations of CR (µ = 728 mg/L ± 48.1 mg/L) and AL (µ = 2568 mg/L ± 171.7 mg/L).

There was however, a tendency for daily total PD excretion (*P* = 0.07), intestinal flow of microbial N (*P* = 0.07) and MCP yield (*P* = 0.07) to differ between the AB10 and MON treatments (See Table 6.1). Mean daily total PD excretion (PDE) across dietary treatments was 358 mmol/d ± 19.8 mmol/d, these results correlate well with the research of Reynal & Broderick (2005), who investigated the effect of the dietary level of RDP on N metabolism in dairy cows and observed a mean total PDE of 383.25 mmol/d, which decreased with decreasing dietary RDP. The results of the present study also correlate with those of Moorby *et al.* (2006) who using the traditional duodenal marker technique observed a total PDE of 334 mmol/d and 370 mmol/d on diets with forage to concentrate ratios of 50:50 and 35:65, respectively, which are similar to the approximately 40:60 ratio fed in the present study. The mean intestinal flow of microbial N observed in the present study was 266 g N/d ± 16.6 g N/d, which was not much different to the values reported by Valadares *et al.* (1999) who reported microbial N values in the range of 297 g N/d to 425 g N/d when using spot urine sampling, nor the study of Brito *et al.* (2007a) who reported values of 219 g N/d to 300 g N/d when evaluating the effect of different protein supplements on MPS by means of duodenal markers. From this value the calculated MCP flow was determined and found to be a mean of 1664 g CP/d ± 103.7 g CP/d across dietary treatments. This value is in accordance with the range of 763 g CP/d to 1959 g CP/d provided by Swanepoel e*t al.* (2015) who compiled the results from various studies in which MCP flow was directly measured by means of duodenal marker techniques. These results suggest that MCP flows estimated indirectly using AL concentrations are biologically sound, which is in agreement with Martín-Orúe e*t al.* (2000) who stated that microbial N flow estimated by means of urinary PD excretion closely reflects relative differences amongst dietary treatments as observed by direct intestinal flow measurements. However, they did report that urinary PD gave consistently lower absolute values. Although within the range provided by Swanepoel *et al.* (2015) the MCP flow values in this study were lower than the values observed in the studies of Swanepoel *et al.* (2015) and Leicester *et al.* (2016) both of which measured CP flow by means of urinary PDE, this was likely due to the substantially lower DMI and milk yields observed in the present study which may have reduced MCP production. Clark *et al.* (1992) summarised the data from 41experiments encompassing a total of 161 diets and found OM intake to be positively correlated ($r^2$ = 0.62) to the passage of microbial N to the small intestine when OM intake was increased. This increased passage of microbial N to the small intestine can be partially attributed to the larger amount of energy supplied by the larger amount of OM fermented in the rumen with higher intakes.

Other possible explanations for the lower MCP yields observed could be the high ruminal pH, as the efficiency of MPS has been reported to be reduced when ruminal pH is lower than 5.5 (Calsamiglia e*t al.*, 2008) which is a possibility in this trial as ruminal pH averaged 5.58 ± 0.029. Unusually high ruminal ammonia was also observed in this study (µ = 22.6 mg/dL ± 1.05 mg/dL) which could have potentially reduced MCP flow as Robinson (1996) reported that ruminal ammonia-N exceeding 11.0 mg/dL decreased bacterial N flow either through negative feedback mechanisms or direct bacterial toxicity. When utilising spot urine sampling and urinary PDE to estimate MCP flows one must keep in mind that spot urine sampling is subject to more

variability than total collection and more measurements are required to reduce error (Chen & Ørskov, 2004), this error risk was reduced in the present study by sampling for urine eight times for each dietary treatment per period.

Research on the effects of dietary buffers on MCP flow has been limited, and as of yet no studies have investigated the effect of Acid Buf on MCP, because buffers are known to improve ruminal pH one could postulate that they could potentially improve or maintain MCP production when fed to animals on acidotic diets as pH below 5.5 can be detrimental to the efficiency of MPS.

As with the ruminal fermentation parameters, dietary DFM's have not been shown to consistently alter MCP flows. As mentioned in Erasmus *et al.* (1992), Newbold (1990) suggested that fungal and yeast cultures may have the potential to affect the flow of protein from the rumen through enhancement of the number and activity of ruminal microbial populations. Studies have reported increased ruminal populations, particularly the fibrolytic and cellulolytic populations, in response to supplementation with various DFM's, which owing to their preference for ammonia as an N source (Bryant 1973) will result in increased utilisation and efficiency of conversion of ruminal ammonia-N into microbial protein. The study of Erasmus *et al.* (1992) in which 10 g of a yeast culture was supplied daily via the ruminal cannula, observed an increased flow of bacterial N to the duodenum of lactating dairy cows, this was also observed by Hristov e*t al.* (2010) who by means of urinary AL excretion found the estimated MCP flow from the rumen to be increased with yeast culture supplementation. In contrast Leicester *et al.* (2016) reported two *S. cerevisiae* based DFM's to have no influence on daily microbial CP flow when supplemented to lactating dairy cows, which is in agreement with the previous studies of Carro *et al.* (1992) and Putnam *et al.* (1997).

Supplementation with ionophores does not consistently alter MCP flows or the efficiency of ruminal MPS, with reports of no change in MCP flow to the duodenum (Ali- Haïmoud *et al.*, 1995; Castillejos *et al.*, 2006; Yang *et al.*, 2007). Whetstone *et al.* (1981) reported significant *in vitro* reductions in net microbial growth and microbial N, whilst *in vivo* Ali-Haimoud *et al.* (1995) reported a tendency towards a reduction in the proportion of duodenal N from bacterial origin. Both these studies observed improved flow of dietary NAN, total peptides and dietary N which is explained by the inhibitory effect of ionophores upon the ruminal microbiome, specifically the inhibition of proteolytic and AA fermenting bacteria, allowing for more dietary N to pass to the duodenum. The reduced MCP flow observed in the present study ($P < 0.10$) may be as a result of the typical mode of action of monensin and was not unusual, however, in conjunction with the significantly greater ruminal ammonia-N concentrations observed with MON as compared to the C treatment, the results don't support an inhibition of proteolytic and AA fermenting bacteria. This suggests that MCP flow was reduced by another factor, potentially the higher ammonia-N concentration as suggested by Robinson (1996).

## 6.2 Bacterial composition and amino acid profile

As previously discussed in *Chapter two*, the ruminally synthesised microbes flowing into the duodenum contribute significantly to the total absorbable protein available to the dairy cow. These microbes supply 40 % to 80 % of the ruminant's daily AA requirement (Sniffen & Robinson, 1987) and for this reason knowledge on the amount and composition of microbial protein, particularly in response to dietary factors, is imperative if nutritionists wish to improve the accuracy of diet formulation for dairy cows. To date many studies have investigated the effects of dietary factors and composition on either the fluid or mixed ruminal microbial community but have failed to differentiate between the microbes associated with the liquid and particulate phases of the rumen. This is short-sighted as the composition of the bacteria isolated from the liquid and particulate phases of the rumen has been shown to differ across studies (Merry & McAllan, 1983; Rodríguez *et al.*, 2000; Sok *et al.*, 2017). With these differences being reflective of variation in metabolic functions (Williams & Strachan, 1984), stage of growth, polysaccharide content, and the bacterial communities associated with each digesta fraction (Czerkawski, 1976; Cecava *et al.*, 1990; Abecia *et al.*, 2013). Numerous studies have also shown bacteria associated with the particulate matter to contribute significantly more, 70% to 80% (Forsberg & Lam, 1977; Craig e*t al.*, 1987a), to the ruminal OM than those associated with the fluid fraction, highlighting the fact that the accurate description of the chemical and AA composition of this fraction is essential to the estimation of microbial AA flow to the duodenum. For this reason, in the present study ruminal bacteria were isolated from both the liquid and particulate phases of the rumen.

### 6.2.1 Chemical composition

The chemical composition of the FAB and PAB for the present study in response to dietary treatments can be found in Tables 6.2 and 6.3, respectively.

**Table 6.2** Chemical composition of the ruminal fluid-associated bacterial (FAB) fraction as affected by supplementation of lactating dairy cows with Acid Buf 10, a direct-fed microbial and monensin, when fed a total mixed ration (n = 16)

| | Treatments[1] | | | | SEM[2] | Contrasts, *P* | | |
|---|---|---|---|---|---|---|---|---|
| | C | AB10 | DFM | MON | | C vs. AB10 | C vs. DFM | C vs. MON |
| Chemical composition | | | | | | | | |
| OM, % of DM | 91.3[c] | 91.2[cd] | 91.0[d] | 91.0[cd] | 0.14 | 0.52 | 0.11 | 0.26 |
| N, % of DM | 10.8 | 10.7 | 10.7 | 10.7 | 0.08 | 0.54 | 0.75 | 0.97 |
| N, % of OM | 11.3 | 11.2 | 11.2 | 11.2 | 0.08 | 0.44 | 0.95 | 0.95 |
| AA, % of DM | 47.0[c] | 46.0[cd] | 44.7[d] | 45.7[cd] | 0.79 | 0.39 | 0.12 | 0.86 |
| Total AA-N, % Total N | 61.2 | 60.0 | 58.6 | 59.7 | 1.12 | 0.49 | 0.20 | 0.85 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve[FE], administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[c, d] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

The chemical composition of the FAB was shown to be unaffected by treatments, with only an observed tendency ($P = 0.09$) for the C treatment to have a greater OM content and a greater total AA content expressed as a percentage of DM ($P = 0.09$) as compared to the DFM treatment. However, when the contrast procedure was employed to compare the C treatment to each individual treatment it was clear that the dietary treatments were unable to alter the chemical composition of the FAB.

**Table 6.3** Chemical composition of the ruminal particle-associated bacterial (PAB) fraction as affected by supplementation of lactating dairy cows with Acid Buf 10, a direct-fed microbial and monensin, when fed a total mixed ration (n = 16)

| | Treatments[1] | | | | SEM[2] | Contrasts, *P* | | |
|---|---|---|---|---|---|---|---|---|
| | C | AB10 | DFM | MON | | C vs. AB10 | C vs. DFM | C vs. MON |
| Chemical composition | | | | | | | | |
| OM, % of DM | 93.3[ab] | 93.2[ab] | 93.5[a] | 92.8[b] | 0.19 | 0.80 | 0.30 | 0.06 |
| N, % of DM | 9.16 | 9.10 | 9.03 | 9.03 | 0.085 | 0.66 | 0.39 | 0.55 |
| N, % of OM | 9.39 | 9.32 | 9.25 | 9.29 | 0.075 | 0.51 | 0.31 | 0.76 |
| AA, % of DM | 43.7[cd] | 41.0[d] | 42.8[cd] | 44.1[c] | 1.09 | 0.14 | 0.76 | 0.27 |
| Total AA-N, % Total N | 65.5[c] | 62.2[d] | 65.8[c] | 68.3[c] | 1.19 | 0.09 | 0.22 | 0.03 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve[FE], administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[a, b] Means within a row with different superscripts differ ($P < 0.05$)
[c, d] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

The chemical composition of the PAB appeared to be somewhat affected to a greater extent by the dietary treatments. Within this bacterial fraction, the DFM treatment displayed a greater OM content than the MON treatment ($P = 0.04$), however, when the contrast procedure was employed to compare the C treatment to each individual treatment, MON only tended to differ in OM content ($P = 0.06$). No treatment differences were observed for the N content of these bacteria, expressed as either a percentage of DM or OM. The AA content of the PAB expressed as a percentage of DM did not differ amongst treatments, but the AB10 and

MON treatments did tend to differ ($P$ = 0.10). The total AA-N content expressed as a percentage of total N was affected by dietary treatments, with the AB10 treatment displaying the lowest value and differing from the MON treatment ($P$ = 0.01) and tending to differ from the C ($P$ = 0.09) and DFM ($P$ = 0.07) treatments. When the contrast procedure was employed to compare the C treatment to each individual treatment, the AB10 treatment was again shown to tend ($P$ = 0.09) to differ from the C treatment with regards to the total AA-N content, whilst the MON treatment was shown to contain a greater content of total AA-N, expressed as a percentage of total N, than the C treatment ($P$ = 0.03).

Although various studies have examined the effect of feeding frequency (Cecava e*t al.*, 1990), level of feed intake (Rodríguez *et al.*, 2000), diet composition (Olubobokun *et al.*, 1988; Shabi *et al.*, 2000; Boguhn *et al.*, 2006) and dietary factors (Yang *et al.*, 2001) on the ruminal bacteria, almost no research exists for the effect of dietary additives on the ruminal microbes. Only the study of Putnam e*t al.* (1997) examined this parameter in lactating Holstein dairy cows fed a yeast culture with either high or low CP diets and found supplementation to have no effect on the chemical composition of mixed bacteria isolated from the reticulo-omasal orifice.

The mean OM content expressed as a percentage of DM across treatments was 91.1 % ± 0.11 % and 93.2 % ± 0.19 % for FAB and PAB respectively. These values are in agreement with the range provided by Clark *et al.* (1992) who summarised data from more than 20 studies and found the composition of the mixed ruminal bacteria to be highly variable, with OM content ranging from 60.8 % to 92.2 %. Albeit slightly higher these values are also similar to those reported in the studies of Olubobokun *et al.* (1988), Cecava *et al.* (1990) and Fessenden *et al.* (2017), despite OM content being strongly influenced by the isolation procedure, i.e. salt content of the wash solution and number of wash steps, followed (Martin *et al.*, 1994). The mean N content, expressed as a percentage of OM across treatments was 11.2 % ± 0.08 % and 9.3 % ± 0.07 % for FAB and PAB, respectively. These values are comparable with the range provided by Clark *et al.* (1992) for mixed bacteria of 7.35 % to 13.2 %, and albeit slightly greater, with the study of Valadares *et al.* (1999) on mixed ruminal bacteria. These values are also in agreement with Cecava *et al.* (1990) and Yang *et al.* (2001) who examined the effects of dietary energy levels and physical factors, respectively, on both fluid and particle associated bacterial fractions. Published literature reporting the total AA content of the ruminal bacteria as a percentage of DM is limited, in the present study values of 45.9 % and 42.9 % were obtained for the FAB and PAB, respectively, these values are higher than those reported in the study of Rodríguez *et al.* (2000), who investigated the effect of feed intake in sheep and reported values of 36 % and 31.8 %, for PAB on low and high feed intakes, respectively and values of 33.8 % and 21.8 % for FAB, on each diet, whilst Fessenden *et al.* (2017), examined mixed ruminal microbes and reported a similar value of 32.5 %. In the present study total AA-N expressed as a percentage of total N was examined, with values of 59.9 % and 65.5 % being obtained for FAB and PAB, respectively. These values are within the range, 54.9 % to 86.7 %, reported in the extensive study of Clark *et al.* (1992) and when compared to other literature, although similar, illustrate the variation in a parameter previously thought of as being constant. Although there are no published studies investigating the effect of feed additives on this parameter, the values obtained are similar to those found in the *in vitro* study of Boguhn *et al.* (2006) who reported values of 67.4 % and 65.2 % for fluid- and particle associated microbes, respectively, when investigating the effect of TMR composition, and the *in vivo* studies of Rodríguez *et al.* (2000) and Yang *et al.* (2001) who reported values of 61.5 % (PAB) and 57.5 % (FAB) when investigating the effect of feed intake, and 73.6 % (PAB) and 66.7 % (FAB) when investigating the effect of dietary factors, respectively.

As previously mentioned, research has shown the fluid- and particle associated bacterial fractions to differ considerably in composition owing to variation in the bacterial communities present within each fraction, each displaying differing growth rates, metabolic functions and hence cellular physiology. For this reason, in the present study the fluid and particulate bacterial fractions were compared to one another, the results of which are presented in Table 6.4.

In agreement with previous research, the findings of the present study found the OM, N, total AA and total AA-N content of the two bacterial fractions to be highly different ($P$ < 0.0001). The higher OM concentration observed in PAB as compared to FAB agrees with literature where this difference has been unequivocally observed, with a high level of significance (P < 0.01) across diverse experimental studies (Merry

& McAllan, 1983; Martín-Orúe *et al.*, 2000; Rodríguez *et al.*, 2000; Yang *et al.*, 2001), with the difference arising directly from the greater ash content of the FAB (Merry & McAllan, 1983; Craig *et al.*, 1987b). Only the study of Cecava *et al.* (1990) has failed to observe a difference in OM content between the two bacterial fractions. The ability of microbes to trap the salt-based wash solution used during isolation is dependent on interstitial space and the degree of cell aggregation, the FAB tend to be more aggregated than the PAB and thus trap more salt in the interstitial spaces of the aggregated microbes, resulting in the lower OM content observed for the FAB (Martin *et al.*, 1994). The higher OM content of the PAB is likely related to the higher lipid and polysaccharide content of the PAB, which although not evaluated in the present study was observed by Merry & McAllan (1983), Craig *et al.* (1987b) and Rodríguez *et al.* (2000), with the study of Rodríguez e*t al.* (2000) determining that an average of 67 % of the difference in OM content of the two bacterial fractions could be explained by the differing lipid content. This higher lipid concentration in PAB could be related to a lower proportion of gram-positive bacteria as these bacterial species contain cell walls with less lipid content than the gram- negative bacterial species (Cummins, 1989). The greater observed polysaccharide content of the PAB as compared to the FAB would too lead to a higher OM content for the PAB whilst decreasing the N content through dilution (Craig *et al.*, 1987b; Yang *et al.*, 2001). This dilution effect explains the greater N content of the FAB fraction observed in the present study, this observation is in agreement with the studies of Merry & McAllan (1983), Martin *et al.* (1994), and Yang *et al.* (2001), however, some have failed to observe a significant difference in N content between the two bacterial fractions (Craig *et al.*, 1987b; Cecava *et al.*, 1990; Rodríguez *et al.*, 2000). The total AA-N, expressed as a percentage of total N, was shown to be greater for the PAB as compared to the FAB in the present study which is in agreement with Yang *et al.* (2001).

**Table 6.4** Variation in the chemical composition of the ruminal fluid-associated bacteria (FAB) and particle-associated bacteria (PAB) across treatments, in lactating dairy cows fed a total mixed ration (n = 32)

| | FAB[1] | PAB[2] | SEM[3] | *P* –value[4] |
|---|---|---|---|---|
| Chemical composition | | | | |
| OM, % of DM | 91.1 | 93.2 | 0.07 | < 0.0001 |
| N, % of DM | 10.7 | 9.08 | 0.046 | < 0.0001 |
| N, % of OM | 11.2 | 9.31 | 0.047 | < 0.0001 |
| AA, % of DM | 45.9 | 42.9 | 0.46 | 0.0002 |
| Total AA-N, % of Total N | 59.9 | 65.5 | 0.63 | < 0.0001 |

[1] FAB = Fluid-associated bacteria
[2] PAB = Particle-associated bacteria
[3] Standard error of the mean
[4] *P*- value, bacterial fractions differ ($P < 0.05$)

The chemical composition of the bacterial fractions has been demonstrated to vary with time after feeding, with Cecava *et al.* (1990) reporting a quadratic effect of time on the concentration of OM and N in the mixed and FAB fractions, whilst the PAB fraction displayed a quadratic effect of time for OM but a linear effect for N content. In this study peak OM and N concentrations were observed 6 hours to 9 hours post feed, with the variation over time being explained as being related to shifts in bacterial species present, synthesis of cell material and dilution effects caused by the storage polysaccharide content. These researchers suggested that to avoid deviation over time one should not base sampling on one fixed sampling time post-feed, for this reason, in the present study, samples were collected at three hours intervals over a 24 hour period, thereafter all time period samples were composited to give one representative sample per sampling period.

### 6.2.1 Amino acid composition

As with the chemical composition, the AA profile of the ruminal bacteria have been shown to be altered, however, results have not been as consistent. Early studies on this topic found the AA profile of the ruminal microbes to be remarkably constant across widely diverse dietary conditions (Weller, 1957; Meyer *et al.*, 1967; Bergen *et al.*, 1968), however, the study of Clark *et al.* (1992) showed considerable variation in the AA composition of the ruminal bacteria. This study evaluated the data from over 20 published research papers and found the chemical and AA composition of the ruminal microbes to be variable, it was acknowledged that part of this variation may have arisen from different sampling methods, isolation techniques, chemical analyses and expression of results, however, upon refinement of the data to include only studies originating from one

laboratory variation in the composition still remained. The observed shift in AA profiles brought about by level of feed intake, dietary composition or dietary factors is likely a direct result of a modification of the ruminal microbiome (Hungate, 1965; Bergen *et al.*, 1968). Although shifts in microbial populations were not investigated in the present study alteration of the AA profile of the FAB and PAB fractions was observed in response to feed additive supplementation.

The AA composition of the FAB as influenced by dietary feed additive supplementation is presented in Table 6.5 and is illustrated for easier visual interpretation in Figure 6.1.

**Table 6.5** Amino acid profile (g of AA/ 100 g of AA) of the ruminal fluid-associated bacteria (FAB) as affected by supplementation of lactating dairy cows with Acid Buf 10, a direct-fed microbial and monensin, when fed a total mixed ration (n = 16)

| | Treatments[1] | | | | SEM[2] | Contrasts, *P* | | |
|---|---|---|---|---|---|---|---|---|
| | C | AB10 | DFM | MON | | C vs. AB10 | C vs. DFM | C vs. MON |
| Essential amino acids | | | | | | | | |
| Arginine | 6.52 | 6.83 | 7.11 | 6.96 | 0.224 | 0.37 | 0.17 | 0.60 |
| Histidine | 2.43$^{d}$ | 2.04$^{de}$ | 1.92$^{e}$ | 2.22$^{de}$ | 0.163 | 0.14 | 0.17 | 0.65 |
| Isoleucine | 5.09$^{e}$ | 5.52$^{d}$ | 5.19$^{de}$ | 5.44$^{de}$ | 0.135 | 0.07 | 0.51 | 0.31 |
| Leucine | 7.34 | 7.50 | 7.49 | 7.72 | 0.145 | 0.49 | 0.73 | 0.16 |
| Lysine | 9.20$^{ad}$ | 8.97$^{a}$ | 8.32$^{b}$ | 8.70$^{abe}$ | 0.173 | 0.40 | 0.01 | 0.54 |
| Methionine | 3.28$^{a}$ | 2.90$^{b}$ | 2.98$^{b}$ | 2.86$^{b}$ | 0.087 | 0.02 | 0.33 | 0.10 |
| Phenylalanine | 4.60 | 5.19 | 4.75 | 4.80 | 0.355 | 0.29 | 0.75 | 0.91 |
| Threonine | 5.83$^{de}$ | 5.83$^{de}$ | 5.99$^{d}$ | 5.59$^{e}$ | 0.119 | 0.97 | 0.32 | 0.08 |
| Valine | 5.88$^{bce}$ | 6.16$^{abd}$ | 6.26$^{a}$ | 5.83$^{c}$ | 0.082 | 0.05 | 0.05 | 0.03 |
| Non-essential amino acids | | | | | | | | |
| Alanine | 7.49 | 7.45 | 7.64 | 7.63 | 0.433 | 0.94 | 0.76 | 0.85 |
| Aspartic acid | 11.9 | 11.9 | 12.1 | 11.5 | 0.21 | 0.95 | 0.47 | 0.13 |
| Glutamic acid | 12.6$^{b}$ | 12.8$^{abe}$ | 13.3$^{ad}$ | 12.9$^{ab}$ | 0.18 | 0.61 | 0.03 | 0.87 |
| Glycine | 6.48$^{ad}$ | 6.11$^{abe}$ | 6.31$^{ab}$ | 6.01$^{b}$ | 0.119 | 0.07 | 0.93 | 0.07 |
| Proline | 3.40$^{de}$ | 3.31$^{e}$ | 3.76$^{d}$ | 3.60$^{de}$ | 0.161 | 0.69 | 0.08 | 0.57 |
| Serine | 3.71 | 3.48 | 3.76 | 3.52 | 0.142 | 0.28 | 0.38 | 0.46 |
| Tyrosine | 4.21$^{abd}$ | 4.01$^{ab}$ | 3.11$^{be}$ | 4.71$^{a}$ | 0.356 | 0.71 | 0.06 | 0.06 |
| Lys: Met | 2.81$^{e}$ | 3.12$^{d}$ | 2.80$^{e}$ | 3.05$^{de}$ | 0.109 | 0.09 | 0.25 | 0.32 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.

[2] Standard error of the mean

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

[d, e] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

The AA composition of FAB was shown to be affected by feed additive supplementation, with 10 out of the 16 AA's analysed being either altered ($P < 0.05$) or tending ($P \leq 0.10$) to be altered by supplementation. The EAA's in this fraction were affected as follows; histidine (His) concentration was greatest for the C treatment, with the His concentration tending to differ between the C and DFM treatments ($P = 0.07$). The Ile content of the FAB tended ($P = 0.07$) to be greater for AB10 as compared to the C treatment with neither treatment differing from the DFM or MON treatments. Lysine content of the FAB differed amongst treatments, with the C treatment displaying the highest Lys content differing from that of the DFM treatment ($P = 0.01$) and tending to differ from the MON treatment ($P = 0.09$), whilst the AB10 and DFM treatments differed ($P = 0.04$) from each other. Methionine content was too, shown to be the greatest for the C treatment and differed from the similar AB10 ($P = 0.02$), DFM ($P < 0.05$) and MON ($P = 0.01$) treatments. The Thr content of the DFM and MON treatments tended ($P > 0.05$) to differ from one another but neither differed from the C and AB10 treatments. The Val content of the FAB was greatest for the DFM treatment, with this treatment differing from both the C ($P = 0.02$) and MON treatments ($P = 0.01$), while the AB10 treatment differed from the MON

treatment ($P = 0.03$) and tended to differ from the C ($P < 0.05$). Of the EAA's Arg, Leu and Phe were unaffected by supplementation. The non-essential AA's (NEAA) present in the FAB fraction were also altered by dietary supplementation, with the exception of Ala, Asp and Ser. The DFM treatment displayed the greatest concentration of Glu, and differed from the C ($P = 0.04$) and AB10 ($P = 0.08$) treatments. The Gly content of this bacterial fraction differed amongst treatments with the C treatment differing from the MON ($P = 0.03$) treatment and tending to differ from the AB10 treatment ($P = 0.07$). The Pro content of the isolated FAB did not differ amongst treatments, although there was a tendency for the AB10 and DFM treatments to differ from each other ($P = 0.10$). The Tyr content of the bacteria isolated from the DFM treatment was the lowest and differed from the MON treatment ($P = 0.02$) but only tended to differ from the C treatment ($P = 0.07$).

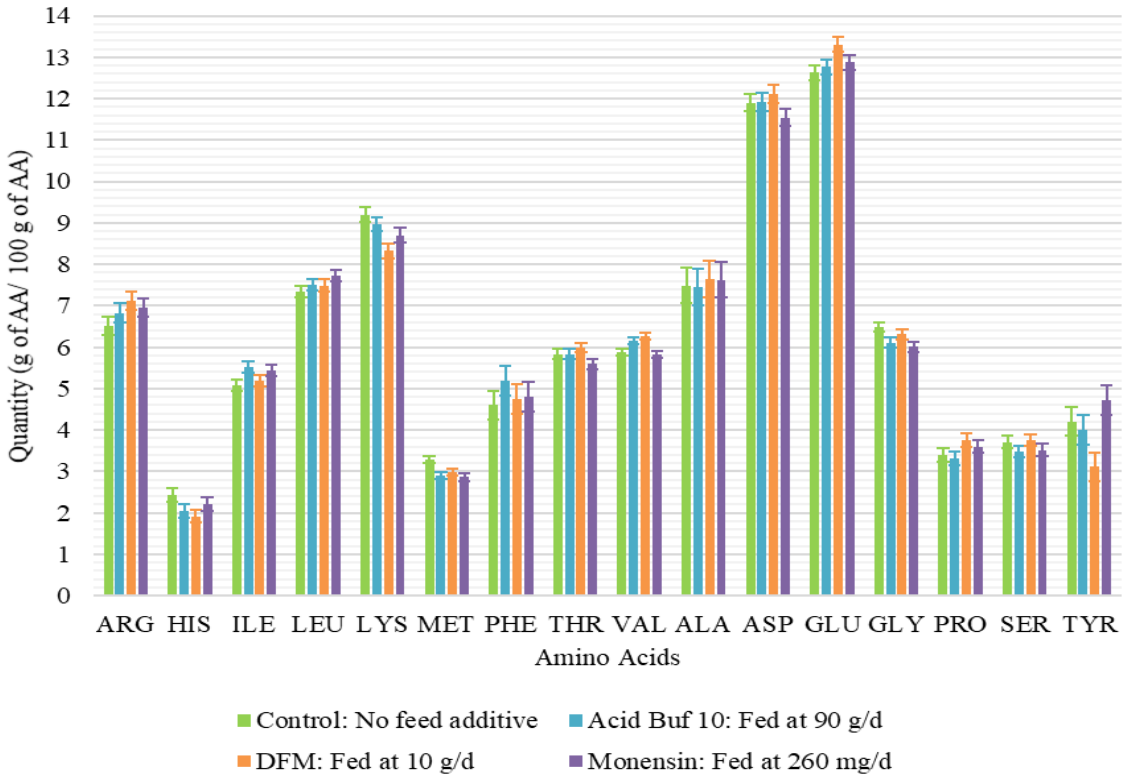


**Figure 6.1** The effect of Acid Buf 10, a direct-fed microbial and monensin on the amino acid composition of the ruminal fluid-associated bacteria (FAB) (g of AA/ 100 g of AA) of lactating dairy cows fed a total mixed ration, *ad libitum*, as compared to the control (n = 16). Results are means ± SEM

The AA composition of the PAB as influenced by dietary feed additive supplementation is presented in Table 6.6 and is illustrated for easier visual interpretation in Figure 6.2.

The AA composition of the PAB differed from that of the FAB and responded differently to feed additive supplementation, with 12 out of the 16 AA's analysed being either significantly altered ($P < 0.05$) or tending ($P \leq 0.10$) to be altered by supplementation. The EAA's in this particulate associated fraction were affected as follows; Arg content of the DFM treatment differed from that of the C treatment ($P = 0.04$) while the Arg content for the MON treatment only tended to be greater than that of the C treatment ($P = 0.09$). The DFM treatment appeared to have the lowest His content, which differed from the C ($P = 0.04$) and MON ($P = 0.02$) treatments but only tended to differ from the AB10 treatment ($P < 0.10$). The Isl content was not affected by treatment but the DFM and MON treatments did tend to differ ($P < 0.10$). The Lys content of the PAB isolated from DFM supplemented cows was the lowest, with the DFM and MON treatments differing from each other ($P = 0.04$) and the AB10 and MON treatments tending to differ ($P = 0.06$). The greatest content of Met was observed for the DFM treatment, and whilst no different to the C or MON treatments, tended to differ ($P < 0.10$) from the AB10 treatment. The concentration of Thr did not differ amongst treatments, however, there was a tendency for the MON treatment to differ from the C treatment ($P = 0.07$). The NEAA's present

in the PAB fraction that were altered by dietary supplementation are as follows. The Ala content tended to be reduced for the MON treatment as compared to the C ($P$ = 0.09) and AB ($P$ = 0.08) treatments. The Asp content of the bacteria isolated from the rumen of cows supplemented with AB10, DFM and the C treatment were similar and all greater than that of the bacteria isolated from cows supplemented with MON ($P$ = 0.01, $P$ = 0.01 and $P$ < 0.05, respectively). Similarly, the concentration of Glu was lowest for the MON treatment and differed from the C ($P$ = 0.003), AB10 ($P$ = 0.01) and DFM ($P$ = 0.002) treatments, all of which were similar. The highest Gly content was observed for the MON treatment, which differed from that of the C ($P$ = 0.02) and AB10 ($P$ = 0.004) treatments, while the DFM treatment differed in Gly content from the C ($P$ = 0.10) and AB10 treatments ($P$ = 0.02). The greatest Ser content was observed for the MON treatment which did not differ from the DFM treatment but was greater than that of the C ($P$ = 0.04) and AB10 ($P$ = 0.02) treatments. The lowest Tyr content was observed for the DFM treatment, which differed from the C ($P$ = 0.01) and MON treatments ($P$ = 0.02), and tended to differ from the AB10 treatment ($P$ = 0.06), while the AB10 treatment tended ($P$ = 0.08) to differ from the C treatment. Overall for the particulate fraction the only AA's not to be affected by dietary supplementation were Leu, Phe, Val and Pro

**Table 6.6** Amino acid profile (g of AA/ 100 g of AA) of the ruminal particle-associated bacteria (PAB) as affected by supplementation of lactating dairy cows with Acid Buf 10, a direct-fed microbial and monensin, when fed a total mixed ration (n = 16)

| | Treatments[1] | | | | SEM[2] | Contrasts, $P$ | | |
|---|---|---|---|---|---|---|---|---|
| | C | AB10 | DFM | MON | | C vs. AB10 | C vs. DFM | C vs. MON |
| Essential amino acids | | | | | | | | |
| Arginine | 5.93$^{be}$ | 6.38$^{ab}$ | 6.99$^{a}$ | 6.74$^{abd}$ | 0.288 | 0.31 | 0.06 | 0.40 |
| Histidine | 2.26$^{a}$ | 2.11$^{abd}$ | 1.69$^{be}$ | 2.34$^{a}$ | 0.155 | 0.54 | 0.04 | 0.12 |
| Isoleucine | 5.47$^{de}$ | 5.46$^{de}$ | 5.30$^{e}$ | 5.77$^{d}$ | 0.172 | 0.98 | 0.45 | 0.12 |
| Leucine | 8.05 | 8.06 | 7.89 | 7.94 | 0.120 | 0.94 | 0.31 | 0.68 |
| Lysine | 7.91$^{ab}$ | 7.66$^{abe}$ | 7.60$^{b}$ | 8.59$^{ad}$ | 0.283 | 0.55 | 0.61 | 0.04 |
| Methionine | 2.98$^{de}$ | 2.85$^{e}$ | 3.13$^{d}$ | 2.99$^{de}$ | 0.099 | 0.41 | 0.13 | 0.98 |
| Phenylalanine | 5.07 | 5.08 | 5.07 | 5.25 | 0.142 | 0.99 | 0.98 | 0.32 |
| Threonine | 5.66$^{e}$ | 5.82$^{de}$ | 5.87$^{de}$ | 6.13$^{d}$ | 0.152 | 0.48 | 0.52 | 0.09 |
| Valine | 6.22 | 6.12 | 6.15 | 6.14 | 0.167 | 0.69 | 0.94 | 0.91 |
| Non-essential amino acids | | | | | | | | |
| Alanine | 7.61$^{d}$ | 7.65$^{d}$ | 7.44$^{de}$ | 6.72$^{e}$ | 0.311 | 0.93 | 0.65 | 0.06 |
| Aspartic acid | 11.4$^{a}$ | 11.6$^{a}$ | 11.6$^{a}$ | 10.9$^{b}$ | 0.16 | 0.34 | 0.65 | 0.01 |
| Glutamic acid | 12.8$^{a}$ | 13.3$^{a}$ | 13.0$^{a}$ | 11.1$^{b}$ | 0.25 | 0.18 | 0.94 | <0.001 |
| Glycine | 6.32$^{bce}$ | 6.17$^{b}$ | 6.57$^{acd}$ | 6.76$^{a}$ | 0.093 | 0.3 | 0.03 | 0.01 |
| Proline | 3.95 | 3.83 | 3.99 | 3.94 | 0.082 | 0.33 | 0.35 | 0.86 |
| Serine | 3.83$^{b}$ | 3.75$^{b}$ | 4.00$^{ab}$ | 4.39$^{a}$ | 0.148 | 0.71 | 0.28 | 0.02 |
| Tyrosine | 4.59$^{ad}$ | 4.15$^{abe}$ | 3.66$^{bf}$ | 4.33$^{a}$ | 0.150 | 0.08 | 0.01 | 0.30 |
| Lys: Met | 2.69$^{de}$ | 2.71$^{de}$ | 2.44$^{e}$ | 2.88$^{d}$ | 0.139 | 0.90 | 0.18 | 0.15 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.

[2] Standard error of the mean

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

[d, e, f] Means within a row with different superscripts tend to differ (0.05 < $P$ ≤ 0.10)

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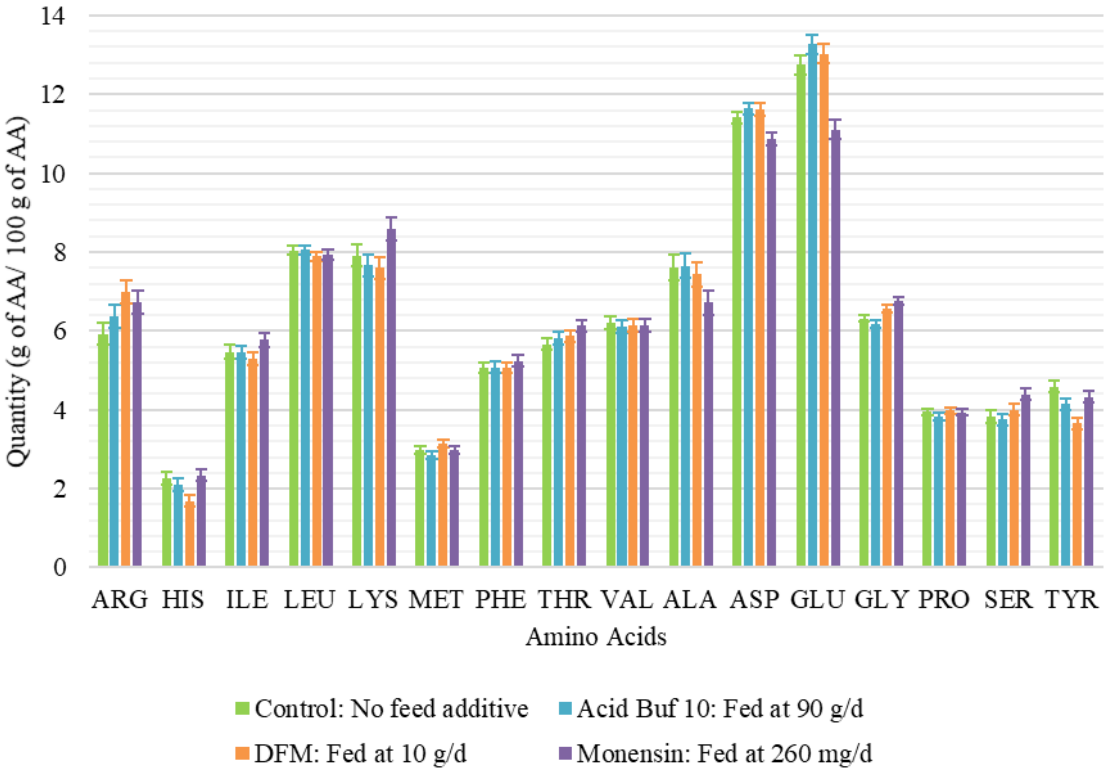


**Figure 6.2** The effect of Acid Buf 10, a direct-fed microbial and monensin on the amino acid composition of the ruminal particle-associated bacteria (PAB) (g of AA/ 100 g of AA) of lactating dairy cows fed a total mixed ration, *ad libitum*, as compared to the control (n = 16). Results are means ± SEM

Overall, the composition of the AA profile for the ruminal bacteria in the present study is comparable to those reported in previous published literature, in particular the data correlates well with the ranges given for mixed ruminal bacteria in Clark *et al.* (1992) and the ranges given for FAB and PAB in Sok *et al.* (2017), which were compiled from a database of 29 and 7 published cattle studies from 1967 to 2014, examining the AA composition of FAB and PAB, respectively. However, the AA content of certain AA's did consistently deviate from those given in previously published studies, with the content of Arg and Met being greater and that of Ser and Tyr being lower, for both bacterial fractions, as compared to the data of Bergen *et al.* (1968), Clark *et al.* (1992), Rodríguez *et al.* (2000) and Sok *et al.* (2017). Research on the alteration of the AA profile of the ruminal microbes has been limited, with the majority of the research available being focussed on nutritional influences such as the effect of the level of feed intake (Rodríguez *et al.*, 2000), diet composition (Bergen *et al.*, 1968; Martin *et al.*, 1996; Shabi *et al.*, 2000; Boguhn *et al.*, 2006), and dietary factors such as forage to concentrate ratio and forage particle length (Yang *et al.*, 2001). Although alteration of the AA profile was observed in some of these studies, the results of which were discussed in *chapter 2*, changes were variable and not always of significant value. Regarding the effect of feed additive supplementation on the AA composition of the two ruminal bacterial fractions, there has to date been only a single published study, that of Putnam *et al.* (1997), in which the effect of a yeast culture fed to lactating dairy cows on the EAA profile of mixed bacteria was examined and found to have no modifying effect. For this reason, one is unable to compare the data obtained in the present study, and this data stands at the forefront for future research on this topic.

In the present study the AA data was also analysed according to the contrast procedure in SAS which allowed for the identification of the main effects of each feed additive on the AA composition of each bacterial fraction as compared to the control treatment. The DFM and MON treatments were shown to have a far greater ability to alter the AA composition of both of the bacterial fractions than the AB10 treatment, which was found to only have an effect ($P < 0.05$) on the AA composition of the FAB. Relative to the control the AB10 treatment reduced the concentration of Met ($P = 0.02$) and increased the concentration of Val ($P = 0.049$), Ile ($P = 0.07$) and Glu ($P = 0.07$) in the FAB fraction. Relative to the control the DFM treatment altered the concentration of

Lys ($P = 0.01$) and Glu ($P = 0.03$) and tended to alter that of Val ($P = 0.05$), Pro ($P = 0.09$) and Tyr ($P = 0.06$) in the fluid-associated fraction. While for the PAB, the DFM treatment gave rise to increased concentrations of Arg ($P = 0.06$), and Gly ($P = 0.03$) and reduced those of His ($P = 0.04$) and Tyr ($P = 0.01$). For both the fluid and particulate bacterial fractions supplementation with MON appeared to have the greatest impact on the alteration of the AA profile. Within the FAB fraction, MON supplementation was shown to reduce the concentration of Val ($P = 0.03$), Thr ($P = 0.08$), Gly ($P = 0.07$) and to tend to increase that of Tyr ($P = 0.06$). The MON treatment appeared to have a greater effect on the alteration of the PAB and was shown to increase the concentrations of Lys ($P = 0.04$), Gly ($P = 0.01$) and Ser ($P = 0.02$) whilst reducing those of Ala ($P = 0.06$), Asp ($P = 0.01$) and Glu ($P < 0.001$).

The study of Erasmus e*t al.* (1992) showed the AA in duodenal digesta to be higher in dairy cows fed a yeast culture and attributed this increase to the increased concentration of certain AA's in the microbial protein of supplemented cows, suggesting that this alteration of AA profile was due to the selective stimulation of the growth of certain species of ruminal bacteria as previously observed by Harrison *et al.* (1988) and Dawson *et al.* (1990). Although Purser & Buechler (1966) investigated the AA composition of the ruminal bacteria and found it to be relatively constant it was noted that when compared, individual species of ruminal bacteria did differ markedly in AA content. In the aforementioned study the AA composition of four of the most abundant rumen bacteria, *S. ruminantium*, *B. fibrisolvens*, *Bacteroides amylophilus* and *Bacteroides ruminicola* were compared, and it was found that there were substantial differences in the concentrations of certain AA's across these species. Erasmus *et al.* (1992) thereby suggested that feed supplements with a selective stimulatory effect on the growth of certain ruminal bacterial species could result in population shifts which could conceivably alter the AA composition of the bacterial fractions. Unfortunately, the present study did not directly examine the effect of dietary treatments on relative shifts in the bacterial populations associated with each bacterial fraction, thus we were unable to draw definitive conclusions on the alteration of the bacterial communities as reflected by the altered AA composition. However, when examining the results overall, it would seem that the buffer treatment didn't give rise to any significant alteration of the bacterial communities associated with either fraction, although the FAB did appear to be affected to a greater extent by supplementation with AB10. This would be expected as buffers do not typically have a direct effect on the ruminal microbial communities and only affect change through alteration of ruminal pH and fermentation, neither of which were notably affected by AB10 supplementation in this study. The effect of ionophore supplementation on the AA concentrations is difficult to explain, ionophores are known to inhibit the fibrolytic bacterial species, which are typically associated with the particle phase of the rumen, whilst enriching the saccharolytic populations which are common to the fluid phase (Legay-Carmier & Bauchart, 1989; Ipharraguerre & Clark, 2003). However, MON appeared to have a greater effect on the PAB fraction were this treatment either tended to increase ($P < 0.10$) or increased ($P < 0.05$) the concentrations of Lys, Thr, Gly, and Ser, and reduced the concentrations of Ala, Asp and Glu. Whereas, for the FAB fraction the MON treatment only reduced the concentration of Val ($P < 0.05$). The DFM treatment appeared to have an almost equal effect on the FAB and PAB fractions, reducing ($P < 0.05$) or tending to reduce ($P < 0.10$) the concentration of both His and Tyr in both fractions, whilst increasing the concentration of Arg and Gly in the PAB fraction and that of Val, Glu and Pro in the FAB fraction. Decreases in the concentration of Lys and Met two of the most important AA's in dairy nutrition were also observed within the FAB fraction with DFM supplementation. Previous research (Harrison *et al.*, 1988; Erasmus *et al.*, 1992) attributed the observed increases in the AA's, specifically Thr, Cys, Ser and Glu, to the stimulation of the anaerobic, fibrolytic, bacterial species, which would be associated with the particulate fraction.

As with the chemical composition, differences in the AA composition of the two bacterial fractions have been well documented. Of the nine EAA's evaluated in this study three were shown to differ ($P < 0.05$) in AA content between the FAB and PAB, these being Leu, Lys and Arg, whilst four of the seven NEAA's evaluated differed ($P < 0.05$), these being Asp, Pro, Ser and Glu, with Gly tending to differ ($P = 0.06$) between the two fractions (Refer to Table 6.7). Compared with the FAB, the PAB fraction had a higher proportion of Leu, Pro, Ser and Gly, in contrast the FAB fraction displayed greater proportions of Lys, Arg, Asp and Glu. The limited literature available on the topic indicates that the differences in AA composition between the FAB and PAB are not consistent with reports of 10 out of 17 (Martin *et al.*, 1996), 15 out of 17 (Yang *et al.*, 2001), and 7 out of 18 of the AA's (Sok *et al.*, 2017) differing between the fractions. The higher Leu observed in the PAB

fraction was in agreement with most of the published studies (Martin *et al.*, 1996; Rodríguez *et al.*, 2000; Boguhn *et al.*, 2006; Sok *et al.*, 2017) as was the higher Pro content in this fraction (Yang *et al.*, 2001; Boguhn *et al.*, 2006; Sok *et al.*, 2017). Whilst the greater proportion of Asp observed in FAB was in agreement with most of studies (Martin *et al.*, 1996; Yang *et al.*, 2001; Boguhn *et al.*, 2006). Of the other AA's observed to differ in the present study some were in agreement with the aforementioned publications but others not, on a whole, reported differences between the two bacterial fractions were somewhat conflicting amongst studies. In brief, as previously discussed, differences in the composition between the two bacterial fractions are reflective of different stages in growth, metabolic activity and species composition of the two fractions, however, one cannot assume that these factors are solely responsible for the observed disparities and the reasons for these remain unclear.

**Table 6.7** Variation in the amino acid profile (g of AA/ 100 g of AA) of ruminal fluid-associated bacteria (FAB) and particle-associated bacteria (PAB) across treatments, in lactating dairy cows fed a total mixed ration (n = 32)

| | FAB[1] | PAB [2] | SEM[3] | *P* –value[4] |
|---|---|---|---|---|
| Essential amino acids | | | | |
| Arginine | 6.86 | 6.51 | 0.112 | 0.04 |
| Histidine | 2.15 | 2.10 | 0.073 | 0.62 |
| Isoleucine | 5.31 | 5.50 | 0.081 | 0.12 |
| Leucine | 7.51 | 7.99 | 0.074 | 0.0002 |
| Lysine | 8.80 | 7.94 | 0.162 | 0.002 |
| Methionine | 3.00 | 2.99 | 0.049 | 0.80 |
| Phenylalanine | 4.84 | 5.12 | 0.130 | 0.14 |
| Threonine | 5.81 | 5.86 | 0.068 | 0.54 |
| Valine | 6.03 | 6.16 | 0.063 | 0.17 |
| Non-essential amino acids | | | | |
| Alanine | 7.55 | 7.36 | 0.196 | 0.48 |
| Aspartic acid | 11.9 | 11.4 | 0.10 | 0.003 |
| Glutamic acid | 12.9 | 12.5 | 0.11 | 0.04 |
| Glycine | 6.22 | 6.45 | 0.080 | 0.06 |
| Proline | 3.52 | 3.93 | 0.058 | < 0.0001 |
| Serine | 3.62 | 3.99 | 0.079 | 0.003 |
| Tyrosine | 4.01 | 4.18 | 0.133 | 0.37 |
| Lys: Met | 2.94 | 2.68 | 0.061 | 0.01 |

[1] FAB = Fluid-associated bacteria
[2] PAB = Particle-associated bacteria
[3] Standard error of the mean
[4] *P*- value, bacterial fractions differ ($P < 0.05$), bacterial fractions tend to differ ($0.05 < P \leq 0.10$)

Martin *et al.* (1996) investigated the effect of sampling time on the AA composition of the ruminal bacteria and found the AA concentration of the different bacterial fractions to be relatively constant over time, and suggested that the AA composition is likely an intrinsic characteristic of each microbial fraction and is not related to metabolic activity. Owing to the difficulty of detaching pure PAB samples from ruminal contents, various techniques, i.e. physiochemical and mechanical methods of fractionation and isolation have arisen over time (See *chapter 3* for detailed description), all of which differ in their ability to detach the PAB. Recoveries were not examined in the present study but have been shown to range from 20% (Martín-Orúe *et al.*, 1998) to 80% (Whitehouse *et al.*, 1994), thus calling into question the true ability of the recovered bacteria to represent the PAB (Legay-Carmier & Bauchart, 1989; Fessenden *et al.*, 2017). For this reason, it is difficult to elucidate if differences are due to actual differences in composition or to either an induced selection of certain bacterial species during the detachment procedure (Martín-Orúe *et al.*, 2000) or feed particle contamination (Martin *et al.*, 1996). When comparing results from various studies on AA composition one must exert caution as much of the variation is likely due to the isolation techniques utilised, with only a small portion of the variation being

an actual difference (Fonseca *et al.*, 2014). However, in the present study the same procedure was strictly followed throughout, thus relative changes to AA composition amongst dietary treatments are likely true.

Dairy cows are known to not have a requirement for protein per se but rather a requirement for AA's particularly the indispensable AA's, with the efficiency of use of the AA's provided by the MP for protein synthesis being determined by the ability of the EAA profile provided by the MP to complement the EAA requirement of the cow (NRC, 2001). Importantly if just a single EAA becomes limiting the utilisation of the other absorbable AA's for protein synthesis is hindered, with a concomitant decline in protein efficiency. The two most limiting EAA's for lactating dairy cows fed conventional diets are Lys and Met (NRC, 2001), for this reason, the Lys to Met ratio serves as a useful indicator as to whether the cow is utilising the AA provided by the diet as efficiently as possible (Ordway & Aines, 2010). In the present study the Lys to Met ratio of both the fluid- and particle- associated bacteria appeared to be influenced by dietary supplementation, with the ratio being higher for the AB10 treatment and tending to differ from the C ($P = 0.09$) and DFM ($P = 0.08$) treatments for the fluid-associated fraction. Whereas for the PAB the Lys to Met ratio didn't differ amongst treatments with the exception of the MON and DFM treatments which tended to differ ($P = 0.07$) from each other. When applying the contrast procedure to compare the C treatment to each individual treatment, none of the treatments were shown to differ from the C treatment for the PAB, while for the FAB the AB10 treatment was still shown to tend to differ from the C treatment ($P = 0.09$). When compared to one another the mean ratio of Lys to Met differed ($P = 0.01$) between the FAB and PAB, with the FAB displaying a greater ratio of 2.94:1, which correlated well with the generally accepted ideal ratio of 3:1. Whereas the mean ratio for the PAB and the individual ratios for each of the treatments were lower than ideal, indicating that the EAA profile provided by the PAB may be inadequate, with the exception of the MON treatment. It must be noted that this ideal ratio is not definite, ranging typically from 2.8 to 3, and can differ with the use of different models and the production targets (Tucker, 2014).

Of the MCP flowing to the duodenum, one can assume that 33.4 % is FAB, 50.1 % is PAB and 16.5 % is protozoal (Sok *et al.*, 2017). Although not examined in the present study nutritional factors have shown the potential to alter the proportions of the bacterial fractions and the protozoa flowing to the duodenum, this data in conjunction with information on the effect of various nutritional factors on the AA composition of each fraction, will allow for the more accurate prediction of AA supply to the lactating dairy cow. Despite the changes in the AA profile amongst the feed additives in the present study being small, many were significant and when combined with knowledge on alterations in the proportion of the fractions flowing to the duodenum, in response to each additive, significant alterations in AA supply are plausible. Particularly with regards to the most-limiting AA's in dairy nutrition, i.e. Lys, Met and His, as these were all shown to be affected by supplementation in this study and have been shown by Clark *et al.* (1992) to vary considerably in passage to the duodenum, with Lys ranging from 26 g/d to 274 g/d and Met from 6 g/d to 142 g/d. This highlights the importance of accurate knowledge on the composition of the microbial fractions, if one is to accurately quantify the passage of microbial AA's to the duodenum.

# CHAPTER 7

# CONCLUSION

Currently research focussed on alteration of the ruminal microbial populations and the AA composition of the various microbial fractions is limited, particularly in response to feed additive supplementation. To date most research has focussed on the effect of nutritional factors such as diet composition, level of feed intake, forage to concentrate ratio and dietary processing on the chemical and AA composition of the ruminal microbial population, however, even this research has been scarce and typically limited to only those microbes associated to the fluid phase of the rumen. Owing to the historical use of ionophores and the ever increasing, wide-spread use of alternative "natural" feed additives, in dairy production, further investigation of the effects of these additives was warranted, particularly with regards to the effect of these additives on the ruminal microbial population which is known to supply up to 80 % of the ruminant's daily AA requirements.

In this study, daily supplementation of the buffer Acid Buf 10, commercial DFM Achieve$^{FE}$ and ionophore Rumensin$^{TM}$ to lactating dairy cows had no effect on feed intake, body condition or lactational performance as was expected, with these parameters being monitored throughout the study although not the focal point of this research. This lack of effect was likely due to the cows in this study being in late-lactation, with beneficial results typically being observed in early-lactation. The efficiency of milk production was not influenced by dietary supplementation, although the DFM did tend to improve efficiency. Milk composition was unaltered by supplementation with the exception of milk protein which was reduced with ionophore supplementation. This lack of production response was a reflection of the inability of the additives to significantly alter ruminal fermentation, in particular ruminal ammonia, total VFA concentration and the molar proportions of the VFA's, under the dietary conditions of this study. Ruminal pH, however, appeared to be improved by Acid Buf 10 supplementation, which despite failing to improve the mean ruminal pH, tended to reduce the total hours for which ruminal pH remained below pH 5.5, the threshold for sub-acute ruminal acidosis, from the 10.1 hours displayed by the control to 6.1 hours. This finding corroborates previous research on Acid Buf in which it has been shown to have a pH stabilisation effect. Dietary additives had no significant impact on microbial protein synthesis, with ionophore supplementation tending to reduce microbial synthesis, as has been previously reported. Supplementation was shown to have an effect on the AA profile of the fluid-associated and particle-associated bacteria, with 9 and 10 out of the 16 AA's being either altered or tending to be altered by supplementation for the FAB and PAB fractions, respectively. The results, however, are difficult to explain as no particular pattern was observed with the changes in the AA profile appearing to be random. This could be due to the feed additives but the design of the trial did not give us the ability to make a concrete conclusion. Overall, monensin appeared to have the greatest ability to alter the AA profile of both the bacterial fractions as compared to the other additives.

Although the feed additives evaluated in this study were shown to alter ruminal pH and fermentation dynamics, results have been variable across diverse experimental studies. As a result of the lack of treatment responses observed in this study it is unclear as to whether Acid Buf 10 and Achieve$^{FE}$ could serve as potential alternatives to ionophores, or work in synergy with ionophores, and further research on both of these additives is warranted due to the currently limited knowledge. The results of this study do suggest that feed additives have the potential to change the AA profile of the ruminal bacteria but further research on the potential changes in the microbial populations within the microbiome and the AA profiles of the various populations are needed to enable us to predict with more accuracy which AA will be affected and to what extent.

Unfortunately, research in this area has been limited, and further studies are required to examine the effects of dietary factors and additives on the relative contribution, quantity and AA composition of the different fractions of ruminally synthesised MCP flowing to the duodenum, as these factors ultimately determine the duodenal AA flow and supply of AA to the dairy cow. Knowledge of dietary-mediated effects on AA composition and duodenal flows of the microbial fractions is imperative if nutritionists wish to improve the accuracy and precision of the estimation of the AA supply available to meet the nutritional requirements of the dairy cow, through incorporation of these dietary-mediated effects into the mathematical approaches

currently used for ration formulation and simulation of nutrient requirements. With the final outcome of improving the prediction of duodenal ratios of EAA, specific to various nutritional situations, to meet the needs of the dairy cow with less dietary CP and increased N efficiency.

# CHAPTER 8

# CRITICAL EVALUATION AND FUTURE RESEARCH

## 8.1 Critical evaluation

The contribution of protozoa to the microbial protein flowing to the duodenum and available to the ruminant is often overlooked, despite studies demonstrating that protozoa can contribute up to 21 % to 25% of the total microbial N flowing to the duodenum (Yáñez-Ruiz *et al.*, 2006) and are richer in EAA's, particularly Lys, than the ruminal bacteria (Sok *et al.*, 2017). Such data indicates the importance of acknowledging the presence of protozoa in the duodenal MCP flow for the correct prediction of the AA present in the duodenal flow and available to meet the nutritional requirements of the cow. Investigation of the AA composition of the ruminal protozoa would have been a beneficial addition to the present study, and although originally planned for, we were unable to include this research due to time and labour constraints.

An additional enhancement to this research would have been the inclusion of a genetic study. The isolation of genomic DNA from each of the bacterial fractions would have allowed for next generation sequencing (NGS) analysis, this technology has revolutionised genomic research and allows for the further elucidation of microbial community structure. Inclusion in the present study would have allowed for the identification of the dominant or signature microbial species present in each bacterial fraction and allowed for the examination of the quantitative shifts in the bacterial communities of each bacterial fraction, thus allowing for the determination of the effect of the feed additives on the ruminal microbiome which would have assisted in the explanation of the observed alterations of AA composition in response to supplementation.

Although sampling directly from the omasal or duodenal contents may have allowed for the isolation of microbes more representative of those serving as microbial protein to the ruminant, investigation of the ruminal microbes is a sufficient first step in the evaluation of the effect of nutritional factors on the composition on the microbes. However, having said this ruminal samples in this study where taken from various regions within the rumen in order to obtain composite samples representative of the entire rumen, but it may have been more correct to sample ruminal contents near the reticulo-omasal orifice, as microbes isolated from this region are likely more representative of the microbes flowing into the abomasum than those isolated from the entire rumen (Shabi *et al.*, 2000).

While fluctuations in ruminal pH were not the focus of this study, the use of indwelling pH meters could have been beneficial to this study, as it would have allowed for a more detailed description of the fluctuations in ruminal pH over time for each of the treatments, and a more accurate determination of the time ruminal pH remained below pH 5.5.

The lack of response to supplementation in the present study was unexpected and likely owed to the following considerations: 1) Cows late in lactation were utilised in this study, in this stage of the production cycle cows are typically in a positive energy balance and not as physiologically challenged as early-lactation cows and thus one cannot expect to observe the same beneficial production responses; 2) Intakes of the Acid Buf 10 and Monensin treatments were less than the optimal inclusion dictated by the manufacturers, as the cows displayed lower DM intakes, 20.1 kg DM/d *versus* 24 kg DM/d, than previously measured and expected for the duration of the experimental period, which could explain the inability of these additives to exert an effect on ruminal dynamics and hence productive performance; and 3) Diet composition, particularly with regards to examining the effect of Acid Buf 10, as the diets were likely well-buffered owing to the substantial inclusion of Lucerne hay which is known for its inherent buffering capacity, thus the cows were likely not experiencing a significant enough acidic challenge to demonstrate the true capability of this buffer, as exhibited by the diminished, non-significant ruminal pH response to this additive.

As a note, from the outcomes of this study it is important to highlight the importance of understanding the statistical analysis and interpretation of a data set when examining results obtained in various studies, and to exert caution when drawing conclusions as results can vary substantially based on the statistical analysis employed.

### 8.2 Future research

This particular study has demonstrated the need for further investigation of the potential of feed additives to alter the ruminal microbiome, the flow of ruminally synthesised MCP to the duodenum, particularly the differential passage and contribution of the fluid- and particle-associated bacterial fractions and protozoa, and the AA composition of these fractions. Considerable uncertainty about the relative contribution of the FAB, PAB and protozoa to the duodenal digesta still exists, and as the AA composition of these different fractions is known to vary, any dietary factor or feed additive capable of inducing variation in the relative contribution of the various microbial fractions will give rise to variation in the AA composition of the MCP flowing to the duodenum (Dijkstra *et al.*, 1997), as would any alteration of the AA composition of individual fractions in response to dietary factors or supplementation. By enhancing our knowledge nutritionists could potentially better predict the influence of various nutritional factors on the flow of individual AA's, thus improving diet formulation and allowing for the more accurate prediction of AA supply and requirements to the lactating dairy cow, with the focus on improving milk production with reduced dietary CP inputs.

This study also highlights the need for further investigation of calcareous marine algae products such as Acid Buf 10, and direct-fed microbials, as these additives are being increasingly used in the dairy industry despite the variable production responses observed across studies. In particular studies on the various direct-fed microbial products which are commercially available is warranted owing to the diverse composition of these products. Specifically, practical on-farm studies are required, in which the product is incorporated into the feed rather than administered via oral dosing or directly into the rumen as neither of these are truly practical situations.

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# APPENDIX

## A.1 Microbial crude protein calculation example

The various steps for the calculation of microbial crude protein production in lactating dairy cows, as explained in detail in *Chapter 4*, are illustrated with the following example. Note that if you have a large data set or will be required to perform these calculations routinely you can construct a spreadsheet template using Microsoft Excel.

[Example] A spot urine sample with a specific gravity (SG) value of 1.028 was obtained from a lactating Holstein dairy cow weighing 589 kg. The urine sample was analysed chemically for Allantoin (AL) (Young & Conway, 1942; Chen & Gomes, 1992) and found to contain 3473.21 mg/L of AL*.

* Note that this value is an adjusted value, the AL concentration obtained from analysis was 57.89 mg/L but was adjusted to account for the 1: 60 dilution factor specific to this study – the dilution factor utilised will differ based on the specifics of each study.

1) *Calculation of urine volume (L/d):*
   Urine volume = 332.66* (((SG -1)*1000) $^{-0.884}$), where SG is the specific gravity of the urine sample.

   Urine volume (L/d) = 332.66* (((1.028 -1)*1000) $^{-0.884}$) = 17.47 L/d

2) *Calculation of daily urinary AL output (mmol/d):*
   Urinary AL output (mmol/d) = [AL] (mmol/L)* urine volume (L/d)

   Urinary AL output (mmol/d) = 21.97*17.47 = 383.73 mmol/d

   Convert [AL] from mg/L to mmol/L as follows;
   (([AL] (mg/L) / (158.12*1000))*1000, where 158.12 is the molecular mass of AL (g/mol)

3) *Calculation of total daily purine derivative (PD) excretion (mmol/d):*
   Total PD excretion (mmol/d) = Urinary PD (mmol/d) + Milk PD (mmol/d)

   Total PD excretion (mmol/d) = 423.64 + 21.18 = 444.82 mmol/d

   Note: To calculate total PD excretion (mmol/d) one must first calculate the daily excretion of urinary PD and the daily excretion of milk PD.

   *3.1) Daily excretion of urinary PD (mmol/d):*
   Urinary PD excretion (mmol/d) = AL output (mmol/d) / 0.906, where 0.906 is a coefficient which expresses the [AL] as a proportion of total urinary PD excretion

   Urinary PD excretion (mmol/d) = 383.73 / 0.906 = 423.64 mmol/d

   *3.2) Daily excretion of milk PD (mmol/d):*
   Milk PD excretion (mmol/d) = Urinary PD excretion (mmol/d)* 0.05

   Milk PD excretion (mmol/d) = 423.64*0.05 = 21.18 mmol/d

4) *Calculation of daily intestinal absorption of microbial PD (mmol/d):*
   Intestinal absorption of microbial PD (mmol/d) = (Total daily PD (mmol/d) – 0.385* (BW (kg) $^{0.75}$)) / 0.85,

where 0.385 is the net endogenous contribution of PD to total PD excretion, and 0.85 is the recovery of absorbed purines as PD in the urine.

Intestinal absorption of microbial PD (mmol/d) = (444.82 – 0.385* (589 $^{0.75}$)) / 0.85 = 469.16 mmol/d

5) *Calculation of intestinal flow of microbial nitrogen (N) (g N/d):*
Intestinal flow of microbial N (g N/d) = (Intestinal absorption of microbial PD (mmol/d)*70) / (0.116*0.83*1000),

where 70 is the N content for purines, 0.116 is the ratio of purine N: total N in mixed ruminal microbes, and 0.83 is the coefficient for microbial purine digestibility.

Intestinal flow of microbial N (g N/d) = (469.16*70) / (0.116*0.83*1000) = 341.10 g N/d

6) *Calculation of microbial crude protein (MCP) production (g CP/d):*
MCP production (g CP/d) = (Intestinal flow of microbial N (g N/d)*6.25,

where 6.25 is a factor utilised to convert N content to CP, as the average N content of protein is 16 % (1/0.16 = 6.25)

MCP production (g CP/d) = 341.10*6.25 = 2131.89 g CP

## A.2 Comparative bacterial amino acid profiles from literature

**Table A.2.1** Amino acid composition of mixed ruminal bacteria (g/ 100 g of AA) (Source: Clark *et al.*, 1992)[1]

| Amino acid | Mean[2] | Minimum | Maximum | SD[3] | CV[4] |
|---|---|---|---|---|---|
| Arginine | 5.1 | 3.8 | 6.8 | 0.7 | 13.2 |
| Histidine | 2.0 | 1.2 | 3.6 | 0.4 | 21.3 |
| Isoleucine | 5.7 | 4.6 | 6.7 | 0.4 | 7.4 |
| Leucine | 8.1 | 5.3 | 9.7 | 0.8 | 10.3 |
| Lysine | 7.9 | 4.9 | 9.5 | 0.9 | 11.9 |
| Methionine | 2.6 | 1.1 | 4.9 | 0.7 | 25.6 |
| Phenylalanine | 5.1 | 4.4 | 6.3 | 0.3 | 6.4 |
| Threonine | 5.8 | 5.0 | 7.8 | 0.5 | 8.9 |
| Valine | 6.2 | 4.7 | 7.6 | 0.6 | 10.1 |
| Alanine | 7.5 | 5.0 | 8.6 | 0.6 | 7.3 |
| Aspartic acid | 12.2 | 10.9 | 13.5 | 0.6 | 4.8 |
| Glutamic acid | 13.1 | 11.6 | 14.4 | 0.7 | 5.3 |
| Glycine | 5.8 | 5.0 | 7.6 | 0.5 | 8.2 |
| Proline | 3.7 | 2.4 | 5.3 | 0.5 | 13.2 |
| Serine | 4.6 | 3.4 | 5.4 | 0.4 | 8.9 |
| Tyrosine | 4.9 | 3.9 | 7.7 | 0.6 | 13.2 |

[1] Table taken from the published paper of Clark *et al.*, 1992, in which data from various other sources were compiled (Weller, 1957; Meyer *et al.*, 1967; Bergen *et al.*, 1968; Hoogenraad & Hird, 1970; Ibrahim & Ingalls, 1972; Williams & Dinusson, 1973; Burris *et al.*, 1974; Ulyatt *et al.*, 1975; Czerkawski, 1976; Storm & Ørskov, 1983; John, 1984; Rooke *et al.*, 1984; Hvelplund, 1986; Cecava *et al.*, 1988; Rooke & Armstrong, 1989; Titgemeyer *et al.*, 1989; Cecava *et al.*, 1990; and Klusmeyer *et al.*, 1991 unpublished data

[2] Mean composition is the average of 441 bacterial samples from animals fed 61 dietary treatments in 35 experiments

[3] Standard deviation

[4] Coefficient of variation

**Table A.2.2** Amino acid composition of ruminal fluid-associated (FAB) and particle-associated (PAB) bacteria (g of AA/ 100 g of AA) (Source: Sok *et al.*, 2017)[1]

| | Fluid-associated bacteria[2] | | | | | Particle-associated bacteria[3] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Amino acid | Mean | Minimum | Maximum | CV[4] | N[5] | Mean | Minimum | Maximum | CV | N |
| Alanine | 7.16 | 5.55 | 8.03 | 8.3 | 22 | 6.52 | 5.40 | 7.59 | 11.6 | 6 |
| Arginine | 4.61 | 3.91 | 5.24 | 7.3 | 20 | 5.01 | 4.42 | 5.79 | 9.3 | 6 |
| Aspartic acid | 11.97 | 11.15 | 12.66 | 3.5 | 21 | 11.53 | 10.89 | 12.11 | 4.2 | 7 |
| Cysteine | 1.56 | 1.20 | 2.82 | 34.3 | 9 | 1.50 | 1.16 | 2.28 | 35.2 | 4 |
| Glutamic acid | 12.83 | 11.31 | 14.60 | 6.8 | 22 | 13.11 | 12.15 | 14.30 | 6.9 | 7 |
| Glycine | 5.55 | 4.88 | 6.47 | 6.6 | 20 | 5.36 | 4.57 | 5.86 | 8.5 | 6 |
| Histidine | 1.87 | 1.47 | 2.49 | 15.4 | 19 | 2.01 | 1.62 | 2.28 | 13.1 | 7 |
| Isoleucine | 5.54 | 4.85 | 6.33 | 6.5 | 22 | 5.68 | 5.14 | 6.07 | 7.0 | 7 |
| Leucine | 7.62 | 7.15 | 8.33 | 4.9 | 21 | 8.14 | 7.93 | 8.32 | 1.8 | 7 |
| Lysine | 7.72 | 6.05 | 9.09 | 10.6 | 22 | 7.43 | 6.45 | 8.52 | 12.2 | 7 |
| Methionine | 2.38 | 1.76 | 3.00 | 16.4 | 14 | 2.33 | 1.98 | 2.58 | 11.5 | 4 |
| Phenylalanine | 5.12 | 4.40 | 6.07 | 7.4 | 22 | 5.60 | 4.52 | 6.42 | 10.5 | 7 |
| Proline | 3.60 | 2.84 | 4.24 | 8.9 | 22 | 3.84 | 3.24 | 4.21 | 8.1 | 7 |
| Serine | 4.50 | 3.73 | 5.36 | 9.6 | 21 | 4.30 | 3.58 | 4.70 | 9.6 | 7 |
| Threonine | 5.62 | 5.07 | 6.32 | 5.8 | 22 | 5.40 | 5.07 | 5.81 | 6.3 | 6 |
| Tryptophan | 1.27 | 1.00 | 1.63 | 25.6 | 3 | 1.28 | 1.10 | 1.47 | 20.6 | 2 |
| Tyrosine | 5.21 | 4.37 | 6.57 | 10.7 | 22 | 5.27 | 4.17 | 6.25 | 14.1 | 7 |
| Valine | 5.91 | 5.11 | 6.67 | 7.9 | 22 | 5.83 | 5.18 | 6.44 | 8.5 | 7 |

[1] Table adapted from the published paper of Sok *et al.*, 2017

[2] Data compiled from Meyer *et al.*, 1967; Ibrahim & Ingalls, 1972; Williams & Dinusson, 1973; Burris *et al.*, 1974; Rooke *et al.*, 1984; Hvelplund, 1986; Rahnema & Theurer, 1986; Cecava *et al.*, 1988; Erasmus *et al.*, 1994; Cozzi *et al.*, 1995; Ludden & Cecava, 1995; Martin *et al.*, 1996; Ludden & Kerley, 1997; Mabjeesh *et al.*, 1997; Volden & Harstad, 1998; Elizalde *et al.*, 1999; Volden *et al.*, 1999; Korhonen *et al.*, 2002; Robinson *et al.*, 2002; Reynal *et al.*, 2003; Zebeli *et al.*, 2008; Fonseca *et al.*, 2014.

[3] Data compiled from Martin *et al.*, 1996; Volden & Harstad, 1998; Volden *et al.*, 1999; Korhonen *et al.*, 2002; Reynal *et al.*, 2003; Zebeli *et al.*, 2008; and Fonseca *et al.*, 2014.

[4] Coefficient of variation

[5] Number of studies

## A.3 Additional data

### A.3.1 Amino acid profile of bacterial fractions expressed as g of amino acid per 100 g of dry matter

**Table A.3.1.1** Ruminal fluid-associated bacterial (FAB) amino acid profile (g of AA/ 100 g DM) as affected by supplementation of lactating dairy cows with Acid Buf 10, a direct-fed microbial and monensin, when fed a total mixed ration (n = 16)

| | Treatments [1] | | | | |
|---|---|---|---|---|---|
| | C | AB10 | DFM | MON | S.E.M. [2] |
| EAA[3] | | | | | |
| Arginine | 3.07 | 3.14 | 3.18 | 3.18 | 0.090 |
| Histidine | 1.14$^{d}$ | 0.93$^{de}$ | 0.86$^{e}$ | 1.01$^{de}$ | 0.082 |
| Isoleucine | 2.39 | 2.54 | 2.32 | 2.50 | 0.091 |
| Leucine | 3.45 | 3.45 | 3.35 | 3.54 | 0.102 |
| Lysine | 4.32$^{a}$ | 4.13$^{ab}$ | 3.72$^{c}$ | 3.98$^{c}$ | 0.071 |
| Methionine | 1.54$^{a}$ | 1.33$^{b}$ | 1.33$^{b}$ | 1.31$^{b}$ | 0.056 |
| Phenylalanine | 2.16 | 2.39 | 2.13 | 2.20 | 0.166 |
| Threonine | 2.74$^{d}$ | 2.68$^{de}$ | 2.68$^{de}$ | 2.56$^{e}$ | 0.063 |
| Valine | 2.76$^{ab}$ | 2.83$^{a}$ | 2.80$^{abd}$ | 2.66$^{be}$ | 0.044 |
| NEAA[4] | | | | | |
| Alanine | 3.52 | 3.42 | 3.42 | 3.49 | 0.210 |
| Aspartic acid | 5.59 | 5.48 | 5.42 | 5.28 | 0.124 |
| Glutamic acid | 5.94 | 5.87 | 5.95 | 5.88 | 0.109 |
| Glycine | 3.05$^{d}$ | 2.81$^{de}$ | 2.82$^{de}$ | 2.74$^{e}$ | 0.096 |
| Proline | 1.60 | 1.52 | 1.68 | 1.65 | 0.092 |
| Serine | 1.74 | 1.60 | 1.69 | 1.61 | 0.069 |
| Tyrosine | 1.99$^{ad}$ | 1.84$^{ab}$ | 1.39$^{be}$ | 2.16$^{a}$ | 0.178 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[3] Essential amino acid
[4] Non- essential amino acid
[a, b, c] Means within a row with different superscripts differ ($P < 0.05$)
[d, e] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

**Table A.3.1.2** Ruminal particle-associated bacterial (PAB) amino acid profile (g of AA/ 100 g DM) as affected by supplementation of lactating dairy cows with Acid Buf 10, a direct-fed microbial and monensin, when fed a total mixed ration (n = 16)

| | Treatments [1] | | | | |
|---|---|---|---|---|---|
| | C | AB10 | DFM | MON | S.E.M. [2] |
| EAA[3] | | | | | |
| Arginine | 2.58$^{b}$ | 2.62$^{abd}$ | 3.00$^{ac}$ | 2.98$^{ac}$ | 0.114 |
| Histidine | 0.98$^{a}$ | 0.86$^{ab}$ | 0.72$^{b}$ | 1.03$^{a}$ | 0.064 |
| Isoleucine | 2.39 | 2.24 | 2.27 | 2.54 | 0.119 |
| Leucine | 3.52 | 3.31 | 3.38 | 3.50 | 0.123 |
| Lysine | 3.45$^{ab}$ | 3.14$^{b}$ | 3.26$^{abd}$ | 3.79$^{ac}$ | 0.176 |
| Methionine | 1.30$^{a}$ | 1.17$^{b}$ | 1.34$^{a}$ | 1.32$^{a}$ | 0.036 |
| Phenylalanine | 2.22 | 2.08 | 2.17 | 2.32 | 0.106 |
| Threonine | 2.48$^{cd}$ | 2.39$^{d}$ | 2.51$^{cd}$ | 2.70$^{c}$ | 0.098 |
| Valine | 2.73 | 2.52 | 2.64 | 2.70 | 0.120 |
| NEAA[4] | | | | | |
| Alanine | 3.33 | 3.14 | 3.18 | 2.95 | 0.135 |
| Aspartic acid | 4.99 | 4.78 | 4.97 | 4.78 | 0.117 |
| Glutamic acid | 5.58$^{a}$ | 5.46$^{a}$ | 5.58$^{a}$ | 4.89$^{b}$ | 0.140 |
| Glycine | 2.77$^{ab}$ | 2.53$^{b}$ | 2.81$^{ab}$ | 2.98$^{a}$ | 0.104 |
| Proline | 1.73 | 1.57 | 1.71 | 1.74 | 0.067 |
| Serine | 1.67$^{ad}$ | 1.53$^{b}$ | 1.71$^{a}$ | 1.94$^{ac}$ | 0.085 |
| Tyrosine | 2.00$^{a}$ | 1.70$^{b}$ | 1.57$^{b}$ | 1.90$^{a}$ | 0.056 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[3] Essential amino acid
[4] Non- essential amino acid
[a, b] Means within a row with different superscripts differ ($P < 0.05$)
[c, d] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

### A.3.2 Amino acid profile of bacterial fractions expressed as g of AA-N per 100 g of dry matter

**Table A.3.2.1** Ruminal fluid-associated bacterial (FAB) amino acid profile (AA-N/ 100 g DM) as affected by supplementation of lactating dairy cows with Acid Buf 10, a direct-fed microbial and monensin, when fed a total mixed ration (n = 16)

| | Treatments [1] | | | | |
|---|---|---|---|---|---|
| | C | AB10 | DFM | MON | S.E.M. [2] |
| EAA[3] | | | | | |
| Arginine | 0.99 | 1.01 | 1.02 | 1.02 | 0.029 |
| Histidine | 0.31$^{d}$ | 0.25$^{de}$ | 0.23$^{e}$ | 0.27$^{de}$ | 0.022 |
| Isoleucine | 0.26 | 0.27 | 0.25 | 0.27 | 0.001 |
| Leucine | 0.37 | 0.37 | 0.36 | 0.38 | 0.011 |
| Lysine | 0.83$^{a}$ | 0.79$^{ab}$ | 0.71$^{c}$ | 0.76$^{b}$ | 0.014 |
| Methionine | 0.14$^{a}$ | 0.13$^{b}$ | 0.12$^{b}$ | 0.12$^{b}$ | 0.005 |
| Phenylalanine | 0.18 | 0.20 | 0.18 | 0.19 | 0.014 |
| Threonine | 0.32$^{d}$ | 0.32$^{de}$ | 0.32$^{de}$ | 0.30$^{e}$ | 0.007 |
| Valine | 0.33$^{ab}$ | 0.34$^{a}$ | 0.33$^{abd}$ | 0.32$^{be}$ | 0.005 |
| NEAA[4] | | | | | |
| Alanine | 0.55 | 0.54 | 0.54 | 0.55 | 0.033 |
| Aspartic acid | 0.59 | 0.58 | 0.57 | 0.56 | 0.013 |
| Glutamic acid | 0.57 | 0.56 | 0.57 | 0.56 | 0.010 |
| Glycine | 0.57$^{d}$ | 0.52$^{de}$ | 0.53$^{de}$ | 0.51$^{e}$ | 0.018 |
| Proline | 0.19 | 0.19 | 0.20 | 0.20 | 0.011 |
| Serine | 0.23 | 0.21 | 0.22 | 0.21 | 0.009 |
| Tyrosine | 0.15$^{abd}$ | 0.14$^{ab}$ | 0.11$^{be}$ | 0.17$^{a}$ | 0.014 |
| Total AA-N, % of DM | 6.58$^{d}$ | 6.41$^{de}$ | 6.27$^{e}$ | 6.39$^{de}$ | 0.103 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.

[2] Standard error of the mean

[3] Essential amino acid

[4] Non- essential amino acid

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

[d, e] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)

**Table A.3.2.2** Ruminal particle-associated bacterial (PAB) amino acid profile (AA-N/ 100 g DM) as affected by supplementation of lactating dairy cows with Acid Buf 10, a direct-fed microbial and monensin, when fed a total mixed ration (n = 16)

| | Treatments [1] | | | | |
|---|---|---|---|---|---|
| | C | AB10 | DFM | MON | S.E.M. [2] |
| EAA[3] | | | | | |
| Arginine | 0.83$^{b}$ | 0.84$^{abd}$ | 0.96$^{ac}$ | 0.95$^{ac}$ | 0.037 |
| Histidine | 0.27$^{a}$ | 0.23$^{ab}$ | 0.20$^{b}$ | 0.28$^{a}$ | 0.017 |
| Isoleucine | 0.26 | 0.24 | 0.24 | 0.27 | 0.013 |
| Leucine | 0.38 | 0.35 | 0.36 | 0.37 | 0.013 |
| Lysine | 0.66$^{ab}$ | 0.60$^{b}$ | 0.62$^{abd}$ | 0.73$^{ac}$ | 0.034 |
| Methionine | 0.12$^{a}$ | 0.11$^{b}$ | 0.13$^{a}$ | 0.12$^{a}$ | 0.003 |
| Phenylalanine | 0.19 | 0.18 | 0.18 | 0.20 | 0.009 |
| Threonine | 0.29$^{cd}$ | 0.28$^{d}$ | 0.30$^{cd}$ | 0.32$^{c}$ | 0.011 |
| Valine | 0.33 | 0.30 | 0.32 | 0.32 | 0.014 |
| NEAA[4] | | | | | |
| Alanine | 0.52$^{c}$ | 0.49$^{cd}$ | 0.50$^{cd}$ | 0.46$^{d}$ | 0.021 |
| Aspartic acid | 0.53 | 0.50 | 0.52 | 0.50 | 0.012 |
| Glutamic acid | 0.53$^{a}$ | 0.52$^{a}$ | 0.53$^{a}$ | 0.47$^{b}$ | 0.013 |
| Glycine | 0.52$^{ab}$ | 0.47$^{b}$ | 0.53$^{ab}$ | 0.56$^{a}$ | 0.019 |
| Proline | 0.21 | 0.19 | 0.21 | 0.21 | 0.008 |
| Serine | 0.22$^{abd}$ | 0.20$^{b}$ | 0.23$^{ab}$ | 0.26$^{ac}$ | 0.011 |
| Tyrosine | 0.15$^{a}$ | 0.13$^{b}$ | 0.12$^{b}$ | 0.15$^{a}$ | 0.004 |
| Total AA-N, % of DM | 6.00$^{ab}$ | 5.65$^{b}$ | 5.94$^{ab}$ | 6.17$^{a}$ | 0.140 |

[1] C = Control diet; AB10 = Control diet with Acid Buf 10 included at 3.75 g/kg DM to obtain an intake of 90 g/d; DFM = Control diet and the direct-fed microbial product, Achieve$^{FE}$, administered directly into the rumen at 10 g/d; MON = Control diet with Rumensin® 200 included at 54.2 mg/kg DM to obtain an intake of 260 mg/d of monensin sodium.
[2] Standard error of the mean
[3] Essential amino acid
[4] Non- essential amino acid
[a, b] Means within a row with different superscripts differ ($P < 0.05$)
[c, d] Means within a row with different superscripts tend to differ ($0.05 < P \leq 0.10$)