Changes in rumen microbial ecology during dietary transition in cattle and sheep: a molecular and metabolic approach.

Submitted for the degree of Master of Philosophy at Murdoch University

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B.Sc. (Agriculture) (Hons)

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

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(Fiona Michelle Jones)

Abstract

Ruminal acidosis is often characterised by decreased ruminal pH below pH 6.0, increased concentrations of ruminal D and L- lactate and volatile fatty acid concentrations in grain fed ruminants, creating an environment for growth of lactic acid producing bacteria such as *S. bovis* and *Lactobacillus spp.* and reduction in cellulolytic bacterial populations e.g. *F. succinogenes*.

This thesis undertook genotypic studies of rumen microbial ecology based on five key bacterial species, *Prevotella ruminantium, Fibrobacter succinogenes, Selenomonas ruminantium, Streptococcus bovis*, and *Lactobacillus spp*. using quantitative real time PCR (qRT- PCR) of 16S rRNA genes. This methodology enabled true genetic monitoring of ecological changes rather than traditional phenotypic microbial culture studies. These genetic studies of rumen microbial ecology were aligned with changes in rumen metabolism.

Application of qRT-PCR methodology was validated for complete and consistent extraction of DNA from mixed rumen samples to ensure reliable enumeration of rumen bacteria, and finally development of primers for use in the qRT-PCR assays. The qRT-PCR methods were then used to monitor changes in rumen microbial ecology in cattle managed under commercial conditions in feedlots rather than experimental conditions. The key species were stabilised in the rumen microbial ecology within 7 days of introduction of cattle to feedlots irrespective of feeding hay and grain separately or via total mixed rations. Moreover, metabolic indicators of high production potential coincided with the stable populations of the key rumen bacterial species *F. succinogenes*, *P. ruminicola* and *S. ruminantium* and no evidence of elevated *S. bovis* populations. Developmental changes in rumen bacterial ecology of steers born during either autumn or

winter/spring showed similar trends in bacterial populations when adapting to feedlot rations irrespective of time of calving. However, the rumen protozoal populations were reflective of the time of calving with cattle born in winter/spring maintaining higher protozoal populations throughout the feedlot period. In commercial dairy herds, rumen metabolic end products were consistently correlated with changes in key bacterial populations. Rumen acidosis was observed in sheep fed lupins at 3 times maintenance. Decreased populations of *F. succinogenes* and increased populations of *S. bovis* with no decrease in rumen pH were observed in sheep fed high-fat soyabean diets.

Molecular techniques such as qRT-PCR used here as well as newer molecular genetic approaches such as next generation sequencing will allow for more comprehensive interpretation of ecological changes in the rumen leading to improved management and productivity of cattle and sheep especially during dietary transitions.

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1 Literature Review

1.1 Introduction

This review describes the Australian cattle industry and its management practice of grain feeding in feedlots where livestock are grain-fed to deal with seasonal gaps in pasture supply and ensure year-round supply of high quality meat products to local and international markets.

While grain feeding optimises livestock energy supply, it also has implications for ruminant health. Optimising production of grain-fed cattle has traditionally been achieved through metabolic and physiological assessments during the introductory period of grain feeding. Monitoring of changes in rumen microbial ecology (particularly overgrowth of lactic acid-producing bacteria) has been most commonly based on phenotyping using subculture techniques for rumen bacteria under laboratory conditions.

In recent times, however, techniques have become available that may allow molecular assessment of rumen bacteria in livestock under field conditions. This review outlines possible applications of molecular methods to monitor changes in rumen bacteria during dietary transition in feedlot, field and dairy feeding systems.

1.2 **The livestock industry in Australia**

The beef cattle industry is a major Australian agricultural industry, ranging from intensively managed livestock holdings in southern Australia through to extensive large-scale cattle stations in the northern pastoral regions. The beef industry (including live cattle) contributes 13% to Australia's total farm export value of $40 billion (ABARES Agricultural Commodities June 2013). Hooper (2010) estimated that about a quarter of Australia's 133,000 farming establishments derived their main income from beef cattle farming while another quarter earned a significant portion of their income from beef cattle

in combination with grain farming and sheep. The 2011-12 Australian Bureau of Statistics indicated that the Australian beef cattle herd totalled 28.5 million head plus an additional 2.7 million dairy cattle and 74 million sheep.

While Australian cattle and sheep are predominantly produced using grass-based feeding systems, many areas become deficient in feed quality and availability at specific periods of the year. For example, in the south west of Western Australia (WA) there is usually a summer/autumn feed gap, during which feed supplementation is required to grow or maintain livestock. Supplementation with grain is used when cattle or sheep are not able to meet marketable weights, or during summer in dryland grazing systems. Grain feeding is also used during drought to carry stock over and reduce weight loss when there is a shortage of pasture. Grain feeding is also used to meet customer demand for grain fed beef products irrespective of the season.

Presently there are 450 accredited commercial cattle feedlots in Australia (ALFA, 2014). About seven per cent of the WA beef industry and 17% of the national beef industry finishes cattle in feedlots, however at any one time usually only two per cent of the Australian cattle population is being fed in feedlots (2011). These figures do not include the sheep industry or the dairy industry, which relies on grain feeding to provide high producing dairy cows with enough energy and protein to maintain milk production. Hassel and Associates (2003) estimated that the cattle and dairy industries use about 35% and 55% respectively of the 1.3 million tonnes of grain fed annually to ruminants.

1.3 **Diets for livestock**

In Australia, cattle and sheep are fed under pasture grazing systems for the majority of their lives. In WA grazing production systems depend on rainfall with most rainfall falling during winter (Figure 1.1). This seasonal rainfall leads to pastures being productive during May/June to December but lacking in quality and quantity for the remainder of the

year (Figure 1.2). To account for seasonal variation in pasture supply and quality ruminants are fed conserved fodder such as hay and silage produced during spring or grain. Beef cattle are generally only grain fed to produce a high quality product or to meet protein and energy deficits in the diet. However, dairy cattle in dryland or irrigated pivot pasture systems are often supplemented with grain in-shed, usually following milking. Grain feeding is also used in WA sheep enterprises to increase fertility (using lupin) and to supply protein during feed shortages via short-term 21-day feedlots to meet market specifications.

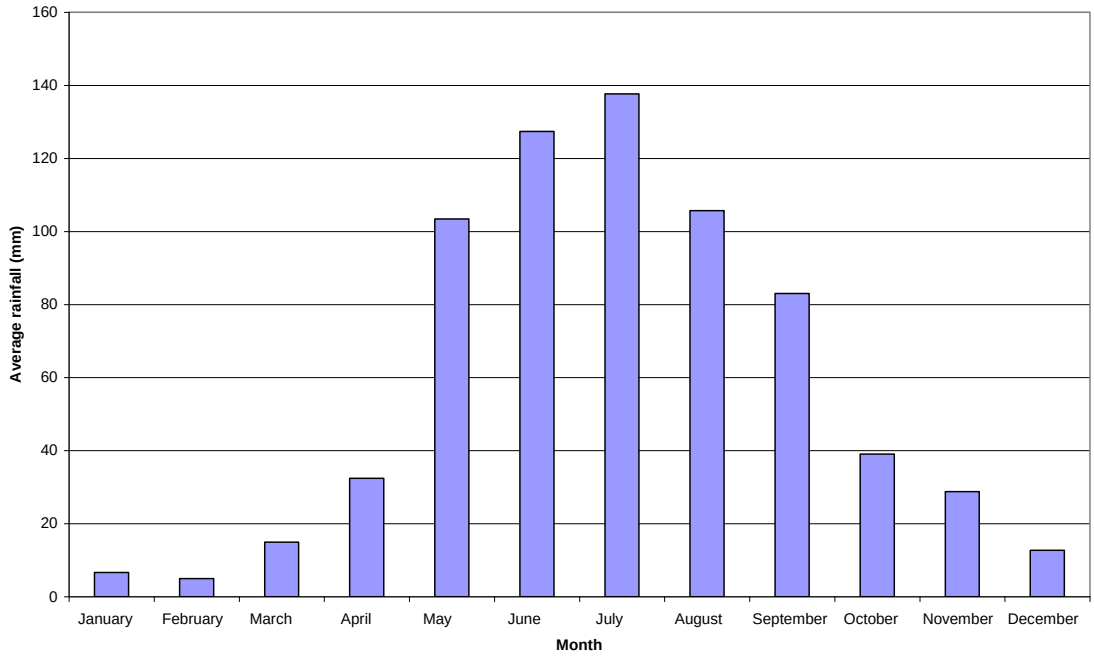


Figure 1.1 Average monthly rainfalls (1990-2010) at Vasse Research Centre, Busselton, Western Australia, which is representative of a Mediterranean environment (Source DAFWA 2011).

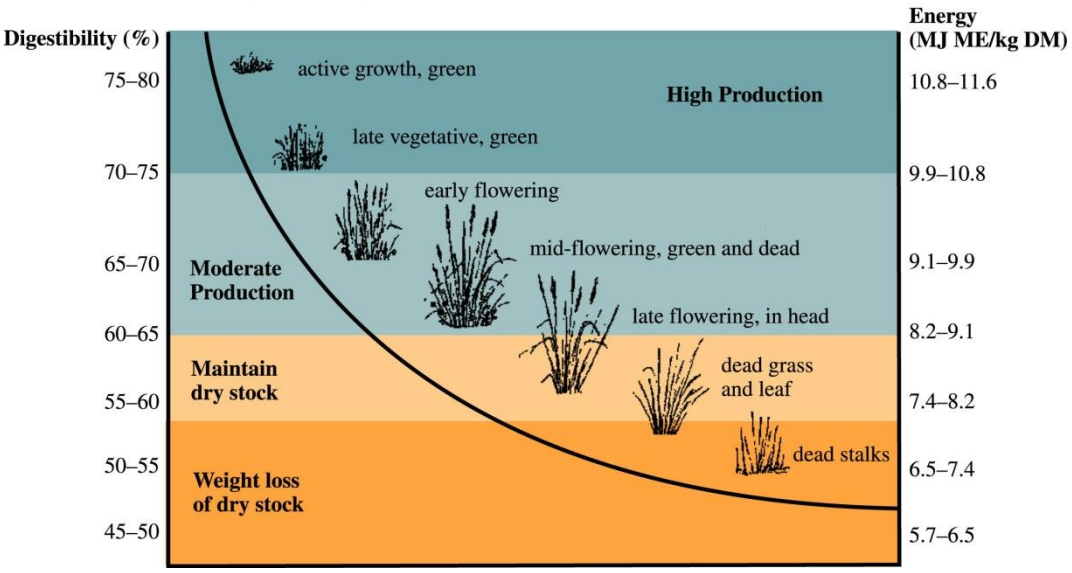


Figure 1.2 Production implications of seasonal changes in digestibility and energy of annual pastures (SGS, 2000).

Grain diets can be fed to ruminants as either a total mixed ration or pellets, or as a separate ration with hay or with dry pasture or standing crops. To get the best production benefits from grain, the characteristics must be matched with the digestive capacity and requirements of the ruminant. As one of the chief factors influencing rumen fermentation is variation in feed composition, it is important to consider diet quality, composition and protein energy balance when formulating diets.

Diets should be formulated to provide the rumen microbes with nutrients that support optimal microbial synthesis and growth for energy and protein supply and supply the host animal with its vitamin and mineral requirements. In order for diets to accomplish this nutrient supply, the pH of the rumen should remain relatively neutral to slightly acid i.e. between pH 6.0 – 7.0). Variation in rumen pH can be minimised by feed management such as addition of feed buffers such as bicarbonate or ionophores (Lean *et al.*, 2007). Management of feeding bunks can heavily impact on feeding disorders under feedlot conditions and reduce animal productivity, even leading to death. Feed bunk management practices such as multiple feedings and consistent time of feed delivery can be used to

reduce variability in intake (Schwartzkopf-Genswein *et al.*, 2003; Pritchard and Bruns, 2003) and the incidence of acidosis. Feeding grain and roughage separately appears to increase the risk of subacute acidosis compared to feeding cattle a total mixed ration (Krause and Oetzel, 2006) and this is particularly an issue when cattle are not adapted to grain feeding. Feeding prime lambs a total mixed ration during introduction to grain based diets reduced growth rates and feed efficiency compared to the same diet fed in a pelleted form (Jones *et al.*, 2000) due to the ability of the animals to select separate dietary components and ingest an imbalance of protein and energy. However, feedlot cattle fed finishing diets containing barley grain and separate roughage were able to self-regulate intake resulting in diets similar in composition, intake level and ruminal fermentation profile to those fed a total mixed ration (Moya *et al.*, 2011).

The rumen microbial population is divided into bacteria that ferment structural carbohydrates (cellulose and hemicelluloses) and use only ammonia as their nitrogen source and those that ferment non-structural carbohydrates (starch, pectin and sugars) and use either ammonia or amino acids as a nitrogen source (Russell *et al.*, 1992). Refining dietary balance is therefore important in optimising animal production (Van Soest *et al.*, 1991). Crude protein content of the diet in grain-fed cattle diets should be about 13-15% and high concentrations of non-protein nitrogen (from urea and sulphate of ammonia) should be avoided due to rapid production of ammonia and poor rumen fermentation (MLA, 2001).

1.3.1 **Importance of the rumen**

Carbohydrate polymers in plants are indigestible to most animals but can be hydrolysed and fermented by a range of organisms in the rumen (Krause *et al.*, 2003a). Rumen fermentation is unique as the efficient breakdown of the cell wall relies on the

relationship between microorganisms that produce fibrolytic enzymes and the host animal providing the anaerobic fermentation chamber (Krause *et al.*, 2003a).

The forestomach is divided into four compartments including the reticulum, rumen, omasum and abomasum (McDonald *et al.*, 2011; Hungate, 1966). The microbial activity of the rumen generates an anaerobic (mainly carbon dioxide (40%) and methane 30-40% and 5% hydrogen, oxygen and nitrogen) environment. The temperature remains fairly static are 38-42 ^{o}C due to the heat that is produced during rumen fermentation (Theodorou and France, 2005). Buffering capacity is provided by the production of copious quantities of saliva containing bicarbonate and phosphate salts, which help maintain the rumen at a pH of 5.5-7 (Pond *et al.*, 1995; Theodorou and France, 2005; Owens *et al.*, 1998; McDonald *et al.*, 2011).

The reticulo-rumen has no sphincter between its two compartments and is considered to a large extent to function as a single entity with chewed food entering the reticulo-rumen where it is subjected to microbial attack as well as mixing and propulsion of the reticulorumen musculature (Dijkstra *et al.*, 2005), which helps with rumen flow and absorption of volatile fatty acids across the rumen wall. The epithelial lining of the reticulum is raised into folds forming honeycomb structure while the rumen is lined with papillae of various sizes for absorption of nutrients (Dehority, 2003) and sort feed particles allowing to go through to the abomasum.

Digesta passes from the reticulum to the omasum via a sphincter. The omasum is filled will laminae (like leaves), tightly packed with finely divided ingesta. The role of the omasum is not well understood but it is known that water, ammonia, VFA and inorganic electrolytes are absorbed in the omasum (Dijkstra *et al.*, 2005; Dehority, 2003; Hungate, 1966). The digesta then passes to the abomasum, which is the equivalent of a monogastric stomach, where protein digestion begins via acid and enzymes excreted into the abomasal

lumen. Mixing of the digesta occurs through muscular contractions. The abomasum exhibits a circadian ultradian rhythm and as a consequence there is relatively continuous flow through of digesta. Distension of the abomasum, which inhibits reticulorumen emptying, is the main stimulus for its emptying (Dijkstra *et al.*, 2005).

The adult ruminant is adapted to digesting grasses and other roughages. Chewed grasses and roughage along with saliva are passed into the rumen. Contractions of the oesophagus pushes the food bolus into the rumen. The muscular wall of the rumen mixes the ingesta while the process of rumination allows the plant material to mix with the saliva enabling the rumen microbes to hydrolyse the plant celluloses, hemicelluloses, pectins, fructons, starches and other polysaccharides. These are broken down to monomeric and dimeric sugars some of which are subject to further microbial action(Hobson, 1997).

The food is diluted with large amounts of saliva with approximately 150 L in cattle and 10 L in sheep. Saliva is essential to the lubrication of feed and pH buffering of rumen. The contents of the rumen are continually mixed by rhythmic contractions of its walls during rumination. Plant material is drawn from the anterior end to the oesophagus and returned by a wave of contractions to the mouth. The major factor inducing the animal to ruminate is the tactile stimulation of the epithelium of the anterior rumen. As a consequence, diets containing little or no coarse roughage may fail to supply the stimulation for rumination (McDonald *et al.*, 2011). These muscular contractions mix fresh feed with microorganisms and wash the epithelium with fermentation fluids so that short chain organic acids can be absorbed (Russell and Rychlik, 2001).

Ruminants do not produce cellulose and hemicellulose fibre degrading enzymes, but they do harbour bacteria, fungi and protozoa that have the ability to breakdown these β-linked structured polysaccharides in diets (Russell and Rychlik, 2001; Krause *et al.*, 2003a). Because cellulolytic bacteria cannot grow on cellubiose at pH below 6.0, pH

sensitivity is a general aspect of growth and not a limitation of cellulases. Cellulolytic bacteria cannot grow with a low intracellular pH, and an increase in pH gradient leads to anion toxicity. (Hungate, 1966) indicated that in modern feeding regimes the rapid fermentation of substrates leads to unstable microflora, acidosis and even death. Acid-resistant ruminal bacteria have evolved with the capacity to let their intracellular pH decrease, maintaining a small pH gradient across the cell membrane, and preventing an intracellular accumulation of VFA anions (Russell and Wilson, 1996).

1.3.2 **Hind gut fermentation**

Most of the work on the ruminant digestive tract has focussed on the rumen, rather than the small and large intestine (Hofmann, 1989). Generally most carbohydrate digestion occurs in the rumen (65-90%) with nitrogen components flowing into the intestines (Pond *et al.*, 1995). Microbial populations in the lower digestive tract including the hindgut and large intestine (caecum and colon) ferment food components that are resistant to endogenous hydrolytic digestion, mixed with considerable amounts of protein substances, producing similar proportions of VFAs as the rumen (Demeyer, 1991).

When starch is not hydrolysed in the small intestine it passes to the large intestine and the colon, which in turn can reduce colonic pH and increase VFA concentrations. Cattle fed hay had a colonic VFA concentration of 25mM, while those consuming a grain ration had a colonic VFA concentration of 80mM (Russell, 1999). Management of grain-based ruminant diets is therefore important. (Reynolds, 2006) studied starch digestion in dairy cows and found considerable capacity for starch digestion in the small intestine at the expense of microbial protein synthesis in the rumen. Some diets are formulated with the aim of increasing the amount of protein not taken up by the microbes so that the protein (called ‘bypass protein’) is instead digested in the hindgut.

1.3.3 **Grains in the diet**

The metabolisable energy, protein and starch content of grain diets affect ruminant productivity via the impact of these dietary components on rumen fermentation (Table 1.1). Starch content varies between cereal grains and influences how fast the grain breaks down in the rumen. Wheat, sorghum and barley have the highest starch level and lowest acid detergent fibre (ADF) of grains commonly fed to ruminants, while lupins are frequently used as a low-starch alternative for ruminant feeding in WA (Table 1.1). Grains are often processed to optimise starch and fibre utilisation. Short particle lengths result in a highly fermentable diet in the rumen. The lack of structural carbohydrates leads to a reduced ruminal pH and increased risk of acidosis (Beauchemin, 2007).

Table 1.1 Nutrient composition and structure of various grains. Adapted from (Sipsas and Seymour, 2008; Beretta and Kirby, 2004; Margan, 1994; Freer and Dove, 1984; Petterson *et al.*, 1997; Rowe *et al.*, 1999).

Chemical composition	**Units**	**Wheat**	**Barley**	**Oats**	**Maize**	**Sorghum**	**Lupin**
Metabolisable energy (ME)	**MJ/kg DM**	13.0	11.6	10.5	13.5	12.4	12.2
Crude protein (CP)	**% CP**	13.0	12.0	11.0	10.0	10.0	32.2
Rumen undegradable protein (RDP)	**% RDP**	18	25	30	55	55	71
Acid detergent fibre (ADF)	**%DM**	2.6	5.3	14.0	2.4	2.8	19.7
Starch	**%DM**	70.3	64.3	58.1	75.7	71.3	1
Grain structure							
Hulls	**%DM**		13.0	25.0			24.0
Testa + pericarp+aleurone	**%DM**	15.0	7.7	9.0	6.0	7.9	

Starch Endosperm	**%DM**	82.4	76.2	63.0	82.0	82.3	
Embryo	**%DM**	2.6	3.0	3.0	12.0	9.8	

1.3.4 **Carbohydrates**

Dietary carbohydrates provide over half the energy requirements for maintenance, growth and production in the form of fibrous feeds containing cellulose, hemi-cellulose and grains rich in starch (Nafikov and Beitz, 2007; Annison *et al.*, 2002). Carbohydrates are the most important source of energy for rumen microbes, with rumen microorganisms fermenting 80-95% in the rumen and the remainder in the small intestine (Nafikov and Beitz, 2007). Soluble carbohydrates are the most common carbohydrate found in forages with starch in the cell contents and insoluble structural carbohydrates in cell wall components constituting 30% of the dry matter in forage.

Starch as both amylose and amylopectin is an important component of many ruminant diets, especially those containing cereal grains. However, too much of these readily fermentable carbohydrates can lower the digestibility of fibre. Starch is digested rapidly in the rumen but more slowly than soluble sugars (Mackie *et al.*, 2002). Some cellulytic bacteria, such as certain strains of *Fibrobacter succinogenes* are also amylolytic (can degrade starch to disaccharide sugars). However, the principal amylase-producing bacteria, including *Selenomonas ruminantium* and *Streptococcus bovis* are the major starch fermenters with a limited ability to use other polysaccharides. These organisms together with soluble sugar utilisers, such as *Megasphaera elsdenii*, occupy a distinct ecological niche in the rumen (Theodorou and France, 2005).

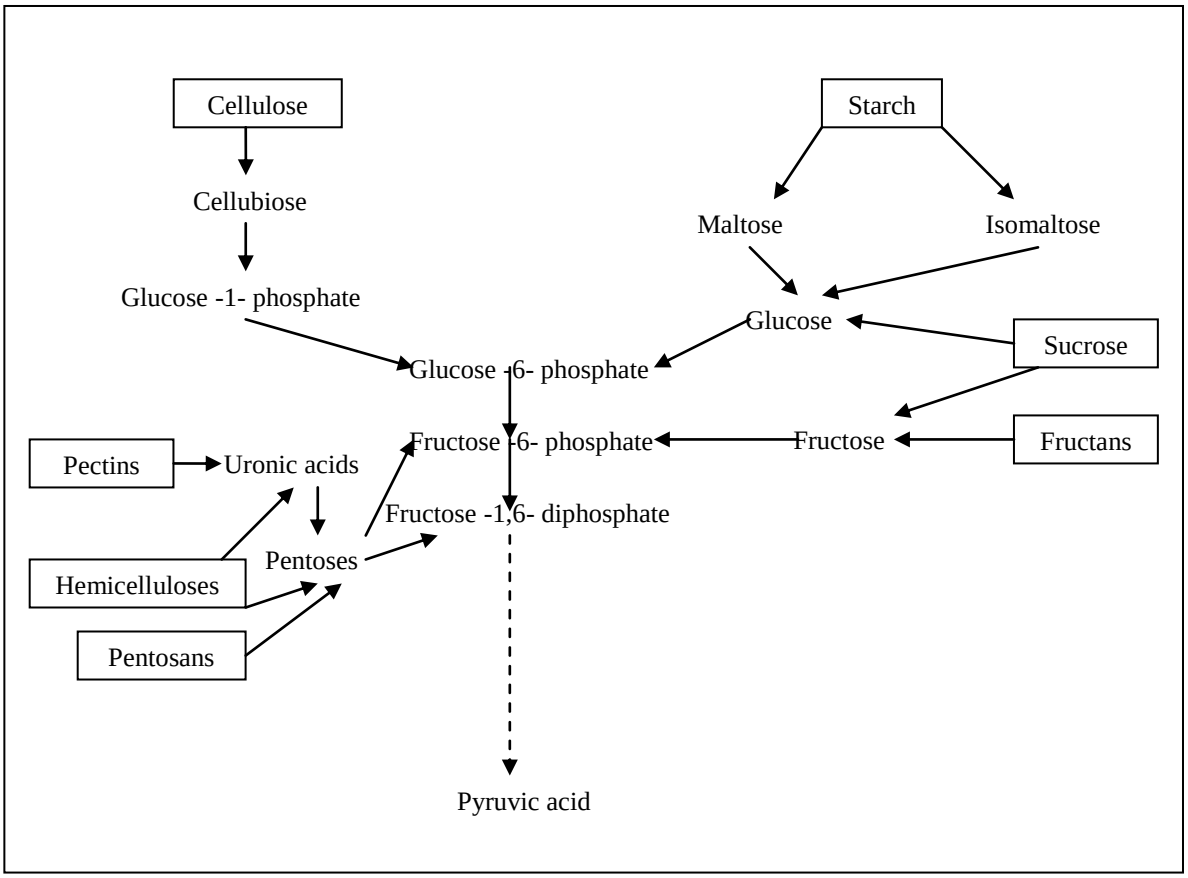


Figure 1.3 Conversion of carbohydrates to pyruvate in the rumen (McDonald *et al.*, 2011).

Hexoses are the main simple sugars produced in the first stage of fermentation of polysaccharides and are taken up and metabolised by microorganisms via the Embden-Meyerhof glycolytic pathway to produce pyruvate (Figure 1.3). The main end products of complete carbohydrate metabolism are short chain fatty acids (acetic, propionic and butyric acids), carbon dioxide and methane (McDonald *et al.*, 2011; Dehority, 2003; Annison *et al.*, 2002) (Fig 1.4).

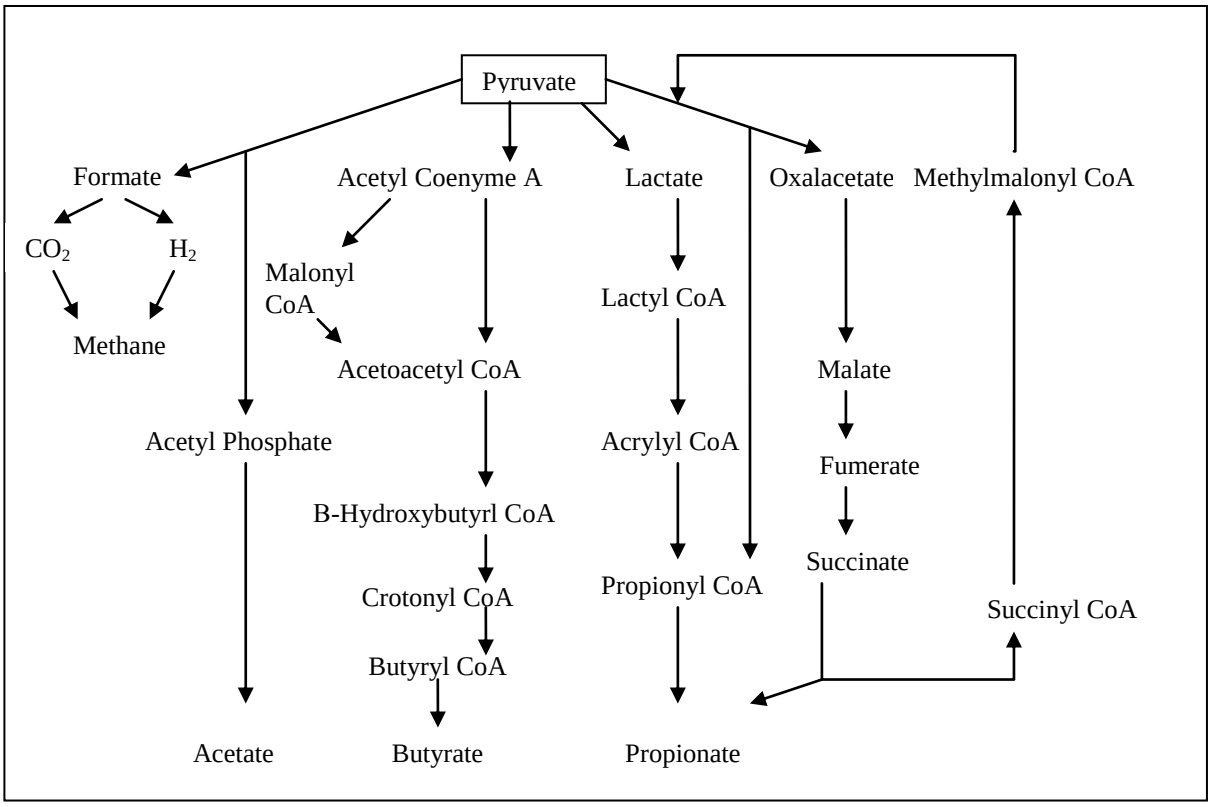


Figure 1.4 Conversion of pyruvate to volatile fatty acids in the rumen (McDonald *et al.*, 2011).

Ruminants use the volatile fatty acids including acetate, butyrate and propionate as their main energy sources. Acetate is transported across the rumen wall unchanged and passes through the hepatic system where it is mostly utilised by peripheral tissues such as muscle, heart and adipose tissue. Propionic acid crosses the rumen wall and is extracted by the liver and converted to glucose via the gluconeogenic pathway and then passed into the hepatic vein to maintain glucose homeostasis. Butyric acid is hydroxylated to D-3-hydroxybutyric acid so that very little butyrate appears in the peripheral circulation. D-3-Hydroxybutyric acid is preferentially utilised by muscle and heart for energy (McDonald *et al.*, 2011).

Ruminants have an obligatory requirement for glucose for particular body functions and depend on the process of gluconeogenesis from propionate to meet their glucose

requirements (Elliott, 1980). Gluconeogenesis is the metabolic pathway by which glucose is synthesised from substrates such as propionate, lactic acid and amino acids (Stryer, 1995). This glucose then enters normal process of carbohydrate metabolism in the body. If the proportion of acetic acid produced in the rumen is high relative to propionate an apparent glucose deficiency can arise. In this situation body tissues are mobilised to meet the energy deficit and as a consequence of the oxidation of fat from adipose tissue, the concentrations of ketone bodies (β- hydroxy butyrate) in the blood will rise as will nitrogen excretion from the breakdown of muscle tissues (Orskov *et al.*, 1991).

1.3.5 **Lipid digestion and metabolism**

Lipids are organic compounds serving an important role in biochemical and physiological functions in plant and animal tissues. They are relatively insoluble in water but soluble in organic solvents (Pond *et al.*, 1995). Plants which are used as a ruminant feed source contain complex mixtures of lipids (phospho- and glycoglycerides, waxes and cutin) at levels of 30-40g/kg DM. Plants are generally low in lipid content but rich in polyunsaturated fatty acids, especially linoleic acid. Concentrate diets vary in their lipid content from 20g/kg DM in wheat to 70g/kg in oats in the form of triglycerides. They are rapidly hydrolysed by bacterial lipases, liberating long chain fatty acids (LCFA) (Annison *et al.*, 2002). If the lipid content of diets is >100g/kg DM (McDonald *et al.*, 2011) or at concentrations above 5-6% of the diet, lipids have an inhibitory effect on forage digestibility (Annison *et al.*, 2002) and ruminant microbial activity is reduced and feed intake falls.

In ruminants, dietary fats are extensively hydrogenated in the rumen before intestinal absorption so that absorbed long chain fatty acids are much more saturated than dietary fatty acids (Doreau and Chillard, 1997) and generally unesterfied (McDonald *et al.*,

2011). The fatty acid composition of ruminant meat and milk is less a reflection of the fatty acid composition of the diet and more of ruminal biohydogenation (Nafikov and Beitz, 2007; Annison *et al.*, 2002).

1.3.6 **Protein digestion and nitrogen metabolism**

All cells require protein for part or all of their life cycle and proteins are highest in concentration in muscle tissues of animals, (Pond *et al.*, 1995). Animals require amino acids for growth, reproduction, lactation and maintenance. Feed protein is partitioned into three fractions; non-protein nitrogen, true protein and unavailable protein (Sniffen *et al.*, 1992).

Protein requirements for feedlot cattle are divided into the ammonia needed for ruminal digestion and the amino acids needed post ruminally (Owens and Gill, 1980). Protein contains about 16% nitrogen and is expressed as crude protein (CP) (where CP = total nitrogen x 6.25) (Freer *et al.*, 2007; Wilson, 1981). Rumen microbes degrade a substantial fraction of the total nitrogenous material in feed, which is referred to as rumen degraded protein (RDP) and is made up of peptides, amino acids and ammonia (Freer *et al.*, 2007) (Figure 1.5). A small amount of protein is referred to as undegradable protein (UDP) because it escapes ruminal breakdown and flows to the abomasum and small intestine where 85% of UDP is made available to the animal (Coleman and Henry, 2002); UDP = CP - RDP. The UDP fraction is termed ‘escape protein’ or ‘protected protein’. The rumen microbes synthesis proteins and other nitrogenous material (microbial protein) for their own needs (Nolan and Dobos, 2005).

Ruminant requirements for essential amino acids are met from microbes grown in the rumen and digested in the small intestine called bypass protein, as well as dietary protein that is intestinally degraded (Leng and Nolan, 1984). While bacteria can use ammonia, it is often produced in excess of bacterial utilisation, excessive ammonia is

produced nitrogen excretion increases, which increases the energy cost of urea synthesis (Russell *et al.*, 1992) (Figure 1.5).

Ruminants are not generally efficient at capturing nitrogen and therefore excess ruminal ammonia is a primary end product of ruminant nitrogen metabolism because the imbalance of protein and carbohydrate in many cases cannot be counteracted (Krause and Russell, 1996). This imbalance needs to be considered when formulating diets for ruminants as nitrogenous compounds can be the most wasteful dietary constituents, it is a challenge to manipulate the diet to improve nitrogen utilisation and reduce excretion.

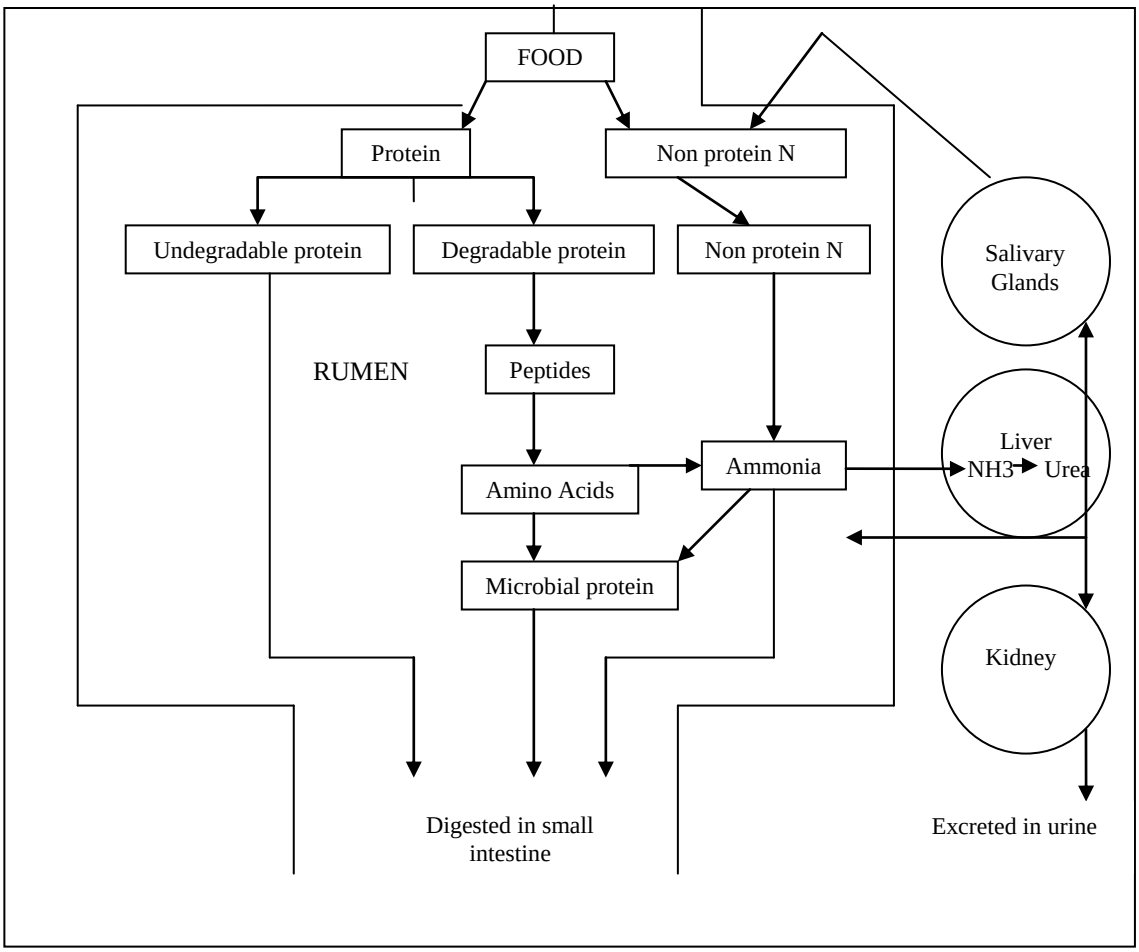


Figure 1.5 Digestion and metabolism of nitrogenous compounds in the rumen (McDonald *et al.*, 2011)

Providing nitrogen to the rumen in appropriate dietary forms and amounts can improve its efficiency of use. It is crucial to consider animal requirements for protein and

metabolisable energy in combination rather than in isolation (Nolan and Dobos, 2005; Russell *et al.*, 1992). Chikunya *et al.* (1996) noted that rumen degradable protein is influenced by carbohydrate supply. Modification of ingested feed proteins by rumen microorganisms has major implications for the supply of amino acids to the intestines and tissues.

Smaller amounts of C4 and C5 branch-chain volatile fatty acids are derived mainly from the fermentation of branch-chain amino acids in dietary protein. Microbial cells are a major source of amino acids but are also a significant source of metabolisable energy for the host animal (Freer *et al.*, 2007).

1.4 **Phenotypic indicators of rumen adaptation**

Phenotypic indicators of rumen adaptation are chemical and biochemical indicators that determine if the rumen is functioning effectively. The indicators have been developed over years of research and are commonly used in rumen studies to determine if rumen fermentation is having a positive or negative effect on the ruminant and if feed is being utilised effectively. The main phenotypic indicators are outlined below.

1.4.1 **Lactic acid**

Lactic acid in ruminants is associated with the metabolism of pyruvate both in the rumen and endogenously (Mackenzie, 1967). Lactic acid has a pKa of 3.86 and is the simplest of the hydroxycarboxylic acids and can exist as two isomers, L- (+) lactate and D - (-) lactate (Ewaschuk *et al.*, 2005). The L- (+) form is identical to that produced in muscle from glycogen (product of anaerobic glycolosis) during exercise and is readily metabolised by the liver and heart. The D (-) form is typically 30-38% of the lactate found in the rumen and is not produced by mammalian tissue (Owens *et al.*, 1998). Lactate is produced in significant amounts when diets are rich in starch and sugars with lactate concentration

increasing to up to 80 mM in the rumen and a decrease in rumen pH from near neutral to 5.5-5.0 (Moller *et al.*, 1997) during periods of acidosis.

Bacteria in the rumen are classified phenotypically as either lactate utilisers e.g. *Megaspharea eldensii* and *Selenomonas ruminantium* which are usually sensitive to low pH, or lactate producers e.g. *Streptococcus bovis* and *Lactobacillus spp.* which are not sensitive to low pH. The relative normal proportions of these two bacterial phenotypes determine if lactate accumulates in the rumen. Under normal rumen conditions lactate concentrations generally do not surpass 5µm, but can exceed 40 mM during severe acidosis (Owens *et al.*, 1998).

In a study undertaken by Hristov *et al.* (2001) where the ruminal L-lactate concentration was not affected by the increased grain content of the diet or by reduced protozoa numbers in the rumen, lactate concentrations remain below 1mmol/L throughout the study.

Lactate concentration could be considered as an indicator of lactic acidosis and rumen function in livestock. However, it is important to note that the process of rumen sampling and saliva contamination can impact lactate concentrations significantly.

1.4.2 Growth rates

Growth rate of livestock is a general indicator of how well the rumen is functioning and if animals are adapting to the feed source supplied. Performance studies reviewed by Brown *et al.* (2006) indicated that animals introduced to a feedlot diet *ad libitum* for 55-90% grain in 14 days or less generally show reduced performance during both the adaptation period and over the entire feeding period.

1.4.3 Rumen pH (power of hydrogen)

Rumen pH is critical to a normal stable rumen environment as it impacts dramatically on rumen physiology, microbial population ecology and the nature and concentration of fermentation products. The rumen pH represents the negative $\log_{10}$ of hydrogen ion concentration in water based solutions. The normal pH range in the rumen is between pH 5.5 – 7.0, while the outer limits are pH 4.5-5.5 and 7.0 to 7.5 (Dehority, 2003). In beef cattle consuming high grain diets, the ruminal pH can range from 5.6 to 6.5 with the average typically around 5.8-6.2. However it can drop below 5.6 for a period during the feeding cycle (Nagaraja and Nagamine, 2007).

The primary ruminal base is ammonia with the major buffers being bicarbonate and phosphate. Removal of lactic acid and volatile fatty acids when absorbed across the rumen wall at optimum concentrations can help stabilise the pH around neutral (Owens *et al.*, 1998). When rumen balance is not maintained such as during increases in lactic acid and VFA production, there can be a downward spiral of rumen pH. Lactic acid is a 10 times stronger acid than the volatile fatty acids (pKa 3.9 vs. 4.9) (Nagaraja and Nagamine, 2007).

Ruminal pH is very responsive to feeding and chewing behaviour, with rumen pH generally decreasing following feeding and increasing during rumination (Allen, 1997). Rumen pH can vary considerably over a day and ruminants have a highly developed system of salivary input to maintain ruminal pH within a physiological range, however if the acid production is more than the system can buffer, rumen pH can decrease drastically (Krause and Oetzel, 2006). Therefore it is important when measuring pH to not just consider the mean pH but also the fluctuations that occur, particularly when at suboptimal concentrations of <5.6 (Nagaraja and Nagamine, 2007).

When ruminants are fed diets lacking in fibre, the physiological mechanisms of homeostasis are disrupted. Salivation in particular decreases, which leads to a decline in

ruminal pH, alteration of microbial ecology and animals becoming more susceptible to metabolic disorders (Russell and Rychlik, 2001). Decreases in ruminal pH decreases dry matter intake, fibre digestibility and microbial yield, which reduces production and increases feed costs (Allen, 1997). Rumen pH in dairy cows fed 65% grain for three weeks decreased to below pH 5.6 for about 4.6 hours, indicating rumen dysfunction and subacute ruminal acidosis (Hook *et al.*, 2011).

Although rumen pH can be used to reflect functional change in the rumen, rumen pH is difficult to measure with any consistency. Bramley *et al.* (2008) showed that rumencentesis and use of cannulated cattle was the best means for assessing rumen pH. However, these methods do not enable sample collection continuously over long periods of time under commercial feedlot conditions.

1.4.4 Volatile fatty acid

Volatile fatty acids (VFAs) principally acetate, propionate and butyrate but also to lesser extent valerate, caproate, iso-butyrate, iso-valerate, 2-methylbutyrate and traces of various other acids are produced in the rumen as end products of rumen fermentation. Acetate and butyrate are used efficiently for fattening animals but do not make a net contribution to the glucose supply, while propionate can be used for gluconeogenesis but can reduce milk fat when present in higher proportions than acetate (Russell *et al.*, 1992).

During the fermentation process energy is conserved in the form of adenosine triphosphate (ATP) and subsequently utilised for the maintenance and growth of the microbial population. Only a small proportion of the potential energy in glucose is captured by the microorganisms (2 moles of ATP per 1 mole of glucose when converted to 2 moles of pyruvate). VFAs are not utilised by the microbes but are a major source of absorbed energy for the host animal (France and Dijkstra, 2005).

Early work showed that a variety of ruminal bacteria produced end products that could not be detected in ruminal fluid. These intermediates include succinate and lactate, which are subjected to secondary fermentation by bacterial species such as *Selenomonas ruminantium* and *Megasphaera elsdenii* (Russell and Rychlik, 2001).

The ratio of VFAs produced is strongly influenced by the diet, with roughage diets producing lower concentrations of propionate to acetic acids and grain-based diets producing a higher ratio of propionate to acetate (Czerkawaski, 1986). Cattle fed on a Timothy hay or high fibre diet had higher concentrations of volatile fatty acids and different ratios of volatile fatty acids than cattle fed a 90% concentrate diet (Lana *et al.*, 1998), there was a large increase in the molar proportion of butyric acid.(Table 1.2).

Table 1.2 The rumen fluid characteristics of steers fed Timothy hay (forage) or a 90% concentrate diets. Adapted from (Lana *et al.*, 1998).

Measurement	Forage Diet	90% Concentrate Diet
Acetate, mM	59.1	55.6
Propionate, mM	12.8	29.8
Butyrate, mM	6.0	40.1
Total VFA, mM	77.9	125.5
Acetate:Propionate Ratio	4.6	1.9
Rumen pH	6.5	5.7

Manipulation of rumen fermentation is commonly used to improved production. Orskov *et al.* (1991) indicated that changes in VFA proportions produced in the rumen only benefitted the energy economy of the animal when they changed the fermentation energy.

1.4.5 Rumen contractions and outflow rate digesta

Rumen solid turnover time in cattle is 1.3-3.7 days and 0.8- 2.2 days in sheep (Dehority, 2003). Factors that influence outflow rate include the intake of concentrates, feed particle size and concentration of solids (Hungate, 1966; Pond *et al.*, 1995).

The rumen has a well-developed pattern of contractions of the various compartments of the reticulo-rumen that circulate the digesta through the different sections of the reticulum, rumen, omasum and abomasum. The contractions are imperative for rumination (Pond *et al.*, 1995), which occurs for up to eight hours a day. Mixing of the rumen reticulum contents aids in inoculating fresh ingesta with a mass of micro-organisms in the fermenting digesta and incorporates the saliva through the rumen contents. This works to enhance absorption by replenishing the fermentation acids absorbed by the rumen epithelium. It also counteracts the flotation of solids during fermentation and assists in movement of digesta to other organs in the digestive tract. When large particles are ruminated, surface area and fermentation rate are both increased. Rumination triggers saliva flow, which maintains a favourable rumen pH for the microbes and the animal (Russell and Rychlik, 2001). The mixing is accomplished by contractions of the wall of the rumen and reticulum, and contractions are coordinated with movement of the other digestive organs (Hungate, 1966). These muscular contractions mix fresh feed with microorganisms and wash the epithelium with fermentation fluids so the microbial short chain organic acids can be absorbed (Russell and Rychlik, 2001).

If ruminants are fed fibre deficient diets, the mixing motions, eructation, rumination and saliva flow decrease and fermentation acids accumulate and rumen pH declines (Russell and Rychlik, 2001). The importance of fibre digestion is supported by the practical observation that cattle usually are fed at least 10 to 15% forage to ensure normal rumen function (Russell and Wilson, 1996).

1.4.6 **Ammonia and nitrogen outflow**

Ammonia in the rumen fluid is the final end product of proteolysis by the mixed rumen populations and is a major source of nitrogen for protein synthesis and the major source of nitrogen by many bacterial species. (Nolan and Dobos, 2005). Rumen ammonia concentrations are usually explained by an increase in microbial protein synthesis and enhanced ammonia assimilation, however work done by Lana *et al.* (1998) indicated that ammonia concentrations were correlated with the deamination rate of amino acids by rumen bacteria. The ability of bacteria from forage fed cows to deaminate amino acids is influenced more by changes in pH than those fed 90% concentrates, indicating there was a difference in the populations of ammonia-producing bacteria.

1.4.7 Ruminal Acidosis

Extracellular and blood pH are maintained by the body's buffering systems of which the bicarbonate system is quantitatively the most important. The addition of significantly large amounts of acid (or alkali) to the blood is necessary for the body's buffering capacity to be exceeded and pH changed. Changes in the normal acid-base balance towards either acidosis or alkalosis can cause ill health. The common cause of acidosis is the excessive, uncompensated loss of bicarbonate ions due to production and adsorption of large quantities of fixed acid such as lactic acid produced from acute carbohydrate engorgement in ruminants (Blood *et al.*, 1983).

When rumen fermentation rate is too high, lactic acid can accumulate in the rumen and blood. Lactate absorbed into the blood can be converted to blood glucose via hepatic gluconeogenesis. Acute and chronic acidosis are significant ruminant production problems that can result from excess ingestion of readily fermented carbohydrates. When production of acids exceeds their rate of removal, rumen pH can decrease to < 6.0, a rumen environment favouring the growth of *S. bovis* or *Lactobacillus spp*. populations as outlined

in Al Jassim and Rowe (1999). This occurs when ruminants are not adapted to readily fermented carbohydrates or to forage that is low in efficient fibre. While high grain diets predispose ruminants to acidosis, some grains pose a greater threat than others. Wheat is generally considered the worst grain as far as development of acidosis, while barley has been observed as the least predisposing cereal grain (Elam, 1976; Bird *et al.*, 1999).

The cascade of physiological effects of acidosis, originating from the initial ingestion of carbohydrate depends upon the intensity and duration of the insult. Most critical is the pH threshold, which not only influences microbial growth rates and shifts in the ruminal populations but also significantly impacts the systematic metabolic state and the ability to catabolise certain metabolites (Nocek, 1997). In theory animals that have been adapted to a grain based diet should show greater resistance to acidosis. However the results from a trial done by (Goad *et al.*, 1998) indicated that there were similar changes in the ruminal fermentation patterns during subacute acidosis regardless of whether the steers were adapted to a grain or hay diet prior to induced acidosis. This suggests that the incidence of acidosis depends on the diet just as much as the previous dietary exposure of the ruminant.

Ruminal pH of 5.6 or below is considered the benchmark for ruminal acidosis; a pH of 5.0 to 5.6 is regarded as subacute or chronic acidosis and a pH below 5.0 or approaching 4.5 is considered acute acidosis (Owens *et al.*, 1998; Kleen *et al.*, 2003; Krause and Oetzel, 2006; Hristov *et al.*, 2001; Nagaraja and Nagamine, 2007). Russell (1999) showed that as ruminal pH decreases there is an increase in VFA concentrations, this decrease in ruminal pH can be exacerbated by reduced ruminal contractions during grain feeding, leading to a reduction in ruminal flow-through and fibre digestion by the microbial population.

Work done by Kleive *et al.* (2003) found that cattle suffering from acute acidosis had a 100-fold increase in *S. bovis* within 24 days of the problem arising. This is supported by (Petri *et al.*, 2013b) who found that under induced acidosis the *S. bovis and Lactobacillus spp.* populations increased when analysed using parallel pyrosequencing technology. However, work by Kleive *et al.* (2003) found no increase in the *S. bovis* population with an increase in the starch content of the diet with the authors concluding that *S. bovis* was possibly not the major starch-utilising bacterium under the imposed dietary conditions. Golder *et al.* (2014) also demonstrated that the *S. bovis* and *Lactobacillus spp.* populations did not increase in dairy cattle that were exhibiting signs of acidosis. Diet changes carried out too rapidly or without proper transition will put animals at risk (Kleen *et al.*, 2003).

Cattle fed forage diets had higher concentrations of *S. bovis* than *Lactobacillus spp.* However, when transferred to a grain diet ruminal pH of the animals declined and *S. bovis* populations reduced while *Lactobacillus spp.* increased (Wells *et al.*, 1997). In this study *Lactobacillus fermentum* appeared to inhibit the growth of *S. bovis* in the rumen (Table 1.3).

Table 1.3 The impact of diet and ruminal pH on most probable numbers (MPN) of *S. bovis* and *Lactobacillus spp.* when grown on MRS medium. Based on duplicate samples from two animals (n=4) (Wells *et al.*, 1997; Russell, 1999).

Diet	**Ruminal pH**	***S. bovis*** **(cells/mL ruminal fluid)**	***Lactobacillus spp.***
100% forage	6.8-6.7	3×10^7	4×10^3
80% cereal and 20% forage	6.0-5.6	2×10^3	5×10^7

Other studies indicate that as pH continues to fall *S. bovis* can no longer grow while *Lactobacillus spp.* increase leading to increasing starch fermentation, the production of

more lactic acid and pH levels as low 5.5 (Al Jassim and Rowe, 1999; Owens *et al.*, 1998; Garrett *et al.*, 1999). Declining ruminal pH also decreases the efficiency with which substrates are converted to VFA. Ruminal pH values of 5.5 to 5.0 with increased VFA concentrations, but normal lactate concentrations (<5m*M*) were indicative of subacute acidosis (Goad *et al.*, 1998; Garrett *et al.*, 1999).

Clinical manifestations of lactic acidosis range from complete anorexia, loss of appetite, diarrhoea, lethargy, staggering, recumberancy and even death. Lactic acid may not consistently accumulate in the rumen fluid, but has been found in transient spikes of up to 20 mM when measured frequently during the day (Kennelly *et al.*, 1999).

Acute acidosis presents significant signs and symptoms, which if caught in time can be treated directly while symptoms of subclinical acidosis are insidious and considerably less overt. Subclinical acidosis is often dismissed as other problems, such as poor forage quality or bunk management and can cause significant economic loss, draining major productive efficiency from dairy herds. The major clinical manifestation of subclinical acidosis is reduced or inconsistent feed intake (Nocek, 1997; Krause and Oetzel, 2006). In many cattle operations the challenge is not the acute acidosis but rather subacute acidosis whereby very little accumulation of lactic acid is detected in the rumen however pH decreases (Nocek, 1997).

Schwartzkopf-Genswein *et al.* (2003) found that subclinical acidosis reduced performance and caused erratic feeding behaviour and intake by cattle resulting in a $15-$20 per animal efficiency loss. Smith (1998) found that although death is the primary concern with ruminal acidosis, illness can cause higher costs due to the extra labour and medication required and the resultant low animal performance.

Rumen acidosis may also have human health impacts because low ruminal and intestinal pH increases the risk of enterphemorrhagic O157:H7 *E.coli* shedding (Russell

and Rychlik, 2001; Steele *et al.*, 2011). This can be combated by feeding cattle a high-forage diet just before slaughter; however such a practice can also cause dark cutting meat.

1.4.8 **Control methods for acidosis**

Control of lactic acidosis has been well researched under induced acidosis conditions using various control methods ranging from grain type to antibiotic use to inhibit lactic acid-producing bacteria (Al Jassim *et al.*, 2003; Al Jassim and Rowe, 1999; Bramley, 2004; Bramley *et al.*, 2008; Coe *et al.*, 1999; Commun *et al.*, 2009; Doust, 1998; Elam, 1976; Gill *et al.*, 2000; Godfrey *et al.*, 1995; Godfrey *et al.*, 1994; Grubb and Dehority, 1975; Holroyd *et al.*, 1996; Horn *et al.*, 1979; Huntington, 1997; Huntington and Britton, 1978; Keunen *et al.*, 2002; Kleen *et al.*, 2003; Knee, 2006; Krause and Oetzel, 2006; Lean *et al.*, 2007; Moya *et al.*, 2011; Nagaraja and Nagamine, 2007; Owens *et al.*, 1997; Rowe, 1988; Rowe, 1999; Rowe *et al.*, 1999; Schwartzkopf-Genswein *et al.*, 2003; Smith, 1998; Walker, 2006; Zorrilla-Rios *et al.*, 1992). Interestingly, the majority of the experiments referenced above were performed under induced acidosis conditions, which Nagaraja and Nagamine (2007) suggested might not reflect feeding conditions indicative of farm-based feeding systems.

Control of acidosis can be targeted at several points in the feeding process, including: the starch level of the grain based feed; grain feeding amount and frequency and; use of feed additives such as ionophores, probiotics or buffers such as bicarbonate and bentonite to counteract low ruminal pH. Options used to control acidosis in cattle are outlined below (Owens *et al.*, 1998; Rowe *et al.*, 2002).

1.4.9 Introduction and feeding management

Grain choice plays an instrumental role in acidosis because rumen fermentation of grain and digestion of starch are influenced by grain characteristics (Rowe *et al.*, 2002; Bird *et*

al., 1999). Grain choice is often influenced by price and availability, with barley and wheat being classed as high-risk grains due to their high starch fermentability (Table 1.1). The speed with which the chosen grain is introduced to the animals can also impact the potential development of acidosis. To avoid acidosis the ruminants must receive enough roughage before consuming the grain diet, which should be introduced in a step wise fashion over time (Rowe *et al.*, 2002; Lean *et al.*, 2007).While it is common practice to include sodium bicarbonate or bentonite into high grain diets to reduce acidosis, the use of such buffering agents is not thought to play a significant role in reducing acidosis because the acidosis development is too advanced by the time of their addition (Rowe *et al.*, 2002).

1.4.10 **Use of feed additives in grain feeding systems**

Feed additives are used to reduce the severity of grain-associated disorders such as acidosis and in some instances have been shown to improve animal productivity and growth rates (Russell and Rychlik, 2001). Common additives include antibiotics such as monensin, lasalocid, virginiamycin and tylosin.

Carboxylic polyether ionophores are produced by strains of *Streptomyces* and have been used extensively as feed additives. They are highly lipophilic and toxic to many bacteria, protozoa, fungi and higher organisms. Russell and Strobel (1989) found that carboxylic polyether ionophores improved production efficiency when fed to growing ruminants. The improved production efficiency Bergen and Bates (1984) has been attributed to::

- Increased efficiency of energy metabolism in the rumen;
- Improved nitrogen metabolism in the rumen and or animal; and
- Retardation of feedlot disorders, especially lactic acidosis and bloat.

The ionophores lasalocid and monensin inhibit major lactic acid-producing bacteria such as *Lactobacillus spp*. and *Streptococcus bovis*. while lactic acid producers that

produced an end product of succinate such as *Selenomonas ruminantium* were not affected (Dennis and Nagaraja, 1981; Schelling, 1984).

Ionophores modify the movement of ions across the membrane of rumen microbes and have greatest impact against gram positive bacteria (Bergen and Bates, 1984). The ionophores monensin and lasalocid have been shown to decrease lactic acid *in vitro* (Dennis and Nagaraja, 1981) with cattle fed monensin displaying lower lactate concentrations and higher rumen pH than cattle on the control diets (Nagaraja *et al.*, 1982).

Antibiotic use has been increasingly restricted in livestock grain feeding regimes (JETACAR, 1999). Virginiamycin is considered the most effective antibiotic for use within grain feeding systems. Work by Godfrey *et al.* (1993) showed including virginiamycin in cattle diets resulted in large liveweight gains, higher chaff intake and a reduction in diarrhoea. Additional work by Godfrey *et al.* (1995) indicated that virginiamycin was highly effective at reducing lactate concentration and acidity during *in vitro* fermentation of rumen fluid during an acute grain challenge *in vivo.* Coe *et al.* (1999) showed that virginiamycin controlled the growth of lactic acid-producing bacteria and moderated ruminal fermentation in high starch diets likely to lead to rapid production of lactic acid. Virginiamycin can no longer be used in long-term feeding regimes and there is a need to evaluate its short-term strategic use (Rowe *et al.*, 2002).

The impact of feed additives on restricting lactic acid-producing bacteria and maintaining rumen pH and rumen lactic acid concentrations will be key to understanding the long-term influence of these feed additives on the rumen microbial ecosystem and how they respond under long-term feeding regimes.

1.5 Other grain feeding disorders

As grain feeding increases within production systems grain disorders will continue to be a problem at a clinical and subclinical level.

Acidosis has implications for dry matter intake, rumenitis, liver abscesses, pulmonary bacterial emboli and laminitis (a diffuse aseptic inflammation of the laminae) (Brent, 1976; Garrett *et al.*, 1999; Owens *et al.*, 1998). The critical link between acidosis and laminitis appears to be the association with a persistent hypo perfusion. Management of acidosis is critical in preventing laminitis (Nocek, 1997).

1.6 Microbial **Ecology**

Ruminants and their rumen microbial population exist in a reciprocally beneficial relationship. In exchange, rumen microorganisms utilise the dietary complex carbohydrates and nitrogen for their own energy requirements via anaerobic glycolysis and anabolic processes. The normal rumen flora and fauna are established quite early in life (McDonald *et al.*, 2011) via contact with an adult animal, usually the mother (Hobson and Stewart, 1997).

The rumen microbial community represents all major groups of microbes, obligatory anaerobic bacteria, ciliate, flagellate protozoa, chytrid fungi archaea and bacteriophages (Mackie *et al.*, 2002; Tajima *et al.*, 1999). The microbial population consists entirely of either obligate (predominant) or facultative anaerobes. The most numerous are the rumen bacteria, which fluctuate markedly in response to dietary offerings and changes (Krause and Russell, 1996; Hungate, 1966; Al Jassim *et al.*, 2003; Rowe, 1999).

Work by (Tajima *et al.*, 2000) showed that the most profound changes in the rumen bacterial population (based on development of clone libraries) occurred during the dietary change from roughage to hay-grain diets. Using PCR amplification and a clone library of the 16S rDNA they analysed the bacterial population on days 0, 3 and 28 following a switch to a high grain diet. Well-known cellulytic bacterial populations remained high over

the first few sampling days (day 0 and 3) but moved to high numbers of *Selenomonas-Succiniclasicum-Megasphaera* by day 28 (Tajima *et al.*, 2000).

Work outlined in Russell and Gahr (2000) indicate that there are 11 groups of microbes based on their substrate and product preference:

1. **Cellulolytic** e.g. *Fibrobacter succinogenes, Ruminicoccus Flavefaciens, Ruminococcus albus* and *Butovibryo fibriosolvens,*
2. **Hemicellulytic** e.g. *Butyrivibrio fibrioslovens, Prevotella ruminicola* and *Ruminococcus spp.*
3. **Pectinolytic** e.g. *Butyrivibrio fibriosolvens, Prevotella ruminicola, Lachnospira multiparus, Succinivibrio dextrinosolvens, Treponema bryantii* and *Streptococcus bovis.*
4. **Amylolytic** e.g. *Bacteroides amylophilus, Streptococcus bovis, Succinimonas amylohilus and Prevotella ruminicola.*
5. **Ureolytic** e.g. *Succinovibrio dextrinosolvens, Selenomonas spp., Prevotella ruminicola, Ruminococcus bromii and Butyrivirbio spp*.
6. **Methanogens** e.g. *Methanobrevibacter ruminantium* and *Methanobacterium formicicum*.
7. **Sugar utilising** e.g. *Treponema bryantii, Lactobacillus vitulinus* and *Lactobacillus ruminus.*
8. **Acid utilising** e.g. *Megasphera elsdenii* and *Selenomonas ruminantium.*
9. **Proteolytic** e.g. *Bacteroides amylophils, Prevotella ruminicola, Butyvibrio fibriosolvens* and *Streptococcus bovis*.
10. **Ammonia producing** e.g. *Prevotella ruminicola, Megasphera elsdenii and Selenomonas ruminantium.*

11. **Lipolytic** e.g. *Anaerovigrio lipolytica, Butyrovibrio fibriosolvens* and *Treponema bryantii.*

S. ruminantium rumen bacteria utilise acid and are ureolytic, producing ammonia from urea. Sawanon *et al.* (2011) suggested the synergy between *S. ruminantium* and *F. succinogenes* improves cellulolytic digestion.

Another major group of rumen organisms are the *Archaea* or methanogens, which convert carbon dioxide and hydrogen to methane (Hobson and Stewart, 1997). While functionally significant to rumen microbial ecology they are numerically inferior; accounting for only 0.5-3% of total microbes (Mackie *et al.*, 2002).

Protozoa are the largest of the rumen microbes in size and represent about 40% of the biomass. The protozoa fall into two orders, Holotrichs and Entodiniomorphs, and are obligate anaerobes, motile and eukaryotic (Mackie *et al.*, 2002). They are able to transform the principal dietary components consumed in the diet into a variety of metabolites that can be utilised by the host ruminant. (Williams and Coleman, 1997) found that protozoa impact on the dry matter content of the rumen digesta, retention time, rumen volume, the rumen bacterial population diversity, VFA concentrations and proportions, pH and ammonia concentration.

Fungi represent up to 10% of the biota in the rumen. They are obligate anaerobes, saprotrophic on ingested feedstuffs and contribute significantly to the ability of ruminants to utilise plant material and ferment structural polysaccharides (Mackie *et al.*, 2002). Fungal hyphae breakdown the structural organisation of plants this allows bacteria to access the plant structural carbohydrates, such as cellulose and hemicelluloses.

The microbial components of the rumen population interact in terms of digestion and metabolism in ruminants. The diversity within the rumen makes it a very complex environment to monitor and interpret.

1.6.1 Bacterial species present in the rumen

Bacterial population numbers being quantified utilising molecular techniques may be higher as previously a majority of bacteria were non culturable under laboratory conditions (Karma, 2005). New microbiological technologies will help better quantify rumen microbial numbers and diversity.

Rumen bacteria have different roles in the complex rumen environment. For example, succinate producing and decarboxylating bacterial species interact in the rumen to produce propionate - the main gluconeogenic substrate for ruminal physiology. The balance between these two organisms is important as it can lead to the accumulation of succinate in the rumen (Wolin *et al.*, 1997). Hungate was a pioneer of rumen microbiology and developed techniques for culturing and isolating rumen microbial ecosystems in the 1950s. These methods enabled a better understanding of the complexity of the rumen microbial environment. The techniques relied on phenotypic characteristics and the ability to culture bacteria using lab-developed media and roll tubes. However, the majority of rumen microbes were not able to be cultured using Hungate's techniques. New molecular methods have enabled a more sophisticated categorisation of the rumen microbial population.

Classification of rumen micro-organisms relied until relatively recently on microscopic and phenotypic differentiation, bacteria were classified using phenotypic characteristics such as cell shape, flagella, respiration vs. fermentation and nutritional attributes (Hungate, 1966) but there is little evidence that these criteria have evolutionary

or phylogenetic significance (Krause and Russell, 1996). Culturable counts are often 10 to 100 fold lower than the total bacterial counts in the rumen (Brock, 1987).

However, the advent of 16S rRNA gene analysis has led to a more sophisticated genotypic categorization. Comparative sequence analysis of 16S rRNA genes (abbreviated to rDNA for the purpose of this thesis) has provided a means of describing microbial communities without the limitations imposed by phenotypic classification based on culture methods and biochemical identification. 16S rDNA sequencing has enabled new genera and species of anaerobic gram negative bacteria to be described and existing taxa to be reclassified (Jousimies-Somer and Summanen, 2002).Ribosomes are complicated structures that have evolved slowly providing a long-term natural history of evolution. The DNA-encoding sequences of ribosomes are relatively free from selective pressure, which means the invariable and hyper-variable regions of rRNA genes can be used to group bacteria into kingdoms, genera and species (Krause and Russell, 1996). Bacterial ribosomes account for approximately 20% of cellular dry matter with each ribosome having a molecular mass of several million daltons. Bacterial ribosomes have a sedimation coefficient of 70, but each ribosomal particle can be further separated into particles of 50s and 30s. The 30s particle is in turn composed of the 16s rRNA genes particle (Neidhardt *et al.*, 1990). Ribosomal genes are relatively complicated structures that, during evolution, have undergone relatively little selective pressure or gene transfer (Woese, 1987). The 18S and 23S rRNA genes are longer and contain more information with most analysis being conducted on the 16S genes region, on which most bacterial phylogeny is based (Stackbrandt and Hippe, 1996). The sensitivity of 16s rRNA genes methodology has been enhanced by polymerase chain reaction (PCR), which can give a visual image of bacteria in their natural environment (Amann *et al.*, 1990).

Similarities in nucleotide sequences serve to relate microorganisms and can be used to identify uncultured microbes in environmental studies. Comparative sequencing of bacterial rDNA indicates there is a high degree of genetic divergence among rumen isolates previously thought to represent strains of a single species. Using the rDNA as a phylogenetic marker gene is now one of the most common methods used to identify genome fragments derived from specific groups of microorganisms that have not yet been cultured or that play an important role in the environment (Acinas *et al*., 2004).

More recent application of metagenomic techniques has added further sophistication to the study of uncultured complex microbial systems (Suenaga 2012). This new metagenomic approach became available after the study reported here was carried out and will be used to interpret and discuss the data collected.

Outlined below are main known and isolated rumen bacteria.

1.6.1.1 *Prevotella ruminicola* (formerly *Bacteroides ruminicola*)

Prevotella ruminicola was the first rumen bacteria cultured and has a high prevalence under all dietary regimes, it can use multiple substrates and is not sensitive to pH changes (Stevenson and Weimer, 2007a)., making it an ideal key bacterium to monitor during dietary regime introductions.

Prevotella ruminicola are gram-negative non-motile rods 0.8-1.0 μm wide by 0.8-8μm long. They grow at low pH, are pleomorphic and degrade the cellulose derivative carboxymethylcellulose but cannot digest native cellulose, Prevotella hydrolyse starch and liquefies gelatine and its fermentation products in glucose medium include succinic, formic and acetic acid. (Russell and Wilson, 1996).

Ammonia (NH_3) is the only low molecular nitrogen source used efficiently by this species for growth (Dehority, 2003). Prevotella appears to be relatively more important in

animals receiving low starch rations. (Hungate, 1966) found that Prevotella constituted 64% of the cultivable starch digesters in animals fed wheat straw but only 10% of the starch digesters in animals fed solely on grain mixture. *Prevotella* species constitute one of the most numerous groups recovered from the rumen (Avgustin *et al.*, 1997; Gardner *et al.*, 1995; Tepsic and Avgustin, 2001) and from regions of the hindgut in many mammalian species (Avgustin *et al.*, 1997). Studies of the 16S rRNA gene copies of cows showed 42 – 60% of the gene copies were representative of the three most commonly isolated *Prevotella* species (Stevenson and Weimer, 2007b; Stevenson and Weimer, 2007a). Research by (Tajima *et al.*, 2001) indicated the 16S rRNA gene copies of *Prevotella* species far exceeded those of the other eight species examined.

1.6.1.2 ***Selenomonas ruminantium***

Bacteria from the species *Selenomonas ruminantium* are Gram negative, curved rods 0.9-1.1µm by 3.0-6.0 µm. These bacteria are motile with up to 16 flagella attached to the middle of the concave side of the cell (Stewart *et al.*, 1997).

Selenomonas ruminantium is detected at highest amount in animals fed on cereal grains. *S. ruminantium* constituted 22-51% of the rumen viable count in animals fed cracked corn and urea (Caldwell and Bryant, 1966). *S. ruminantium* converts ruminal lactate to VFA (Krause and Oetzel, 2006) and is a starch digesting bacterium isolated in the rumen, though not all strains are amylolytic. *S. ruminantium* has been observed in direct microscope examination in sheep at 1.5- 428 x 10^6 /mL (Hungate, 1966).

There appear to be few propionate producing species in the rumen except *S. ruminantium*, which is capable of decarboxylating succinate (Wolin *et al.*, 1997). Lactate fermentation by *S. ruminantium* and other species as well as conversion of lactate to acetate and propionate can be an important feature of rumen fermentation when fed a high grain diet. Slyter (1976) found that free glucose inhibited lactic acid metabolism with pure

cultures of *S. ruminantium* and slowed the rate of lactic acid utilisation, suggesting that *S. ruminantium* had a preference for glucose rather than lactate in a pure culture. *S. ruminantium* has complex enzymatic machinery and although it is a key lactate utiliser and propionate producer, *S. ruminantium* is also a lactate producer and some strains produce L-lactate while others produce D-lactate from simple sugars. Interestingly when *S. ruminantium* runs out of substrate this bacterium switches into lactate and converts it to propionate

1.6.1.3 *Mitsuokella multiacidus*

Bacteria from the species *Mitsuokella multiacidus* are non-flagellated, straight Gram-negative rods. The prevalence of these bacteria in the rumen is not known although they have been reported in other gut habitats Hobson (1997). This species is closely related to *S. ruminantium* based on the 16S rRNA gene, indicating the importance of qRT-PCR assay development to reduce cross amplification. Due to resource restrictions the use of *S. ruminantium* was considered the more important bacteria to monitor in this study.

1.6.1.4 *Megasphaera elsdenni* (formerly *Peptostreptococcus elsdenni)*

Megasphaera elsdenni is an anaerobic Gram negative coccus, 1.2 to 2.4μm in diameter, which can digest soluble sugars (glucose, fructose and maltose) and some amino acids (Dehority, 2003). Work done by Hristov *et al.* (2001) indicated that reduced protozoa numbers did not impact on L-lactate concentrations and this may have been linked to enhanced activity of *M. elsdenni*. This is supported by work done by Kleive *et al.* (2003) in which steers fed on a rapidly adapted grain diet and a non-grain diet. *M. elsdenni* was not detected in steers without grain in the diet while they established a high lactic acid utilising population in the rumen of cattle.

While this bacterium is an important component of the rumen in grain-fed cattle, it was decided that this population would not be monitored during this study due to monetary and time constraints.

1.6.1.5 *Fibrobacter succinogenes* (formerly *Bacteroides succinogenes*)

There are three major cellulolytics in the rumen: *Fibrobacter succinogenes, Ruminococcus flavefaciens* and *Ruminococcus albus* of which *Fibrobacter succinogenes* is the most prevalent and was therefore chosen as a key bacterial species to be monitored during this study. *Fibrobacter succinogenes* is non-motile, anaerobic and non-spore forming. It forms Gram-negative rods, which generally vary in diameter from 0.3-0.5 μm and in length from 1-2 μm. *Fibrobacter succinogenes* are very pleomorphic and can vary in shape. *F. succinogenes* ferments only cellulose, glucose and cellubiose with its primary end products being acetic, succinic acids (Dehority, 2003) and formic acid (McDonald *et al.*, 2011).

Fibrobacter succinogenes is pH sensitive and degrades cellulose slowly due to the methods the bacterium uses to break down cellulytic material. Active cellulose digestion involves adherence of cells to the fibres via a glycoprotein glycocalyx (Costerton *et al.*, 1981),this protects cells from protozoa grazing and cellulolytic enzymes from degradation by ruminal proteases, retaining the cellodextrin products for use by the cellulolytic bacteria (Weimer, 1996). Cellulytic bacteria only grow in environments that have a favourable rumen pH – one that does not go below pH 6 for long periods. They are also particularly difficult to culture because they are obligate anaerobes and are very slow growing and therefore the sub culture techniques make them very difficult to quantify.

The importance of cellulolytic bacteria for feedlot cattle is not clear; however, they are thought to play a role in keeping the rumen population stable. Cellulolytics such as *F.*

succinogenes disappear or are decreased in number in cattle with acidosis (Slyter, 1976).

Lactobacillus spp.

Lactobacillus spp. are predominant lactic acid-producing bacteria in the rumen. Under acidic conditions (pH 5.7) there can be 10^4 *Lactobacillus spp* per mL but when pH drops to 4.5 numbers can rise to 10^9/mL. *Lactobacillus vitulinus* is a non-motile D-lactate producer while *Lactobacillus ruminis* is a motile L-lactate producer (Stewart, 1992).

On high forage diets *Lactobacillus spp.* are generally in lower numbers than S. bovis ($3 \times 10^{7/mL}$ versus $4 \times 10^{3/mL}$) (Wells *et al.*, 1997). They are also more resistant to low rumen pH. However, when ruminants are introduced gradually to grain there is a dramatic increase in Lactobacillus populations. *Lactobacillus spp*. can produce D- as well as L-lactate, however most do not produce large amounts of acetate and ethanol when glucose is the fermentation substrate (Kandler and Weiss, 1986).

1.6.1.6 *Streptococcus bovis*

Streptococcus bovis has been widely studied in relation to grain poisoning and acidosis in ruminants. *Streptococcus bovis* bacteria are gram-positive, non-motile and ovoid to coccal in shape and chains are sometimes formed and older cells may stain gram negative (Hobson, 1997). This bacterium is widely recognised in many studies as the main lactic acid-producing bacterium in cattle, sheep and horses. However, *S. bovis* is not normally a predominant ruminal bacterium, but rather is an opportunist bacterium that can outgrow other species when diets are high in soluble carbohydrates (Hungate, 1966). In cattle and sheep, the *S. bovis* populations remain low under normal feeding (e.g. high roughage diet), but increase significantly following dietary change from roughage to concentrate (Ghali *et al.*, 2004; Jarvis *et al.*, 2001). Using PCR-based techniques and 16S probes (Reilly *et al.*, 2002) found that *Streptococcus* populations were relatively stable on fresh forage diets but were significantly affected when protein in the diet was low and

carbohydrate was available at supplemental concentrations. The accepted paradigm for lactic acidosis assumes that *S. bovis* is present at low concentrations in the rumen on high fibre diets but at high concentrations in high grain diets. While this is commonly observed in the rumen ecosystems, the magnitude of difference between high fibre and high grain diets is usually less than one log unit (Krause *et al.*, 2003b). *Streptococcus bovis* is known to have low proteinase activity and therefore a diet consisting of low nitrogen and low carbohydrate can limit the growth of the streptococci (Reilly *et al.*, 2002) and is indicative of a decrease in ruminal ammonia concentration.

Lactate produced by *S. bovis* is regulated by the activity ratio of lactate dehydrogenase to pyruvate formate-lyase, which in-turn responds to energy supply or the intracellular pH (Asanuma and Hino, 2002). *S. bovis* is resistant to low pH in the rumen as it can control its intracellular pH environment (Russell, 1998).

1.7 **Bacterial interactions**

The presence of many substrates capable of supporting anaerobic microbial growth underpins species diversity of the rumen. Metabolic products from one microbial species may become sources of energy for other species. It is the extent of these microbial interactions that regulate the concentrations and activities of individual microbe species and the fermentation products they generate from dietary substrates (Wolin *et al.*, 1997).

1.8 **Rumen Protozoa**

Protozoa are present in the rumen at 10^3-10^6 cells/mL of rumen fluid (McAllister *et al.*, 2006) and represent approximately 50% of the microbial biomass in the rumen. Protozoa have been shown to be important but not essential to rumen function (Jounany, 1991). They are classified into two groups based on morphology. Isotrichidae (commonly called holotrichs) are ovid organisms covered with cilia that generally do not ingest food

particles and cannot utilise cellulose. The second protozoa grouping contains the ophryoscolecidae (or oligotrichs), which vary considerably in size and shape and can ingest food particles and utilise simple and complex carbohydrates including cellulose and vary in size to the entodiniomorphid protozoa (Hungate, 1966; McDonald *et al.*, 2011).

Work by (Hristov *et al.*, 2001) indicated grain-fed feedlot cattle were virtually free of protozoa or had dramatically reduced populations. Brown *et al.* (2006) showed that protozoa numbers peaked with a diet of about 60-70% concentrate. In cows transitioned onto a 65% grain and 35% hay diet with subacute acidosis induced in week one, there was a significant increase in protozoa at week three followed by a significant decrease by week six (Hook and Steele, 2011).

Despite a substantial decline in total protozoa numbers in the rumen of cows fed on a 95% concentrate compared to a 62% concentrate diet, ruminal pH did not decrease below 5.5 and L-lactate concentrations did not increase. This suggests that if an economically feasible method were developed to (Hristov *et al.*, 2001) control protozoa in feedlot cattle, it might be possible to reduce the recycling of bacterial nitrogen within the rumen and improve efficiency of protein utilisation without a concomitant increase in the incident of acidosis (Hristov *et al.*, 2001). Brossard *et al.* (2004) found that sheep on a 60% wheat and 40% alfalfa hay diet had increased numbers of entodinimorph protozoa. Hristov *et al.* (2001) found that reducing the rumen protozoa population by 42% did not affect the concentration of L-Lactate in the rumen.

1.9 **Changes in rumen bacterial ecology**

The rumen is ever changing, and this has been demonstrated predominantly with changes in nutrition (Tajima *et al.*, 2001). Steers adapted to a grain diet prior to induced subclinical acidosis had higher numbers of lactate utilising bacteria than steers adapted to a hay diet prior to the induced acidosis. However lactate-using bacteria increased in both

groups over time following grain challenge (Goad *et al.*, 1998). Culturing and counting bacterial species

Traditionally bacteria have been classified using phenotypic characterisations, including cell shape, flagella, respiration and fermentation and nutritional attributes with little evidence that these were criteria for evolutionary or phylogenetic significance (Krause and Russell, 1996). Traditional methods of enumerating and identifying microbial populations within the rumen are time consuming and cumbersome and methods that involve culturing and microscopy can be inconclusive (Denman and McSweeney, 2006). The enumeration of specific species of bacteria in the rumen ecosystem is difficult with conventional techniques due to the large number of biochemical techniques required and the imprecision of these techniques. In addition, many rumen microbes cannot be cultured in the laboratory (Karma, 2005). Outlined below are some of the techniques used to identify and quantify rumen bacteria.

1.9.1 **Isolation methods for bacteria from rumen samples**

Understanding of ruminal ecology historically was based on those microorganisms that can be quantified and characterised using culture-based techniques (i.e. substrate utilisation and fermentation products). However, these microorganisms have commonly been found to represent only 10 –15% of bacteria observed using direct microscope examination of rumen fluid via traditional anaerobic plating techniques (McAllister *et al.*, 2006).

Traditional methods used to enumerate ruminal bacteria have relied on culture samples on semi-defined media. Once cultured, the bacterial colonies are then counted, purified and characterised using an array of techniques including microscopy, substrate utilisation and fermentation product assays, enzyme production and membrane fatty acid analysis (Krause and Russell, 1996). However, these methods can be inaccurate and

cumbersome as many bacterial populations fail to grow on cultures and a large number of colonies is required to attain statistical significance (Krause and Russell, 1996).

Stewart *et al.* (1981) carried out key research into cellulytic bacteria. They isolated ruminal *F. succinogenes* from a cow and assessed its ability to attack cotton fibres and powdered filter paper. All the cellulytic isolates were cultured on the cotton fibre substrate but not the cellulose agar. This highlighted the selectivity of different strains under culturing and why it is difficult to accurately quantify and characterise the full spectrum of a bacterial species from initial rumen samples.

Difficulty in culturing rumen bacteria often confounds enrichment and enumeration techniques for bacteria. In general, the culturable count is ten to a hundred fold lower than the total count (Brock, 1987).

Most bacteria in natural ecosystems are viable but not be to be cultured which complicated isolation and curtails the number of actual species, particularly as isolation may not always be reproducible in vivo (Tajima *et al.*, 1999). Using molecular technologies to identify and count bacterial species is becoming common practice in complex environmental samples such as rumen fluid.

1.9.2 **Counting of bacteria for quantification**

The technology available to count bacteria has advanced from the traditional counting method using microscopes to the most probable number technique (MPN), which has lower precision than direct counting using the coulter counter (Dehority *et al.*, 1989).

Four counting methods were evaluated by Fiala *et al.* (1999). These were (a) the manual method, using a Helber bacteria counting chamber, (b) a coulter EPICS Elite flow cytometer-based method (c) counting using the portable microcyte flow cytometer and (d)

a coulter principle-based method. All these techniques gave adequate precision in measuring total cell density with no systemic differences between the methods (Fiala *et al.*, 1999).

Bacterial size can be determined using the coulter counter. Baker (1990) found that organisms in an aqueous environment displace their volume in fluid so that the size of the organism can be expressed as its equivalent spherical diameter. This method contributes to classification of rumen microorganisms by size.

Enriched bacterial growth media can over-estimate bacterial counts and do not enable the phenology of bacterial populations to be assessed. This limits the capacity to gain a full picture of the rumen population. Molecular technologies have progressed rumen microbial studies beyond culture methods. The issues associated with the culturing of rumen microbes has been overcome with the introduction of a new approached called metagenomics, in which the microbial DNA is extracted from the rumen samples and sequenced independent of cultivation (Attwood *et al.*, 2008)

1.10 **Use of molecular tools to identify rumen microbiota**

Traditional phylogeny and enumeration methods for ruminal bacteria are tedious and inaccurate. In contrast, modern methods of bacterial classification do not require *in vitro* culture and can potentially detect a single cell (Krause and Russell, 1996). To obtain a good representation of the spectrum of rumen bacteria in a rumen fluid sample it is important to use molecular tools to achieve this.

Molecular technology is becoming increasingly important in establishing the changes that occur in the rumen and other ecosystems. Molecular technology considered in this study included fluorescent in situ hybridisation (FISH), denaturing gradient gel electrophoresis (DGGE) and quantitative real time polymerase chain reaction (qRT-PCR). Of these techniques quantitative real time polymerase chain reaction was used to target key

bacterial species based on 16s rRNA genes. Since this study was undertaken metagenomics has progressed considerably enabling the diversity within samples to be more easily quantified (Petri *et al.*, 2013c; Kittelmann *et al.*, 2013; Morgan and Huttenhower, 2014b; Nikolaki and Tsiamis, 2013; Golder *et al.*, 2014). These modern molecular techniques rely on a higher level of population analysis at the species level than that undertaken during this study, which aimed to study the overall population complexity rather than specific key species. Modern molecular methods include genome sequencing, pyrosequencing, proteomics and transcriptomics (Krause *et al.*, 2013) and techniques such as terminal restriction fragment length polymorphism (T-RFLP), which is a DNA fingerprinting technique used to compare complex microbial communities and next generation sequencing (NGS) (de la Fuente *et al.*, 2014). de la Fuente *et al.* (2014) concluded that earlier molecular techniques were still valuable in the study of microbial diversity and complex environments. However, the use of next generation sequencing provides a more cost effective alternative with a higher level of detail compared to single members of a microbial population.

Metagenomics have progressed to enable massive parallel sequencing techniques that allow for rapid and economical DNA sequencing (Wang and Qian, 2009). Krause *et al.* (2013) note that the new technology of pyrosequencing can potentially elucidate bacterial interactions with their ruminant host to enhance animal health and productivity. At the time of this study (2003-2006) sequencing was expensive and difficult, which impacted on sequence quality however it did provide valuable information with regards to population changes of key species only previously monitored through culturing techniques.

Polymerase chain reaction (PCR) is commonly used as a standard method in diagnostic and research laboratories and is now an essential tool in laboratory research.

PCR reactions detect PCR products at the end stage of exponential amplification Denman and McSweeney (2005). However qRT- PCR is now widely accepted as it is rapid, sensitive and reproducible with minimal risk of carryover contamination (Mackay, 2004). Quantitative or real time PCR is not performed at the end of the reaction, but rather during exponential amplification, which in theory will result in the doubling of product with each cycle (Rasmussen, 2001). Quantitative PCR allows the entire reaction to be viewed and product being generated throughout all stages of the reaction. SYBR Green is used as it binds to double stranded DNA, therefore as the reaction progresses the amplicons produced leads to a higher fluorescence (Denman and McSweeney, 2005). To test the purity of the amplicon a dissociation curve is undertaken to ensure the melting curve of the DNA is in one single sharp point and there are no non-associates products or primer dimers (Denman and McSweeney, 2005).

Real time PCR has allowed quick throughput methods, however it is important to note that qRT-PCR is only as reliable as the controls and standards that are developed in the analysis (Mackay, 2004). This highlights that when developing techniques to monitor bacterial populations it is important to constantly test the accuracy of controls, standards, and DNA extraction, and have well developed primers.

1.10.1 **qRT-PCR using SYBR Green**

SYBR Green is widely used in real-time PCR applications as an intercalating dye and is included in many commercially available kits. The binding of SYBR Green to double-stranded DNA is not specific, so reactions need to be optimised to reduce the amplification of nonspecific products. The use of a melting curve analysis eliminates the necessity for agrose gel electrophoresis because the melting of the specific amplicon is analogous to the detection of electrophoretic band (Giglio *et al.*, 2003). (Giglio *et al.*, 2003) found that with increasing demand for high throughput analysis the characteristics of

SYBR Green may reduce optimisation times by avoiding the use of or limiting concentrations of SYBR Green in assays that target G + C rich targeted regions.

SYBR Green has been reported in the literature since 1997, with little attention to other intercalating dyes for this application, which at times have been shown to have limited dye stability. In addition, the concentration of the dye can be affected by the melting temperature (Monis *et al.*, 2005). (Monis *et al.*, 2005) compared SYT09 to SYBR Green I and found it easier to convert conventional assays to RT PCR and for DNA melt curve analysis.

1.10.2 **Sequencing**

Recent progress in sequencing has allowed researchers to rapidly analyse the 16S rRNA genes on which most analyses of bacterial physiology are based (Krause and Russell, 1996). In recent years the 16S rRNA gene sequence information has been used to characterise the diversity of microorganisms within the rumen ecosystem. Unlike methods based on specific genes sequences, rRNA based methods have been developed on the basis of bacterial phenology. Consequently their specificity is more appropriate for the evaluation of taxonomic diversity (McAllister *et al.*, 2006).

Studies of 16s rRNA genes indicate that the diversity of ruminal bacteria has been greatly underestimated with traditional methods of phylogeny and stymied by fastidious growth requirements making enumeration tedious and inaccurate. Bacterial diversity is therefore thought to be 100-1000-fold greater than the previously 5000 recognised in *Bergeys manual of systematic bacteriology* (Krause and Russell, 1996).

The biotechnological approach has allowed glimpses into what the unique ruminal environment and importantly the changes that can occur in it over a very short period of time. The development of these molecular techniques has broadened our knowledge of the

rumen environment on both an ecological and functional level. Procedures such as RT-PCR can be used to monitor changes such as dietary transition or antimicrobial agents with a degree of sensitivity and precision that was previously impossible (McAllister *et al.*, 2006). Work done in soils by Janssen (2006) indicates that, based on clone libraries, the nine bacteria thought to comprise the population in the soil actually represent less than 5% of the total bacterial population. This highlights that under a complex environment like the rumen we are not effectively documenting and describing ruminal changes through culturing. Advances in DNA sequencing technologies and bioinformatics are allowing a better understanding of complex microbial communities such as the rumen, Morgavi *et al.* (2013) outlines how recent metagenomics was able to provide detailed information about physiology of the species being monitored within the rumen

1.10.3 **Use of Molecular techniques to identify rumen microbial population change.**

Tajima *et al.* (2001) monitored bacteria of cattle fed on a commercial diet under laboratory conditions. Samples obtained via fistulation prior to their morning feeding showed the fibrolytic bacterium *F. succinogenes* fell 20 fold in the 3rd day of introduction to the grain diet with a further 57-fold decrease at day 28. Another fibrolytic bacterium *R. falvifaciens* decreased by 10% at day 3 but remained at that level until day 28. *P. ruminicola* increased seven-fold on day 28.*P. bryantii* increased 263-fold and on day 3 and remained 10-fold higher on day 28 than at day 0. *S. bovis* increased 67-fold on day 3 however on day 28 it decreased in comparison to the hay diet. *S. ruminantium* increased eight-fold during the diet switch but stabilised with only a two-fold increase at day 28. This indicates that there is a need to monitor bacteria over time rather that at one point of sampling.

The Tajima *et al.* (2001) study using 16S qRT- PCR formed the basis of development of techniques for my study with adaptations to deal with issues of cross reactivity with bacteria due to primers (outlined in the primer development section).

Analysis of the community structure and bacterial diversity of steers fed either corn or hay was undertaken by Kocherginskiaya *et al.* (2001) using denaturing gradient gel electrophoresis (DGGE), which were further analysed by excising, reamplification and sequencing and also random shotgun sequence libraries. Kocherginskiaya *et al.* (2001) concluded that populations recovered through DGGE were consistently less diverse than those recovered by random sequencing, which also had substantially higher species richness. The species richness was higher in the corn diet for both methods.

Rapid fragment length polymorphism (RFLP) can be used to examine bacterial diversity in the rumen. Krause *et al.* (2003b) used RFLP to determine the *Lactobacillus spp* isolates cultured throughout the digestive tract. rDNA sequencing of rumen fluid collected from animals fed a diet of haylage/corn silage/concentrate rations indicated several novel bacteria that had not before been isolated or characterised by 16S rDNA (Whitford *et al.*, 1998). Sequences that clustered with *P. ruminicola* represented the majority of the clones isolated. Similarity varied from 94-97%. They analysed the species of *P. ruminicola* likely to be recognised by strain 23 signature. The work indicated that the presence of the signature strain alone might not predict strain relatedness. Their work indicates that *Prevotella ruminicola* like 16S sequences represent the most numerous sequences; however this cannot be used for quantification.

These different techniques give an insight into the rumen environment at a point in time without the need for culturing and the added influence of a non-appropriate growth medium for the targeted bacterial species. These techniques have also enabled scientists to determine the relationship between bacteria or microbes present in mixed environmental

samples (Janssen, 2006). Since this project there has been large jumps in the ability of metagenomics with McCann *et al.* (2014) outlining in their review that pyrosequencing of the 16 rRNA gene could reveal the taxonomic identify of bacteria and archaea to the genus level. While who genome shotgun sequencing is able to predict the functional capacity of the microbiome which is very exciting in the understanding of such a complex system.

1.10.4 **Phylogenetic relationship between bacterial strains**

The phylogenetic tree is an inferred evolutionary relationship between biological species. The introduction of direct retrieval and sequence analysis of some target genes, mainly those of rRNA is used to evaluate genetic diversity and phylogenetic relationships of microorganisms without culturing (Tajima *et al.*, 1999). It has also allowed for major reorganisation among anaerobic taxa (Jousimies-Somer and Summanen, 2002). There is no exact 16S similarity limits defining specific taxa, in general species definition requires species similarities greater than 98% and molecular analysis has allowed the diversity in the rumen population to be explored.

Work by Wright *et al.* (2004) comparing the methanogen population and their relationships was undertaken using a universal methanogen primer for a PCR reaction and the restriction enzyme HaeIII along with rapid fragment length polymorphism (RFLP). The authors then sequenced the product and were able to identify that there was potentially a new order of methanogens to be confirmed with further study. This highlights the potential that new molecular technologies have in identifying diversity within populations such as the geographical or possible dietary differences in species diversity found by (Wright *et al.*, 2007).

1.10.5 **Primer design**

The design of primer oligonucleotide sequences are nucleotides that serve as a starting point for DNA amplification and are the key to success of molecular techniques. The PCR primers work by annealing to targeted regions of DNA to amplify a targeted section of single stranded DNA. The process starts with the reaction containing the DNA being heated to approximately 95 ^{o}C, which melts the double stranded DNA to single strands. The temperature is then lowered to approximately 60 ^{o} C to allow the primers to bond to the targeted regions (forward and reverse primers) which are then amplified. When designing primers for use on the real time PCR the shorter the amplicon length the better the consistency of the assay. Denman and McSweeney (2006) had product lengths of 120-130 base pairs (bp) without cross reactivity. Primers designed by Tajima *et al.* (2001) were used to monitor a variety of bacteria in the rumen that varied from 485 to 869 bp. However, this primer length can be an issue because it can reduce the chance of cross reactivity as well as primer dimers in which non-targeted areas are replicated impacting on the qRT-PCR outputs. The (Tajima *et al.,* 2001) primers were designed to monitor cattle going onto concentrate diets. The *S. bovis* primer also picked up *S. equinus* and *S. ruminantium* as well as *M. multiacida* due to the similarity between their 16S rRNA gene region. Therefore, it is important to recognise the requirements for cross reactivity checks when developing primers.

The shift in metagenomics studies in the last 10 years into new sequencing techniques, has allowed for the discovery of the biosphere in environmental samples and a better understanding of non-culturable populations that may have not been identified in earlier studies (Highlander, 2012). A study by Klindworth *et al.* (2013) showed that commonly used single pair of primers exhibited significant differences in the overall coverage and phylum spectrum with only 10 of the 512 primer pairs evaluated usable for

the new sequencing technology. Further recent studies by Dorn-In *et al.* (2015) outlined that with the new sequencing approach that many primers that targeted the 16s rRNA genes region allowing for sequencing of the total bacterial population also amplified the DNA of plants and other archaea and eukaryotic cells potentially misevaluating the targets with non-target DNA. Fredriksson *et al.* (2013) found that using two different primer pairs on the same wastewater samples generated different results in species diversity which is similar to the rumen environment.

While metagenomics technology has progressed since my study, it has however allowed for a focus on the principles of key species variation using qRT-PCR that has dominated rumen studies since Hungates period all be it based on a culturing. The molecular technology this study implemented is a key step in understanding how these key species change during commercial feeding conditions.

1.10.6 **Sequencing**

Sequencing allows the determination of the sequences of nucleotides (G+C or A+T) and these allow the genes that exist to be identified along the DNA. Sequencing is a enables ease of molecular analysis of samples, with the costs of the technique having dropped by two orders of magnitude since early 2000 These lower costs have seen the method shift from use solely by large sequencing centres into the hands of individual researchers (Shendure and Ji, 2008). The ability to source sequences online through databases such as Genbank has also made analysis of a variety of populations more accessible.

Since this study the advancement of metagenomics has allowed for rapid and economical sequencing of large numbers of samples, making it a more viable way to analyse bacterial ecosystems (Rothberg and Leamon, 2008).

1.10.7 **DNA extraction techniques**

DNA extraction is crucial for molecular techniques, particularly when estimating cells/mL. If DNA extraction is not completed correctly, extrapolation of data can be incorrect or inconsistent and may not be representative of the population diversity that occurs in the sample being analysed. DNA extraction from environmental samples can lead to poor DNA yield or inhibitory substances in the extracted DNA (Yu and Morrison, 2004). Yu and Morrison (2004) reported a DNA extraction technique that improved DNA yield by more than six times. The extraction of gram positive and gram-negative bacteria can be very different and it is therefore important to achieve a consistent and complete extraction of pure cultures and rumen samples. While much work has been done on DNA extraction it appears that plant materials such as tannins or plant polysaccharides or lignin which bind tannins may inhibit PCR quality DNA (Krause *et al.*, 2001). This is often as issue with rumen samples which can be high in varied feed sources being consumed by the ruminant at the time.

Many DNA extraction techniques for rumen and environmental samples are outlined in the literature (Sharma *et al.*, 2003; Anderson and Lebepe-Mazur, 2003; Miller *et al.*, 1999; Chaudhuri *et al.*, 2006). These studies suggest that each situation requires some adjustment to the extraction method to deal with the variation in each sample

1.11 **Aims**

The aims of this study have several dimensions. Firstly, to monitor the key bacterial changes use qRT- PCR under field conditions in feedlot cattle, standard dairy cows in a shed and sheep. Secondly to determine if these key bacterial changes link with metabolic changes in ruminal pH, volatile fatty acid concentrations and molar proportions, and L- and D-lactate concentrations in cattle or sheep during introduction to grain diets. Thus far most studies on grain-induced acidosis have been conducted under experimental,

controlled conditions; a major point of difference for this study will be the use of practical, commercial feedlots for screening different feeding regimes representative of the normal feeding strategies in place under present Western Australian beef industry practice.

Changes in rumen microbial ecology will be monitored using molecular qRT-PCR techniques focusing on selected bacterial species within the rumen of cattle and sheep. The molecular techniques will be cross-referenced with traditional culturing methods and traditional metabolic indicators of rumen acidosis. The qRT-PCR technique was chosen due to the high precision of this approach, its novelty at the time of the study, and the ready access to appropriate equipment at the Murdoch State Agricultural Biotechnology Centre (SABC) at Murdoch University. Studies by Tepsic and Avgustin (2001) indicated that other molecular techniques available at the time such as FISH were not suitable due to the intense florescence of feed particles as well as the difficulty in counting bacteria adhered to the feed particles.

Undertaking a molecular quantification of changes in rumen bacterial populations using qRT-PCR required several methods to be developed. These included establishing the relationship between traditional Coulter counter values and turbidity (spectrophotometer reading) to develop the standards for quantification in the qRT- PCR techniques. Moreover, it was essential to extract DNA of sufficient quality and yield from pure cultures of each bacterial species as well as mixed populations present in rumen samples. Finally, and most importantly was the development of appropriate and effective primers suitable for the RT-PCR reactions so that the primers targeted the desired rumen bacteria within the rumen samples.

1.12 **Hypotheses under test**

The hypotheses under test in this study were:

1. The molecular technique of quantitative real-time polymerase chain reaction (qRT-PCR) can be developed using pure cultures of rumen bacteria as reference to monitor the changes in population ecology of rumen bacteria in mixed rumen samples collected under practical commercial feeding regimes.
2. Changes both microbial and biochemical will be similar with separate feeding of roughage and grain compared with a total mixed ration of cattle
3. Time of calving has a long-term influence on the rumen microbial ecology subsequently established in new-born cattle.
4. Feed additives such as antibiotics will reduce the incidence of acidosis through the bacterial ecology established in the rumen during any grain introduction.
5. Feeding grains with low starch content e.g. lupins or soybeans will not predispose ruminants (sheep in this instance) to acidosis.
6. Fibre utilising rumen bacteria (*Fibrobacter succinogenes*) populations will decrease during grain feeding and any associated reduction in rumen pH. This supports the finding of Tajima *et al.* (2001) were the *F. succinogenes* population declined 3 fold in day 3 and 57 fold in day 28 following a dietary shift form hay to grain.
7. Lactic acid utilising rumen bacteria (*Selenomonas ruminantium*) populations will increase with an increase in the grain component of the diet.
8. *Prevotella ruminicola* will be the most prevalent bacteria in the rumen during dietary transition. This is expected as the bacterium is known to be predominant in the rumen following shifts from hay to grain (Tajima *et al.*, 2001).
9. *Streptococcus bovis* will increase significantly and possibly pathologically during introduction to grain-based diets.

10. If increases in *Streptococcus bovis* are linked with a decrease in ruminal pH, then *Lactobacillus spp.* will also increase significantly.
11. Metabolic changes in the rumen can be related to changes in the molecular ecology during dietary transitions in cattle and sheep.

2 Materials and Methods

2.1 Introduction

This chapter describes in detail all instruments and solutions that were common to the collection of rumen and faecal samples from cattle. It also describes the general materials and methods that were consistent between the experimental chapters including techniques used to quantify the bacterial standards utilised in the qRT-PCR technologies and development and refinement of the qRT-PCR reactions utilised during this study.

2.1.1 Collection of rumen, urine and faecal samples during field trips

The following equipment and items were required and assembled to ensure all samples were collected consistently on each field trip.

2.1.1.1 Equipment

The equipment used included the following

- Engel ® fridge/freezer for storage and transport at controlled low temperature for rumen and faecal samples
- 2 x metal pumps initially designed for collection of gastrointestinal samples from horses (plus spare seals) (Plate 1 (A)) were adapted for rumen collection
- 2 x metal mouth gags (40mm width x 400mm length) (Plate 1(B))

- 2 x 1.5m length x 20mm diameter rumen sampling tubes with each of the ends sanded to produce smooth surfaces, to reduce the chance of oesophageal damage (Plate1(D))
- Brass attachment assisted sampling tubing for sampling drops into rumen contents at a representative location (Plate 1(C))
- 2 x 10L buckets to wash sampling equipment between collections from each animal
- 2 x Gilson auto pipettes; 1000µl and 5000µl for dispensing rumen samples
- Transportable aluminium table [1.2 m x 0.8 m] for use in cattle yards
- Arlec top-pan electronic scales to weigh faecal samples (1kg, 5g increments)
- Portable Orion pH meter – Model 250A with TPS pH ORP and reference electrode (Brisbane, Australia) to measure rumen pH.

Consumables

- Pipette tips for pipetting rumen fluid into protozoa vials (1000µl tip ends had been cut off 1 to 2 mm from the end, allowing for excess fibrous material to be dispensed)
- 20L of fresh tap water
- Latex gloves and long sleeve, pregnancy examination gloves
- 10mL centrifuge tubes labelled for samples collected from each animal
- 5mL screw top storage vials for collection and storage of protozoa
- M^{c}Cartney tubes labelled for faecal samples
- Permanent marker pens (black) for vials
- Sterile, deionised water (sterilised each field trip) ensuring no additional bacterial growth not related to the faeces in the M^{c}Cartney tubes
- 0.1M sulphuric acid (sterilised for each trip) to stop metabolic activity in faecal samples

- Standard buffer solutions (pH 4, 7 and 10) used to standardise the pH meter prior to use on each field trip. These were tested prior to each field trip and replaced as necessary.

All vials were pre-weighed and spare vials were taken.

2.1.1.2 Labelling of vials

Collection identification included location, date and sample type and animal tag number and sample number. These were printed onto self-adhesive labels and placed onto the vials. If the vials were to be placed in a -80^{o}C freezer for any length of time, then sticky tape was placed around the label to ensure they remained secure.

All samples collected on field trips were dispensed into 10mL clip-top, plastic centrifuge tubes, then centrifuged at 2000 rpm for 3 minutes in a bench-top centrifuge, after which the supernatant was then dispensed into 5mL polypropylene vials using Pasteur pipettes. All vials were labelled with:

- The property name (e.g. Manton)
- The date the samples were taken
- The day this was from the first day of sampling e.g. 0,3,7,14 or 21
- The samples for analysis that was to be carried out
    - DNA extraction
    - D-lactate
    - L-lactate
    - Volatile fatty acids
    - Ammonia assays
    - Spare sample.

Protozoal vials were labelled with an A or B both on the vial body and the lids (using black permanent marker pen); this was important as they were weighed prior to the field trip to calculate the correct dilution factors using the final weight was taken.

2.1.1.3 Rumen sampling of cattle

Rumen samples were taken from cattle via stomach tubes. A metal gag was placed in the mouth of the cattle and then a 1.5 m tube with a brass attachment on the sampling end to strain excess fibrous material from the sample and drop to the lower point in the rumen to reduce saliva contamination. This was inserted through the gag, down the oesophagus and into the rumen slowly while waiting for the swallowing reflex to assist passage and thus to protect against forcing the tube. A metal pump (Plate 2.1(A)) was then used to draw rumen fluid through the tube. The tubing was pinched off to ensure that the rumen sample did not run back into the rumen or lungs. The tube and gag were removed slowly to ensure that the oesophageal lining was not damaged. The rumen fluid was then dispensed into a plastic 100mL beaker.

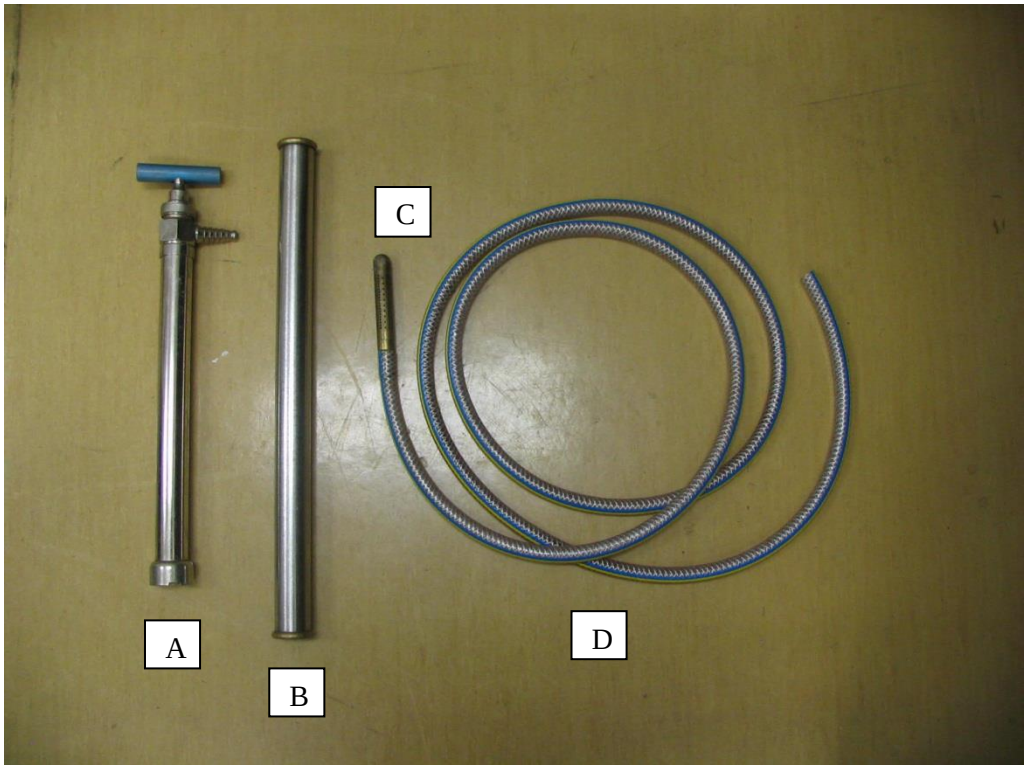


Plate 2.1: horse pump (A), metal gag (B) and brass attachment (C) on plastic sampling tubing (D) [20 mm diameter x 1.5 m length]

2.1.1.4 Rumen pH

The rumen samples were placed into plastic beakers and the pH measured immediately in the field using an Orion portable pH meter model 250 A (Thermo Electro Corporation, Ohio USA) with a TPS pH ORP and reference electrode probe.

2.1.1.5 Handling of rumen samples

The rumen samples from each animal were dispensed into two or three, 10 mL centrifuge tubes and then placed in an Engel® freezer at -10^{o}C for transport in the car. These samples were transported back to the Murdoch University laboratory and centrifuged on a cool spin centrifuge for 3 min at 800 x g. They were then dispensed into 5 mL polypropylene tubes that would be used for analysis of D (-) - and L (+)-lactate, bacterial DNA extraction, ammonia and volatile fatty acid (VFA) analysis and stored at -80 ^{o}C.

2.1.1.6 Depigmentation of rumen fluid for lactate assays

Rumen fluid was depigmented for use in L (+) - lactate and D (-)-lactate assays. This is achieved by placing 0.5 mL of 0.15 M barium hydroxide into a 2 mL microcentrifuge tube. One millilitre of the spun down rumen fluid and 0.5 mL of 5 % zinc sulphate was added, and the mixture mixed thoroughly. The tube was allowed to stand for 5 min and then spun down in a microcentrifuge (Sigma 113, Germany) for 7 min at 5 000 x g. The supernatant was then removed using a Pasteur pipette and placed into a 2 mL microcentrifuge tube for assay analysis.

2.2 **Phenotypic measurements**

2.2.1 **Faecal samples**

2.2.1.1 Scoring faeces

Faeces were scored using the 5-point scoring system developed by Bramley, (2004):

1. Firm cowpat, well formed, no evidence of excessive liquid component.
2. Less formed than above but still holding shape, may contain some whole grain.
3. Softer less formed and evidence of more liquid may contain grain.
4. Minimal formation of cowpat on ground may contain grain.
5. No formation of cowpat on ground, scouring on ground as cow walks, may contain grain.

2.2.1.2 Collection of faecal samples

Samples of faeces were taken directly from the rectum using long examination gloves and placed into a clean, plastic beaker. Samples were into two 30 mL M^{c}Cartney tubes, 5 g of faecal matter dispensed with 8 mL of sterile deionised water into each tube. The pH was then measured using a portable pH meter (Orion portable meter model 250) calibrated using pH 4.0, 7.0 and 10 standards. One millilitre of a 5% glucose solution was added to one faecal sample and this was then incubated at room temperature for 2 hrs. Then a 26.5-gauge needle was used to puncture the lid of the McCartney tube to release any accumulating pressure due to gas production. The contents were then incubated for a further 20 hrs at 37 ^{o}C and pH was remeasured. One millilitre of 0.1M sulphuric acid was added to the second McCartney tube which was then frozen for later analysis of D-lactate.

2.2.1.3 Depigmentation of faecal samples

Faecal samples were defrosted and mixed. The McCartney vials were then emptied into 10 mL clip top centrifuge tubes. These were then spun down at 500 x g for five minutes, 5 mL of the supernatant was removed and placed into another 10 mL centrifuge tube with1mL of 0.15 M barium hydroxide and left to stand for 5 min. Then 1 mL of 5 % zinc sulphate was added, and mixed thoroughly. These samples were then spun down again at 500 x g for 5 min. The supernatant was removed immediately and placed into 5 mL polypropylene vials for use in the assay.

2.2.2 **Rumen and Faecal L-lactate and D-lactate**

Both L- and D-lactate concentrations were measured in the spectrophotometer Shimadzu UV 1201 using end-point assay at 340 nm. Both lactate assays were adapted from (Brandt *et al.*, 1980). The rumen fluid was depigmented as outlined in 4.2.1 prior to assay.

The quantities are outlined in appendix 8.1. The standards were set up in duplicate and samples were set up in triplicate. The samples were set up in disposable cuvettes Sarstedt curvettes (cat no D 51588, Nümbrecht, Germany). The assay was set up in the quantities as outlined with the addition of lactate dehydrogenase (Roche cat no 127876) for L (+) lactate analysis or D (-) lactate dehydrogenase (Roche, cat no 11585436001) for D (-) lactate analysis. The cuvette was then covered with Para film and mixed; reading was then taken in the spectrophotometer at 340nm. Then 5µl of the required the specific form of lactate dehydrogenase depending on whether D or L lactate was being analysed, was added and mixed again and left for 2 hrs. The cuvettes were placed in the spectrophotometer for a second reading at 340 nm.

2.2.3 **Rumen ammonia**

Samples for ammonia analysis were dispensed as in 2.3.2 and stored at -80 ^{o}C. They were then analysed as outlined in appendix 8.2 using Boehringer Mannheim ammonia kit (cat. No. 125857 (19 x 2.0mL).

2.2.4 **Volatile fatty acid analysis**

The samples were defrosted and 1mL of the rumen fluid was then placed into a 2mL microcentrifuge tube. The pH was adjusted to less than pH 3 using a drop of concentrated sulphuric acid to maintain the protonated form of the volatile fatty acid. These samples were then frozen in a -20^{o}C freezer and taken on ice to The Western Australian Department of Agriculture and Food animal health laboratory and submitted for analysis. They used the procedure of Analysing Fatty Acids by Packed Column Gas Chromatography (Appendix 8.3).

2.2.5 **Protozoa counts**

2.2.5.1 Preparation of rumen samples

Five mL polypropylene vials were weighed and then 1 mL of formal saline was added and then each vial was re-weighed. When the rumen samples were collected, 1 mL was placed into its corresponding vial and the vials re-weighed. This enabled the weight of the rumen sample to determine the dilution factor.

2.2.5.2 Counting protozoal samples

The rumen samples were counted using an Olympus microscope CX31, (Tokyo, Japan) on 40x magnification under a counting chamber 1/400mm^2 and a depth of 0.1mm, a counter was used to quantify the numbers protozoa in the sample.

2.2.5.3 Running gels from PCR product (2% agarose)

Agarose gels were run at 2% agarose using Sigma Agrose (A9539-10G) 1 g of agarose to 50 mL of 1x TAE buffer. The machine BioRad Powerpac 300 (California, USA) was run at 80 V for 60 minutes.

2.3 **Development and validation of molecular techniques**

Quantitative real time PCR (qRT-PCR) can be used as a method to quantify bacterial cell numbers in complex environmental samples (Stevenson and Weimer, 2007b). This methodology assumes copies of the targeted gene are present in every bacterial cell, and part of that gene can be copied through appropriately designed primers. The total number of copies of amplified product produced within a fixed number of cycles is directly proportional to the number of copies present in the starting sample. The total copy numbers are then compared to a dilution series of standards for that bacterial species. Verification of the qRT-PCR process therefore requires another standard that can be compared on a cells/mL basis. One methodology for absolute counts of bacterial cells (cells/mL) in a sample is the Coulter counter method which works on the principle that as a particle passes through a fixed aperture, it changes the resistance of the two electrodes located on either side of the aperture. This resulting voltage pulse is proportion to the size of the particle and is counted (Swanton *et al.*, 1962). This requires that a standard suspension of bacterial cells is diluted in series. This standard will then allow the extrapolation of a cells/mL value in the qRT-PCR methodology. This methodology was utilised to determine the relationship between readings of cells/mL on a Coulter counter and the absorbance reading assayed using 600nm wavelength on a spectrophotometer. These relationships will then be used as reference points for the enumeration of cells during qRT-PCR on a cells/mL basis.

The next step requires a consistent extraction of DNA, the main difficulty in extracting DNA from mixed ruminal contents was the high concentration of organic matter in the form of plant material and by-product feeds, making extraction and purification of DNA from whole rumen contents difficult (Stevenson and Weimer, 2007b). Validation of a standard methodology required consistent extraction of DNA from both standard bacterial cultures and bacteria in rumen samples.

2.3.1.1 Cultivation of pure cultures of rumen bacteria

Pure cultures of each bacterial species were established in carbohydrate medium (M 10) based on rumen fluid as outlined in Appendix 8.4. The bacteria were cultured by inoculating 0.5 mL of the pure culture, using a 1 mL syringe and 16.5 G needle, into 10 mL of M 10 rumen fluid medium, stored overnight at 39.7 ^{o}C inside the anaerobic chamber and then cultured for at least 1.5 days to 2 days depending on growth rate of each bacterial species e.g. *F. succinogenes* grew at a slower rate than *S. bovis*. Samples (2.5 mL) from each primary culture of each species were maintained and stored in 2.5 mL of rumen fluid medium, glycerol mix cryoprotectant (Appendix 8.5) at –20 ^{o}C and also –80 ^{o}C. Subsamples were removed and used as needed. All subsequent samples for establishing pure cultures were sourced from these stored cultures to ensure that each strain was always obtained from the same source and not grown in a continuous culture, reducing the chance of cross contamination. *Lactobacillus spp*. was sourced from The University of Western Australia and grown in their carbohydrate-based media (M 10) made from the same protocol as used at Murdoch University laboratories. However, all of these cultures were grown in the laboratories at UWA using the same equipment rather than at Murdoch University to comply with the Australian Quarantine Inspection Services (AQIS) regulations.

Table 2.1 Pure bacterial cultures used in this study and used for enumeration outlined in the following table.

Bacteria	**Strain**	**Source**
Fibrobacter succinogenes ssp. succinogenes	S 85	CSIRO, Livestock Industries, Brisbane
Streptococcus bovis	S 5	University of Queensland, Gatton campus
Selenomonas ruminantium	JW 13	CSIRO, Livestock Industries, Brisbane
Lactobacillus spp.	YE 07	University of Western Australia

Prevotella ruminicola	23	CSIRO, Livestock Industries, Brisbane

The inoculation of each bacterial species was undertaken in an anaerobic chamber [198cm x 81cm x 102cm] equipped with two pairs of gloves, and one airlock (Coy Lab product number 12430 and Coy incubator model number 77, Michigan, USA) (Plate 3.1). The atmosphere inside the anaerobic chamber was kept anaerobic at approximately 96% carbon dioxide and 4% hydrogen (Hamdorf, 1998). However, no gas meter was available to measure exact gas concentrations within the chamber, so proportions of carbon dioxide and hydrogen could not be confirmed.

Plate 3.1 Coy anaerobic chamber in operation, Murdoch University Laboratory.

2.3.1.2 Quantification of rumen bacteria

The pure cultures were the source of standards for quantification on a cells/mL basis by qRT-PCR. There were three steps in this process: firstly, calculating cells/mL of bacteria present in both pure cultures through the Coulter counter and the possible

relationship to the turbidity measurement. Secondly extracting the DNA from pure cultures for use as standards and from the bacteria mixture in the rumen samples in qRT-PCR then thirdly comparing the results from the qRT-PCR from standard curve of pure cultures and the mixed populations in the rumen samples with the corresponding Coulter counter results to determine the cells/mL of that particular bacterial species in the rumen samples taken from cattle or sheep.

2.3.1.3 Enumeration of bacteria

After rumen bacteria were cultured for a period of up to 48 hours, the concentration in cells/mL of bacteria present in the culture was determined using the Beckman Multisizer^{TM} Coulter Counter® (California USA). This cell concentration was then correlated with a turbidity reading in a spectrophotometer at 600 nm. These two determinations of cells/mL were both undertaken prior to DNA extraction. The remainder of the sample of pure culture that had been put through the Coulter counter was frozen and used later for DNA extraction and subsequent used as a standard in qRT-PCR.

2.3.1.4 Turbidity of rumen bacteria measured spectrophotometrically

Rumen bacteria were cultured in Hungate tubes (Bellco Biotechnology, New Jersey, USA. Hungate tubes catalogue number 2047-16125), kept in anaerobic chambers at Murdoch University laboratories or in the case of *Lactobacillus spp*. after the appropriate incubation period at UWA was transported to CSIRO Centre for Mediterranean Agriculture at Floreat, Western Australia.

Absorbance was measured in a visible range spectrophotometer (Jenway 6300, Staffordshire, UK) set at wavelength 600 nm, zeroed against water. The reading of a culture medium blank from the same batch of medium in which the bacteria had been cultured was taken in each set of assays and then subtracted from each sample reading of

the pure cultures to account for any variation in the turbidity of the culture medium. The pure cultures and uncultured media were used in a series dilution to 1 mL total volume, starting with 1 mL of pure culture, then 0.9 mL of pure culture reducing by 0.1 mL increments and topped up to 1 mL with distilled, carbon filtered water in 2mL disposable cuvettes. Paraffin wax paper was placed over the cuvettes after which they were inverted three times prior to being placed in the spectrophotometer (600 nm). Additional serial dilutions of the pure culture were undertaken in an attempt to generate readings at increments of 0.1 absorbance unit, this was necessary for all pure cultures. The absorption values of the series dilution of the culture medium were subtracted from the corresponding pure culture series dilution to ensure an accurate net absorption value. After each reading was taken on the spectrophotometer, the same samples were then passed through the Coulter counter to determine the cells/mL.

2.3.1.5 Enumeration of rumen bacteria in the Coulter counter

The enumeration involved the calibration of the Beckman Coulter counter machine using 2μm beads (Beckman part number 6602792, California, USA).

The bacterial enumeration in the Coulter Counter was performed on 200 μl samples of bacterial culture diluted to 50 ml in 0.5% ultrafiltered formyl-saline run in triplicate for each bacterial culture until the readings were within 1% of each other. Formyl-saline and culture medium were used as assay blanks.

The dilution factors for samples placed in the spectrophotometer and the counter (diluted with ultra-pure formal saline) were calculated and the measurements were averaged and multiplied by these dilution factors. This then resulted in a cell per mL value for the rumen bacteria cultured in the Hungate tubes.

2.3.1.6 Extraction of DNA

Consistency and repeatability was achieved through empirical testing and refinement of DNA extraction techniques used on rumen samples from cattle and sheep. DNA extraction using bead beater method S. Denman, CSIRO Brisbane (*pers comm.*)

There were no modifications made to the protocol developed by Dr S. Denman. This protocol was utilised throughout the thesis as it resulted in the most consistent and complete extraction of pure cultures and mixed rumen population samples.

1. Using a 1mL pipette transfer 1.5mL rumen fluid into a 2mL flat bottom tube (put in details screw top flat bottom with seal) containing a mix of 0.1mm (Cat # 11079101Z) and 1mm (Cat # 11079110Z) diameter sterile zirconium beads (Daintree Industries Pty Ltd, Tasmania, Australia). Spin for 5 minutes at 14,000 x g (approx. 14800 rpm).
2. Discard the supernatant. Resuspend the pellet in 1000µl cell lysis buffer, 100µl potassium acetate solution, 100µl ultrapure water.
3. Placed into a Mini-bead beater (Biospec Products, Bartlesville, OK, USA) and shaken vigorously for 2 minutes on level 4.5.
4. Place at 70^{o}C for 2 minutes.
5. Spin at 20^{o}C for 15 minutes at 14 000 x g (approx 14800 rpm)
6. Transfer 300µl of supernatant to a new tube and add 300 µl of glass milk. Mix on a rotating table for 5 minutes.
7. Spin at 10 000 x g for 1 minute, discard supernatant.
8. Add 500µl of cold ethanol wash. Vortex and spin at 10 000 x g (10 600 rpm) for 1 minute and discard supernatant.
9. Final spin for 20s to remove residual ethanol.

10. Add 110µl deionised water. Vortex and spin 10 000 x g (approx. 10 600 rpm) for 1 minute and transfer 100µl of supernatant to new tube.

Reagents and materials required

Cell lysis buffer

0.2% SDS (Sodium Dodecyl Sulphate)

100mM Tris-HCl

5mM EDTA (Ethylenediaminetetra acetic acid disodium salt)

200mM NaCl

Potassium acetate solution

29.44g potassium acetate

11.5mL glacial acetic acid

Preparation

Dissolve 29.44g potassium acetate in 70mL double distilled (dd) water. Add 11.5mL glacial acetic acid and make up to 100mL with dd water. pH should be ~ 5.5-6.0.

Cold ethanol wash

70% ethanol (keep at -20^{o}C)

Binding matrix (glassmilk)

5g silicon dioxide (0.5-1.0µm diameter; sigma cat # S5631)

50mL 3M Guanidine isothiocyanate

Preparation

Suspend 5g silica in 50mL water. Centrifuge at 2000 x g (approx. 2120 rpm) for 5 minutes. Discard supernatant. Resuspend in water to a volume of 50mL. Adjust pH below 7 using 2µl concentrated HCl (Silica should start to precipitate). Leave to sediment for 2 days and discard supernatant. Repeat sedimentation process twice. Centrifuge at 3000 x g

(approx. 3180 rpm) for 5 minutes and remove residual water with pipette. Resuspend silica pellet in 30mL of 3M guanidine isothiocyanate. Check pH is approximately 6-6.5.

2.3.1.7 Quantification of DNA

DNA was quantified by measuring absorbance at three wavelengths (260nm, 280 nm and 320 nm) using a UV-visible spectrophotometer (Shimadzu UV mini 1240, Kyoto Japan). Ideally, absorbance readings at 260nm ranged between 0.15 and 1.0. After measuring absorbance at wavelength 280 nm, the ratio (Absorbance260 nm/Absorbance280 nm) should fall between 1.6 and 2.0 for purity. Values outside this range were an indication of contaminates and high concentrations of protein in the extracted sample, both of which can influence the qRT-PCR outputs. Any contamination of particulate matter in the sample can be confirmed by the absorbance at 320nm.

For quantification, 40 µl of the extracted DNA and 160 µl of 10 mM Tris HCl, pH 8.5 was pipetted into a semi-micro quartz cuvette. The solutions were gently mixed through the pipette tip and then gently tapped on the bench top to ensure that no air bubbles were present in the sample which can influence absorbance readings.

2.3.1.8 Statistics

For each bacterial species cultured, a linear regression between the Coulter counts and turbidity (spectrophotometer reading at 600 nm) values for each dilution sample was fitted using the data analysis toolpak in Excel. If there was a strong relationship signified by a significance level less than 0.05 and an R^2 value close to 1, then this linear equation was used to determine the cells/mL for each bacterium in future cultures for DNA extraction**.**

Table 2.2 Linear regression relationship between Beckman Coulter counts for each bacterium, significance values (P value) and coefficients of determination (R^2)

Bacteria	P-value	R^2
Selenomonas ruminantium JW13	0.260	0.95
Prevotella ruminicola 23	0.103	0.99
Fibrobacter succinogenes S85	0.087	0.94
Streptococcus bovis Sb5	0.059	0.96
Lactobacillus acidophilus YE07	0.087	0.98

It was noted in the culturing of the bacteria *F.succinogenes,* a cocci shaped bacteria, that prolonged culture of the bacteria resulted in clumping which tended to block the aperture of the Coulter counter. Also the pure bacterial culture of *S. bovis* clumped during time of culture and therefore, culturing time was decreased as this seemed to reduce the incidence of clumping decreasing the potential for miscounting of the bacteria due to blocking of the aperture of the Coulter counter.

2.4 0ptimisation of Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) Assays

This section of the materials and methods outlines the importance of two stages in the development of the qRT-PCR reaction to quantify bacteria in the rumen samples taken from cattle and sheep under various feeding regimes. Firstly, the enumeration of bacterial populations was assessed and standards for bacterial quantification developed to be used in a qRT-PCR reaction. Secondly methods for the extraction of DNA were developed for these standards and rumen samples so that there was confidence that DNA extraction was optimised from these standards. This then allowed for quantification of rumen samples for this study. The qRT-PCR methodology was chosen for this study as it can be used to sensitively quantify absolute abundance of rumen microbes (Deng *et al.*, 2008) with reproducible results (Mackay, 2004; Reilly *et al.*, 2002). The 16S ribosomal gene is a

widely used targeted gene to quantify bacteria in the area of rumen microbiology (Denman and McSweeney, 2005) therefore primer design was based on this for the research project.

This chapter outlines the optimisation of qRT-PCR assay conditions to extrapolate known cells/mL from the standard to unknown bacterial populations in the rumen samples. In addition, this chapter outlines the procedures for evaluating some published primers published in the literature by Tajima *et al.* (2001) and new primers designed for this study to quantify targeted bacterial species in terms of cells/mL. Subsequently, these primers formed the basis for the optimisation of qRT-PCR assay conditions for temperature cycles, and primer concentrations and DNA concentrations, using a Corbett Rotorgene 3000. The development of standards was important to ensure that the bacteria cultured in this study were truly representative. Previous work by (Nadkarni *et al.*, 2002) showed that a DNA standard was important to ensure a more accurate determination of the total bacterial load due to variations in the 16S rDNA copy number with the ability to allow calculation of the amount of template present in the sample (Mackay, 2004).

Quantitative analysis was performed using a Corbett Rotorgene 3000 (now owned by Qiagen), Concorde NSW, Australia to quantify the relative abundance of the 16S rRNA genes of *Prevotella ruminicola, Fibrobacter succinogenes, Selenomonas ruminantium, Streptococcus bovis* and *Lactobacillus spp.* (not analysed in all studies) and the total bacterial populations using primers outlined in Table 4.1. The quantification of DNA from each sample was performed using the Qiagen Quantitect™ SYBR® Green PCR kit (Cat no 1018379). The standards and samples were assayed in 25 µl reactions with 12.5µl of master mix and the remainder ultrapure water and primers and 1µl of DNA template.

Equipment

- Eppendorff Multicycler, Hamburg Germany

- Corbett Rotorgene 3000 – 72 well system, Corbett Rotorgene (now owned by Qiagen), Concorde NSW
- Eppendorff Automated pipette, Hamburg Germany.

Consumables

- Qiagen HotStarTaq master mix kit (cat no 1017657)
- Qiagen Quantitect™ SYBR® Green PCR kit (Cat no 1018379)
- Proligo primers desalted from Sigma (all diluted to 200 pica mole concentration)
- 200µl tubes PCR flat cap thin walled fisher biotech (Part # 321-02-051)
- 200µl tubes dome capped PCR tubes (Part # 3211-00)
- 100µl tubes Corbett research 0.1mL tubes and caps (Part # 3001-002)
- 0.5-10 µl filter pipette tips maxymum recovery™ axygen (Part # 302-06-151)
- 300µl filtered PCR clean pipette tips (Eppendorf TIPS Filter 20-300µl Part #0030077083)
- Promega 100bp ladder (Part #G210A), Madison USA
- Promega 6x loading dye (blue/orange), (Cat # G1881), Madison USA.

2.4.1.1 Primer design

Primers were designed using public domain software programs to target specific bacterial species. All primer sequences were based on the 16S rRNA gene region of the rumen bacterial DNA as they had the largest number of sequences for the rumen populations. These 16s regions were searched through the GenBank (www.ncbi.nlm.nih.gov/genbank), to determine what sequences that were available for the 16S rRNA gene regions of the specific bacteria being quantified. These sequences are then placed into a text file for further primer design. Different strains were aligned and assessed using the Clustal program (Clustal W (1.4) big'n'Fat copy 1) [which is available

only on Mac computer systems] to determine the potential for cross reaction with the bacteria under investigation and any other spurious cross-reactions. This process allowed resolution of the regions which were similar between the different bacterial 16S rRNA gene regions and consequently where a primer alignment may occur. Therefore, to select a region that was unique to a particular bacterium, the BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi) program was searched to determine if alignment related to other bacteria or if it was specific to that bacterial species based on the sequences in the database. To test the bacterial primers the program, Amplify 3 version 3.1.4 (http://engel.genetics.wisc.edu/amplify) was used. Amplify 3 simulated the PCR reaction and tested the bacterial primer and determined if the chosen primer regions would anneal to the desired 16s rRNA genes region. In addition, Amplify 3 assessed the extent of any cross reacting between any of the bacteria as well as primer dimmers that may have occurred in the reaction of the bacterial DNA being quantified. Using this sequence of software assessment, primers were tested on all pure cultures to ensure that there was no cross reactivity when quantifying a particular bacterium.

Three published primers (Denman and McSweeney, 2006; Tajima *et al.*, 2001) were used as outlined in the results section (Table 4.1). The primers in Table 4.1 were the final primers used as part of this study, but numerous other primers were extensively tested but not utilised for the final analysis of the samples in this study.

2.4.1.2 Standards for qRT-PCR reaction

Standard cultures for DNA extraction and primers were set up for each bacterium, with *S. ruminantium* also used as a standard for the total bacterial primer. Each bacterial culture was quantified on a cells/mL basis and the DNA extracted as outlined in chapter 3. The standard DNA solutions for each culture were diluted as follows (1:10; 1:100; 1:1000; 1:10000; 1:100000; 1:1000000) for incorporation into the qRT-PCR reaction.

2.4.1.3 Development and validation of qRT-PCR reaction

Contamination from spurious DNA was the main concern in establishing valid PCR analysis. The primary requirement for setting up a qRT-PCR reaction was a Polymerase Chain Reaction (PCR) clean area in the laboratory, used only for PCR analysis. The surrounding workbench area was cleaned with 80% ethanol and disposable laboratory matting was placed on the bench. This matting was removed and disposed of after each PCR reaction setup. A seventy-two well holder for 0.1µl tubes was placed under UV light in the laminar flow for five minutes prior to setting up the PCR reactions to reduce the chance of cross-contamination. New latex gloves were used for each reaction setup. The master mix was made up of all solutions as outlined in table 4.3, minus the DNA content of the reaction. Twenty-four µl of the master mix was placed into each of the seventy-two, 100µl tubes. DNA was stored at -20^{o}C, thawed and added to the reaction. Since the quantity of DNA added was only small (1 µl), the pipette tip was placed in the master mix and the mix was taken up and down through the pipette tube. The pipette tip was then touched on the side of the 100µl tube to ensure that all reaction reagents are left in the tube. All solutions are also mixed by the spinning motion of the Corbett Rotorgene 3000.Each analysis consisted of a negative control, 6 standards also used as positive controls and triplicates of each experimental sample.

2.4.1.4 Optimisation of reaction conditions

The optimisation of qRT-PCR reaction conditions included the determination of the temperatures for dissociation, annealing and product extension and primer concentration. The aim was to optimise conditions for qRT-PCR to obtain a reaction efficiency as close to a value of 1 as possible (Corbett-Research, 2004). A value of one indicated that the reaction was working efficiently and that the conditions including temperatures, primer

concentration and DNA concentration were at optimum for the reaction. Reactions with efficiency of less than 0.98 were excluded and reanalysed.

2.4.1.4.1 Annealing temperatures

Primers were tested firstly using an Eppendorff multicycler looking at annealing temperatures that ranged 10^{o}C either side of the T_m (melting point) for the respective primers. The PCR products were then run on 2% agrose, using the Qiagen HotStarTaq master mix kit (cat no. 1017657), as outlined in the chapter 2.10, general materials and methods. The agarose gels were then photographed under ultra violet to determine which of the regions was producing the highest fluorescence, indicative of the region with the greatest amount of amplified DNA being present in that well. Each specific region corresponded to a temperature on the multicycler. These amplifying temperatures were then used in starting to optimise the RT-PCR reactions.

2.4.1.4.2 Melt curves/Dissociation curves

Melt curves or dissociation curves were generated at 0.1 ^{o}C increments to 95 ^{o}C to determine if there were any cross-reactions or different-sized PCR products (Corbett-Research, 2004) being produced using the targeted primers.

2.4.1.4.3 Primer concentration

Various primer concentrations were tested in optimisation reactions evaluated through the reaction efficiency (i.e. close to one and greater than 0.98) on the Corbett Rotor gene 3000. All of the primers were diluted in a laminar flow to 200 pica moles concentration before being put in the reactions at the appropriate concentrations.

2.4.1.5 Determination of cells/mL in experimental samples

A set of standard dilutions, quantified as cells/mL were programmed into the settings on the Corbett Rotorgene 3000 (Corbett-Research, 2004). From this a standard curve was generated requirement of an R^2 value close to one and reaction efficiency of great than 0.98. The standard curve allowed the Corbett Rotorgene program to extrapolate the concentrations in cells/mL in the rumen samples during the qRT-PCR reaction.

Table 2.2 Forward and reverse primers developed and utilised during qRT-PCR for analysis of rumen samples.

Bacteria targeted primer	**Forward**	**Reverse**	**Amplicon Size**	**Source**
Prevotella ruminicola	GGTTATCTTGAGTGAGTT*	GGCCGCTCACAGTATATCG	211	*(Tajima *et al.*, 2001)
Lactobacillus spp.	CGTTCCCTTCGGGGAC	CACCTTCCTCCGGTTTGTCA	162	This study
Fibrobacter succinogenes	GTTCGGAATTACTGGGCGTAAA	CGCCTGCCCCTGAACTATC	121	This study
Streptococcus bovis	CTAGCGGGGGATAACTATTGG	GTGCACTTTCCACTCTCTCACAC	345	This study
Selenomonas ruminantium	CGTGATGGGATTGAAACTGTC	CTCCGGCACAGAAGGGGTCG	236	This study
Total Bacterial primer	CGGCAACGAGCGCAACCC#	CCATTGTAGCACGTGTGTAGCC#	Approx 145	#(Denman and McSweeney, 2006)

(*# indicates published primers)

The reaction conditions that allowed for improved reaction optimisation, when analysed using a Corbett Rotorgene 3000, are shown in Table 4.2.

Table 2.3 Optimised reaction conditions for primers developed (Table 4.1) using a Corbett Rotorgene 3000.

Bacteria	**Initial**	**Number**	**Annealing and product**	**Final**	**End of**

targeted primer	denaturation	of cycles	elongation				reaction
Prevotella Ruminicola	95°C 15 min	45	95°C 15 sec	53°C 30 sec	72°C 30 sec	72°C for 10 min	14°C α
Lactobacillus spp.	95°C 15 min	45	95°C 15 sec	62°C 15 sec	72°C 15 sec	72°C for 10 min	14 °C α
Fibrobacter succinogenes	95°C 15 min	45	95°C 15 sec	62°C 60 sec	n/a	72°C for 10 min	14 °C α
Streptococcus bovis	95°C 15 min	45	94°C 15 sec	54°C 60 sec	n/a	72°C for 10 min	14 °C α
Selenomonas ruminantium	95°C 15 min	40	94°C 15 sec	52°C 30 sec	72°C 30 sec	72°C for 10 min	14 °C α
Total Bacterial primer	95°C 15 min	45	95°C 15 sec	60°C 60 sec	n/a	72°C for 10 min	14 °C α

The concentrations of primer that consistently resulted in reaction efficiencies nearest to one are shown in Table 2.3.

Table 2.4 Optimised concentrations of SYBR® Green PCR solution, primers, ultrapure water and DNA for RT-PCR reactions outlined in table 2.2 and 2.3 using a Corbett Rotorgene 3000.

	Prevotella ruminicola	*Lactobacillus spp.*	*Fibrobacter succinogenes*	*Streptococcus bovis*	Total Bacterial	*Selenomonas ruminantium*
Quantitec™ SYBR® Green PCR solution (µl)	12.5	12.5	12.5	12.5	12.5	12.5
Forward primer (µl)	1.0	1.3	0.75	0.8	0.4	0.75
Reverse primer(µl)	1.0	1.3	0.75	0.8	0.4	0.75
Ultrapure water(µl)	9.5	8.9	10.0	9.9	10.7	10
DNA(µl)	1	1	1	1	1	1
Total	25	25	25	25	25	25

3

Changes in rumen parameters of cattle under commercial feedlot conditions during introduction to grain based diets.

3.1 Introduction

Introduction of grain-based diets during the first phase of dietary transition in feedlots can be problematic for cattle producers due to the incidence of ruminal acidosis and subclinical acidosis, usually associated with the unadapted consumption of a large amount of readily fermentable carbohydrates in the grain component of the ration. Most of the previous research on the introduction of grain-based diets to cattle and sheep focused on the induction of ruminal acidosis under experimental conditions (Elam, 1976; Godfrey *et al.*, 1994; Godfrey *et al.*, 1995; Rowe *et al.*, 1999; Al Jassim and Rowe, 1999; Horn *et al.*, 1979; Slyter, 1976). Nevertheless, extension materials developed by veterinarians, consultants, feed companies and government agriculture departments have used this experimental approach as the basis to provide practical information for producers about the likely incidence and progression of acidosis and possible feeding management to prevent or reduce the incidence of acidosis (Knee, 2006; Walker, 2006; Schwartzkopf-Genswein *et al.*, 2003). Another consequence of the experimental approach was the recommendation of inclusion of antibiotic feed additives such as the virginiamycin and ionophores such as monensin, lasalocid, and narasin in grain-based diets for feedlot cattle. Moreover, Lean *et al* (2007), using a combination of these studies of experimentally-induced acidosis and observations of cattle in feedlots, recommended the following feeding and management strategies for prevention or reduction of acidosis under commercial conditions:

- Cattle should have access to hay on arrival to feedlot before induction
- Inclusion of ionophores or virginiamycin at the recommended dose
- The starter rations should not include more than 50% grain

- Cattle should be adapted to the diet slowly with changes grain concentration implemented gradually
- Avoid fluctuating feed intake
- Ensure rations are consistently mixed
- Monitor the level of fines in the diet
- Ensure there is enough roughage of sufficient chop length (mean 5-10cm long).

More recently, Nagaraja and Nagamine (2007) have contrasted the amount of research concentrating mainly on experimentally-induced acidosis with the lack of corresponding studies to evaluate the strategies and consequences of grain-feeding under commercial feedlot conditions.

Consequently, this study examined rumen function and physiology in cattle during the introduction of grain-based diets at two commercial feedlots that used two quite different feeding management strategies. The first feedlot was located in a mixed farming region near Donnybrook in the South West of WA. This feedlot fed grain and roughage separately and did not use antibiotics such as virginiamycin. The second feedlot, located in the wheatbelt of WA, fed grain and roughage as a total mixed ration with incorporation of virginiamycin from introduction until day eleven of feeding. While the feedlots had distinct differences in feeding strategies, we also aimed to monitor any incidence of ruminal acidosis, and other rumen and faecal parameters such as rumen pH, D- and L-lactic acid, volatile fatty acids, and faecal scoring under these commercial conditions. Another point of difference with previous studies was the use of RT-PCR to monitor changes in the molecular ecology of the rumen bacterial populations during introduction to grain diets under commercial conditions.

The following parameters and observations assessed during this study were:

1. Monitor changes in rumen ecology and metabolism in cattle fed under commercial feedlot conditions were hay and grain were fed separately in one feedlot and another fed as a TMR in another feedlot.
2. The addition of any feed additive such antibiotics or ionophores will reduce the incidence of acidosis through the bacterial ecology established in the rumen during any grain introduction.
3. Fibre utilising rumen bacteria (*Fibrobacter succinogenes*) populations will decrease during grain feeding or any associated reduction in rumen pH.
4. Lactic acid utilising rumen bacteria (*Selenomonas ruminantium*) populations will increase with an increase in the dietary grain component.
5. *Prevotella ruminicola* will be the most prevalent bacteria in the rumen during dietary adaptation.
6. *Streptococcus bovis* will increase significantly and possibly pathologically, during introduction to grain-based diets.
7. If increases in *S bovis* are linked with a decrease in ruminal pH.
8. Metabolic changes in the rumen can be related to changes in the molecular ecology during dietary transitions in cattle and sheep.

3.2 **Materials and Methods**

3.2.1 **Feedlot one**

Feedlot one was a property operated by Mr John Fry in the Donnybrook region of Western Australia (389111.06E; 6275931.14N). Cattle were monitored in the feedlot from early December, which utilised a feed management regime of grain and roughage fed separately. The first group of cattle were purchased at saleyards, so no previous background information on dietary history was available. Another group of cattle were also

introduced into the feedlot herd throughout the sampling period from other properties commissioned to 'background' the cattle i.e. establish a consistent, low rate of growth, usually about 0.6 kg LW per day. Therefore the randomly sampled cattle being monitored were at different stages of introduction right from the first sampling. No virginiamycin or other feed additives was incorporated into the dietary regime in this feedlot.

3.2.1.1 Sampling of cattle

Eight animals were initially selected at random for continuous monitoring within the herd. Another eight animals were randomly selected sampling day to assess any impact on the rumen population of the rumen sampling technique itself. Rumen and faecal samples were collected from each of these sixteen animals on days 0, 2, 7, 14, 21 and 50 from day of introduction to their diets by methods outlined in chapter 2.3. Cattle were not kept off food or water prior to sampling.

3.2.1.2 Feeding regime

Cattle on the Fry property were fed once daily on a diet consisting of pasture hay (fed as hay bales in a bunker) and a grain mixture fed separately in self-feeders. The diet aimed to supply 32% roughage throughout the feeding period with a variation in the grain mixture as outlined in table 3.1 with a reduction of oats over time and increasing barley, while lupins were kept constant at 10% of the ration (Table 3.1).

Table 3.1 Changes in the grain component of a mixed grain (68%) and pasture hay (32%) ration fed separately but *ad libitum* without incorporation of virginiamycin near Donnybrook.

Day from introduction	**Grain Type**	**Percentage of ration**
0-4	Lupins	10
	Barley	20
	Oats	70
4-7	Lupins	10
	Barley	40
	Oats	50
7 onwards	Lupins	10
	Barley	75
	Oats	15

The pasture hay was 61.8% dry matter digestibility with 8.9 MJ ME/kg DM and 7.8% crude protein while the final grain component of the ration was 80.8% dry matter digestibility with 11.7 MJ ME/kg DM and 16.0 % crude protein.

3.2.2 **Feedlot Two**

Feedlot two was a property near Yealering (559613.84 E; 6405783.83 N), operated by Alan and Kelly Manton. The cattle were placed into the feedlot in March after being backgrounded on tagasaste (*Chamaecytisus palmensis*) and stubbles. Wheaten straw (chopped to approximately 10 cm in length) and grain was milled in a Renn roller and mixed in a Supreme 400 tub grinder with the inclusion of virginiamycin as Eskalin^{TM} marketed by Pfizer animal health at the recommended dose of 0.1% of the total mixed ration for eleven days from introduction. The resultant total mixed ration was then offered in feed troughs.

3.2.3 **Sampling of cattle**

Samples of rumen fluid and faecal matter were collected from two groups of eight animals each. One group was sampled for the duration of the study while another set of samples was taken from eight steers randomly selected from the herd at each sampling. Sampling of cattle selected randomly at each collection was undertaken to determine if sampling itself impacted on the rumen microbial population.

Cattle were sampled after feeding from approximately 8 am on the sampling days. Cattle were brought straight into the yards with no time off feed or water. Sampling methodology is outlined in the general materials and methods section (2.4.2)

3.2.3.1 Feeding regime

Cattle were fed a total mixed ration made daily on farm, based on the rations below at approximately 6-7am each morning (Table 3.2).

Table 3.2 Composition of a total mixed grain based ration fed *ad libitum* with virginiamycin in Yealering feedlot.

Day	**Lupins (%)**	**Barley (%)**	**Wheat Straw (%)**	**Lime (%)**	**Minerals (%)**	**Virginiamycin included**
1-14	12.3	30	56.6	1	0.1	Yes#
14-28	15	37.6	46.3	1	0.1	No
28-32	15	45	39	1	0.1	No
52-54	16	51	32	1	0.1	No

Virginiamycin was fed until day 11.

The dietary components feed quality are hay at 47.3% dry matter digestibility and 6.5 MJ ME/kg DM and 3.3% crude protein, lupins were 91.4% dry matter digestibility, 13.4 MJ ME/kg DM and 35.1% crude protein. While the barley grain component of the

ration was 80.7% dry matter digestibility (DMD) with 11.7 MJ ME/kg DM and 10.8% crude protein.

3.2.4 **Statistics**

All data from this study, except pH values and faecal scores, displayed log-normal distributions and were log-transformed (log_{10}) prior to statistical analysis, with total bacterial population log-transformed to log_{100}. A linear mixed model which included a fixed effect comparing the randomly and continuously sampled groups, a fixed effect for sample date and an interaction between sample type and date was fitted to each variate using the REML procedure in GenStat (edition 14). The model also included an autoregressive covariance structure between sample dates. All fixed effects were tested using F-statistics or Wald statistics. If there was no significant difference between sample types ($P<0.05$) all samples were used to calculate averages at each sample date which were compared using 5% least significant differences (5% LSD).

Correlations between variates were compared to zero using a two-sided test. The matrix of correlations between logarithms of the counts of individual bacteria was used to construct a biplot which showed the relationships between bacterial counts and how sample counts varied across sample dates.

3.3 **Results**

The results are separated into rumen parameters including measurements that were traditionally used for estimates of rumen acidosis: rumen pH, rumen D- and L-lactate and volatile fatty acid concentrations, faecal measurements and then bacterial population changes using qRT-PCR.

3.3.1 **Feedlot One**

Feedlot one which fed the two component of the diet separately had no growth rates available during the sampling period.

3.3.1.1 Rumen parameters

Rumen pH (Table 3.3) decreased from 7.1 to 6.7 (LSD 5%) from day 0 to day 3 for the continuously sampled group, returning to levels similar to day 0 on day 21 (Table 3.3). Only one steer had a rumen pH below six for two sampling periods (days 14 and 21). There were significant correlations between rumen pH and the rumen concentrations of L-lactate (R=-0.49) (Figure 3.1) and acetic acid (R=-0.54) (Figure 3.1) ($P<0.05$) during the sampling period.

Table 3.3 Rumen pH (±SEM) of steers (n=8) in feedlot 1 with hay and grain fed separately. Means (±SEM) with the same superscript are not significantly different ($P<0.05$) whereas values with different super scripts are significantly different ($P>0.05$).

Day of sampling	**0**	**3**	**8**	**14**	**21**	**50**
rumen pH	7.08^{a} (0.13)	6.70^{b} (0.15)	6.69^{b} (0.11)	6.61^{b} (0.18)	6.84^{ab} (0.16)	6.73^{ab} (0.10)
field faecal pH	6.00^{a} (0.00)	6.77^{b} (0.11)	6.85^{b} (0.10)	6.78^{b} (0.12)	6.81^{b} (0.13)	5.88^{a} (0.17)
post ferm faecal pH	4.98^{a} (0.14)	4.81^{ab} (0.17)	4.52^{bc} (0.11)	4.77^{abc} (0.13)	4.93^{abc} (0.10)	4.65^{bc} (0.11)

The faecal pH taken in the field increased from pH 6.0 to 6.8 with a steady decline until day 54, decreasing to pH 5.9. There was a significant correlation between the field faecal pH and total bacterial counts, *P. ruminicola* populations and acetic acid concentrations. The faeces were further fermented (Table 3.3) with glucose as outlined in chapter 2.2.4 to determine the ability of the food source to cause acidosis. There was a significant decrease in faceal pH post-fermentation from a mean pH of 4.9 at day 0 to 4.5 on day 8 (LSD 5%). There was then a significant increase to post-fermentative faecal pH 4.9 by day 21 (similar to day 0). There was no significant correlation between post fermentative pH and any of their other rumen parameters measured.

Rumen L-lactate concentrations (Figure 3.1) remained low throughout the introduction period while steers were sampled. However, there was a significant difference in L-lactate concentration between days 3 and 7 over the sampling period (LSD 5%). The L-lactate concentrations were variable as shown by the large SEM for concentrations on day 14 and 21. There were correlations between rumen L-lactate concentrations (Figure 3.1) and valeric acid (R=-0.34) (Figure 3.5) concentrations and rumen pH (R=-0.48) ($P<0.05$).

Rumen D-lactate concentrations decreased from day 0 (LSD 5%) to 7 of sampling. There was a correlation between D-lactate and propionic acid concentrations (R=0.42) ($P<0.05$) and *S. bovis* populations (R=0.30) ($P<0.05$) during the sampling period.

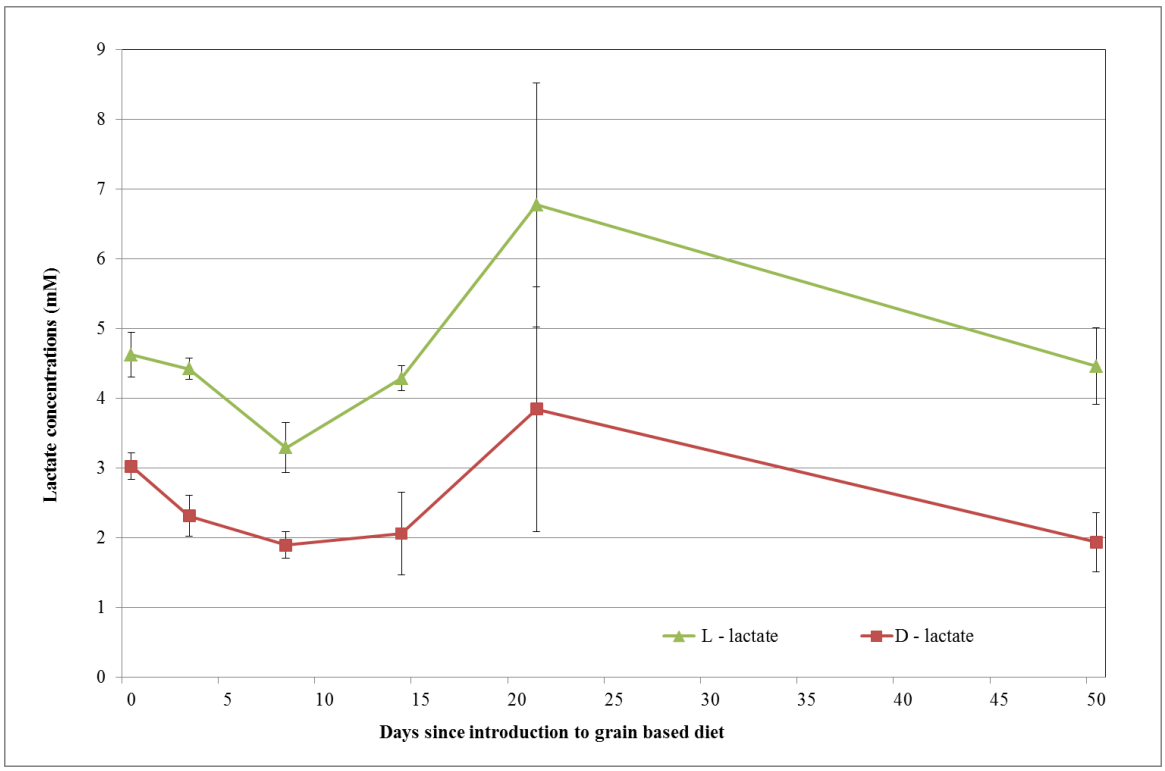


Figure 3.1 Rumen D and L-lactate concentrations (mean mM ± SEM) of steers (n=8) in cattle in feedlot 1with hay and grain fed separately with hay and grain fed separately.

Rumen total volatile fatty acid (VFA) concentrations increased from 76 mM to 109 mM by day 3 (LSD 5%), then remained significantly high over the remainder of the sampling period (Figure 3.3). Total VFA concentrations were correlated to the *F. succinogenes* (R=37) ($P<0.05$) populations over the introduction period.

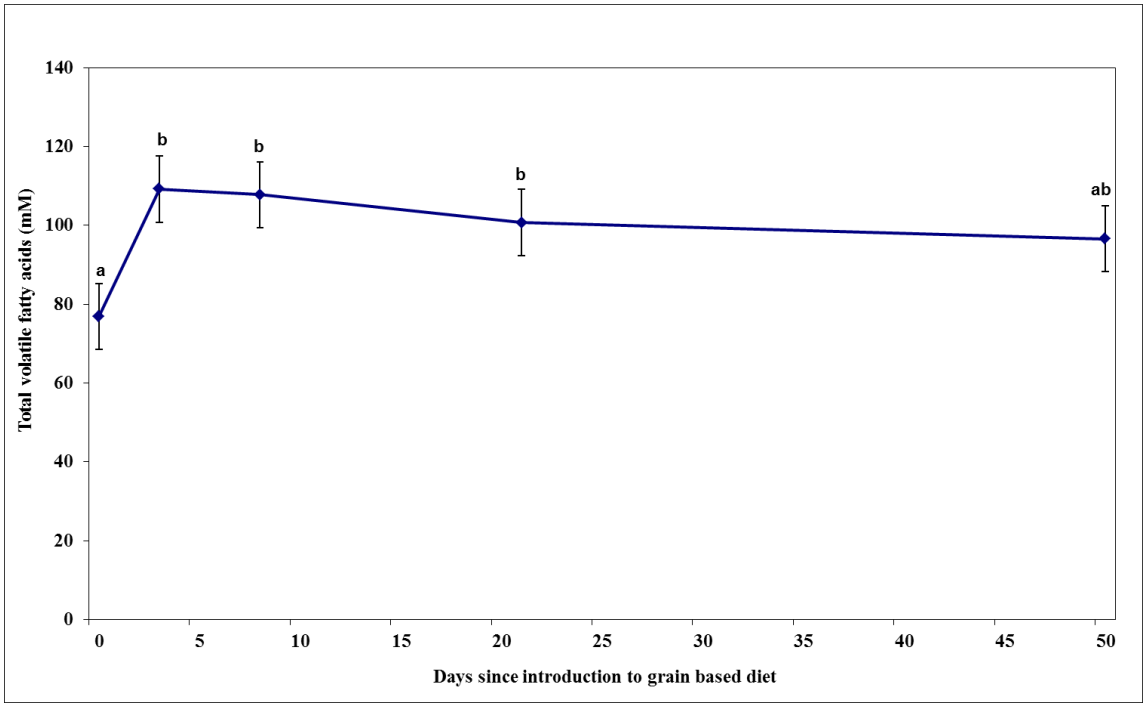


Figure 3.2 Rumen volatile fatty acid concentrations (mean ±SEM) in rumen of steers (n=8) on feedlot 1 with hay and grain fed separately hay and grain fed separately. Means ±SEM with the same superscript are not significantly different (P<0.05) whereas values with different superscripts are significantly different (P>0.05).

Acetic acid concentrations in the rumen also followed a similar trend to total VFA concentrations (Figure 3.4), increasing significantly from 46 mM to 66 mM over the three sampling days (LSD 5%). There was a correlation between acetic acid concentrations and the rumen populations of *F. succinogenes* (R= 0.41), *P. ruminicola* (R=0.37), the total bacterial populations (R=0.36) and rumen pH (R=-0.54) (P<0.05) over the sampling period.

Propionic acid concentrations in the rumen also increased from 18 mM (day 0) to 22 mM (day 3) (Figure 3.3). Propionic acid concentrations were correlated with acetic acid concentrations (R=0.66) and *F. succinogenes* populations (R=0.36) over the sampling period (P<0.05).

Butyric acid concentrations in the rumen increased significantly (LSD 5%) from days 0 to 3 (9 mM to 15.5 mM) (Figure 3.3). Butyric acid concentrations were correlated with acetic acid (R=0.64), caproic acids (R=0.47), iso-butyric acid (R=0.48) and valeric acid concentrations (R=0.73) over the sampling period.

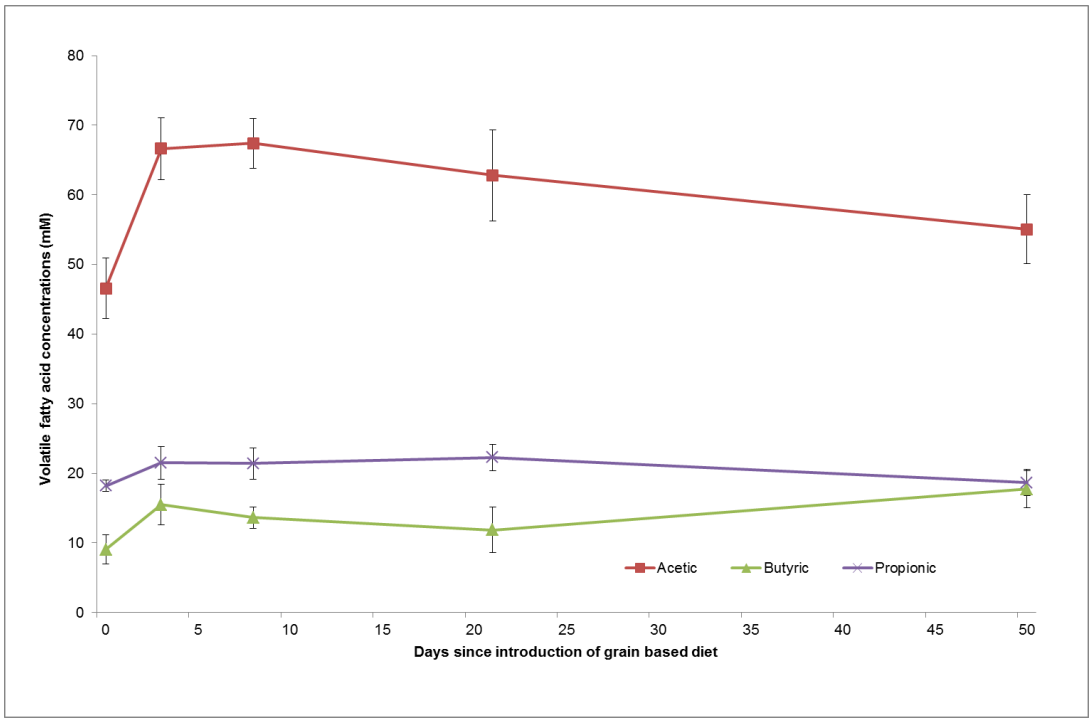


Figure 3.3 Rumen acetic, propionic and butyric acid (mM mean±SEM) concentration (n=8) in steers from feedlot 1 with hay and grain fed separately.

Iso-butyric acid concentrations decreased from day 0 to 3 (LSD 5%), increasing from day 3-8 and returning to concentrations similar to day 0 at days 21. (Figure 3.5). Iso-butyric concentrations were correlated with acetic acid (R=0.51), butyric acid (R=0.48) and total volatile acid concentrations (R=0.48) over the sampling period ($P<0.05$).

Iso-valeric acid concentrations at day 0 were different from day 3 over the sampling period (Figure 3.5). Iso-valeric acid concentrations at day 3 were higher than those taken at day 21 and 50 (LSD 5%). Iso-valeric acid concentrations were not

significant correlated to other rumen parameters during the period of sampling in this commercial feedlot (P>0.05).

Valeric acid concentrations were greater than day 0 (LSD 5%) for the remainder of the sampling period with day 50 being higher than day 0, 3 and 21 (Figure 3.4). Valeric acid concentrations were correlated with acetic (R=0.53), butyric (R=0.73) and caproic acids (R=0.80) concentrations as well as the populations of the lactic acid utiliser *S. ruminantium* (R=-0.36) during the grain feeding period (P<0.05).

Caproic acid concentrations (Figure 3.4) at day 0 were lower than day 8, 21 and 50. Caproic acid concentrations on day 50 were higher than on all other sampling days (LSD 5%) (Figure 3.4). Caproic acid was correlated with butyric (R=0.47), valeric (R=0.80) and L-lactate concentrations (R=0.46) during the grain introduction period (P<0.05).

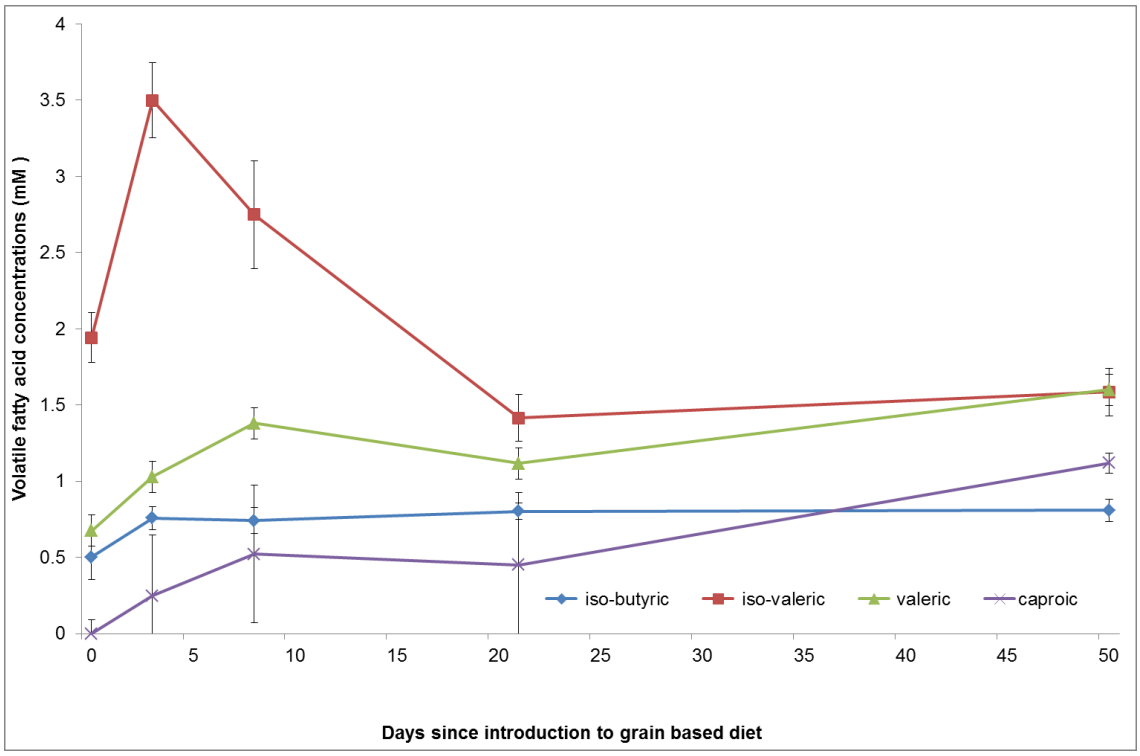


Figure 3.4 Iso-butyric, iso-valeric, valeric and caproic acids (mean±SEM) of steers (n=8) taken at approximately 8am, 1-2 hours post feeding during dietary transition over 54 days on feedlot 1 with hay and grain fed separately.

The rumen ammonia concentrations (Figure 3.5) were constant from day 0 to 3 then declined to their lowest level on day 21 (0.8mM) ($P<0.05$) There was a correlation between rumen ammonia concentrations and *S. ruminantium* (R=0.50) and *P. ruminicola* (R=0.46) numbers ($P<0.05$) during the sampling period.

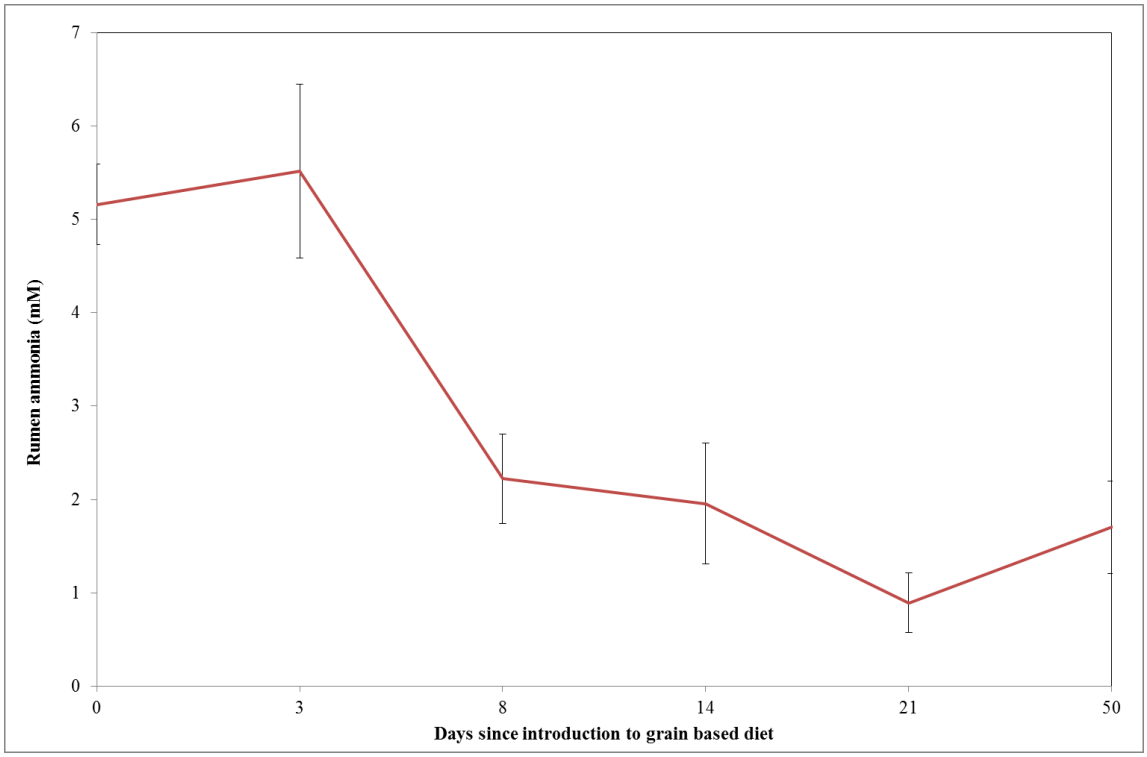


Figure 3.5 Rumen ammonia (mean±SEM) of steers (n=8) from feedlot 1 with hay and grain fed separately. Means ± (SEM) with the same superscript are not significantly different ($P<0.05$) whereas values with different super scripts are significantly different ($P>0.05$).

3.3.1.2 Faecal parameters

The faecal scores (Figure 3.6) for the continuously sampled group showed that on entry the steers had soft faeces that were holding shape, with a slight increase in faecal

score by day 3. However, by day 8 and 14 faeces were firmer and taking shape with lower faecal scores than previous samplings (P<0.05) The faeces continued to have reasonable cowpat formation on the ground with some indication of grain in the faeces at day 21 but by day 54 there was more indication of firm cowpat formation. There were correlations between faecal scores and *S. ruminantium* concentrations (R=0.36) (P<0.05).

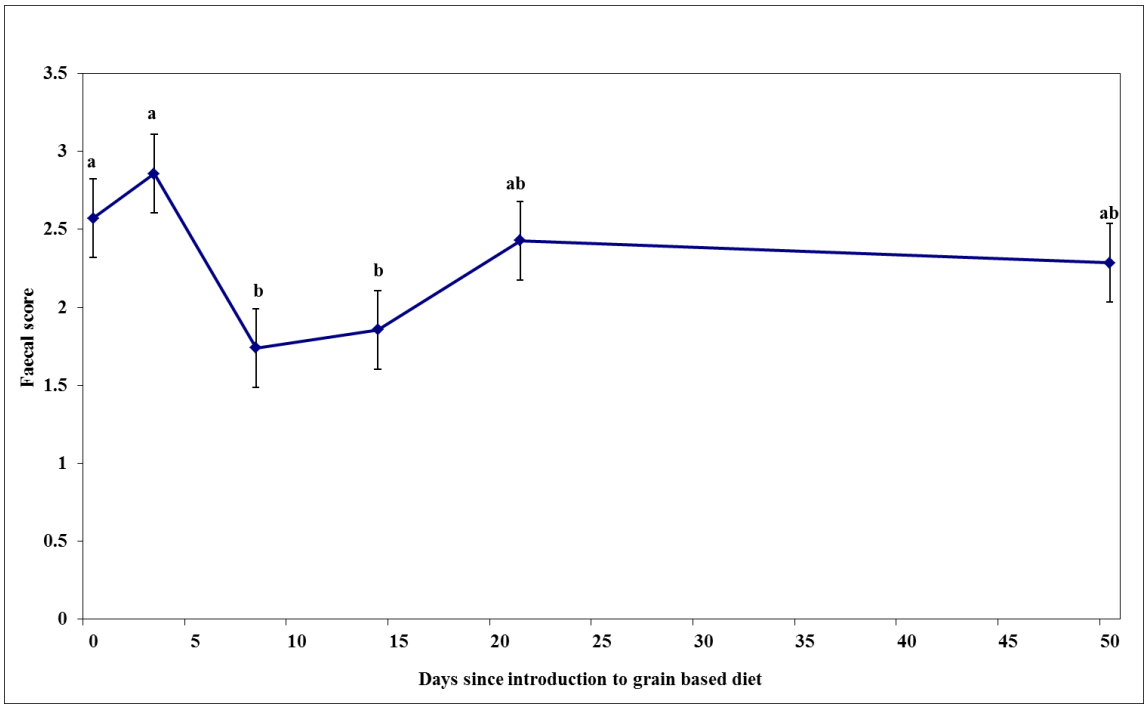


Figure 3.6 Faecal scores (mean±SEM) of steers (n=8) from feedlot 1. Means (±SEM) with the same superscript are not significantly different (P<0.05) whereas values with different super scripts are significantly different (P>0.05).

3.3.1.3 Rumen bacterial parameters

Total bacterial population

The average total bacterial population for the continuously sampled group increased 14.5 fold from days 0 (1.17×10^9 cells/mL) to 3 (2.89×10^{10} cells/mL), then decreased to the same level on day 8. There was a significant correlation between acetic

acid concentration (R=0.31) and *P. ruminicola* (R=0.67) (P<0.05) during the sampling period.

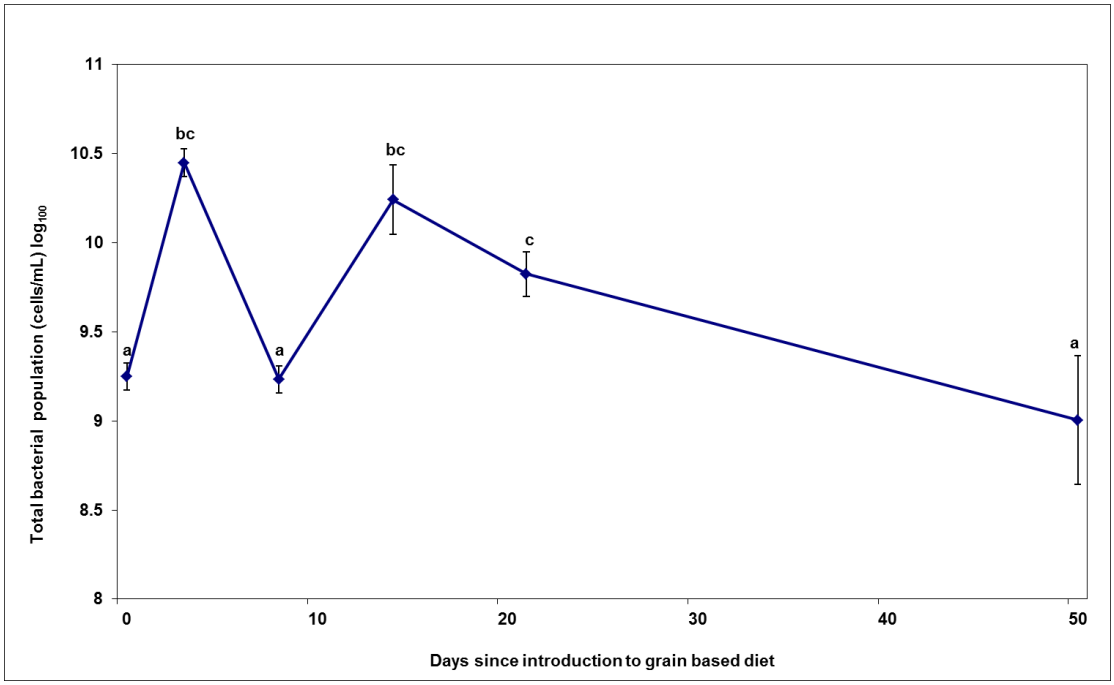


Figure 3.7 Total bacterial cells (cells/mL (log_{100}) (mean±SEM) for steers (n=8) from feedlot 1 with hay and grain fed separately with hay and grain fed separately. Means (±SEM) with the same superscript are not significantly different (P<0.05) whereas values with different super scripts are significantly different (P>0.05).

The average population of *F. succinogenes* (Figure 3.8) for the continuously sampled group decreased for days 0 (2.67 x 10^7cells/mL) to day 3 (9.84 x 10^5 cells/mL log_{10}) with a 16-fold increase at day 8 (2.38 x 10^6 cells/mL log_{10}), then a 23-fold increase by day 14. The population then decreased to near the same concentrations as day 3 and again for the last sampling at day 50. There were significant correlations between *F. succinogenes* and the measurements of total VFA (R=0.37) and acetic acid (R=0.41) (P<0.05) during the sampling period.

The *P. ruminicola* population (Figure 3.8) increased ($P<0.05$) fluctuated until (LSD 5%) by nearly 100 fold on days 0 (5.08×10^{8} cells/mL) to 3 (5.49×10^{10} cells/mL), and then decreased seven fold by day 8 back to day 0 values. The population then increased 67 fold from day 8 (7.03×10^{9} cells/mL) to 14 (4.76×10^{11} cells/mL) and equated to the same level as day 3, on day 21, remaining constant until day 50. There were significant correlations between *P. ruminicola* and total bacterial populations ($R=0.74$) as well as acetic acid ($R=0.37$) concentrations ($P<0.05$) during the sampling period.

The average population of *S. ruminantium* (Figure 3.8) lowered ($P<0.05$) (LSD 5%) from days 0 (7.01×10^{9} cells/mL) and 3 to day 8 eleven fold then increased to day 14. The population then decreased steadily over day 21 and 54 to the same level as day 8. There were significant correlations between *S. ruminantium* and valeric acid ($R=-0.37$) concentration ($P<0.05$) during the sampling period.

The average *S. bovis* population (Figure 3.8) increased steadily until day 14 (36.32×10^{6} cells/mL $\log_{10}$) which then decreased until day 54. There were significant correlations between *S. bovis* ($R=-0.39$) and rumen pH as well as D- lactate ($R= 0.44$) concentration ($P<0.05$).

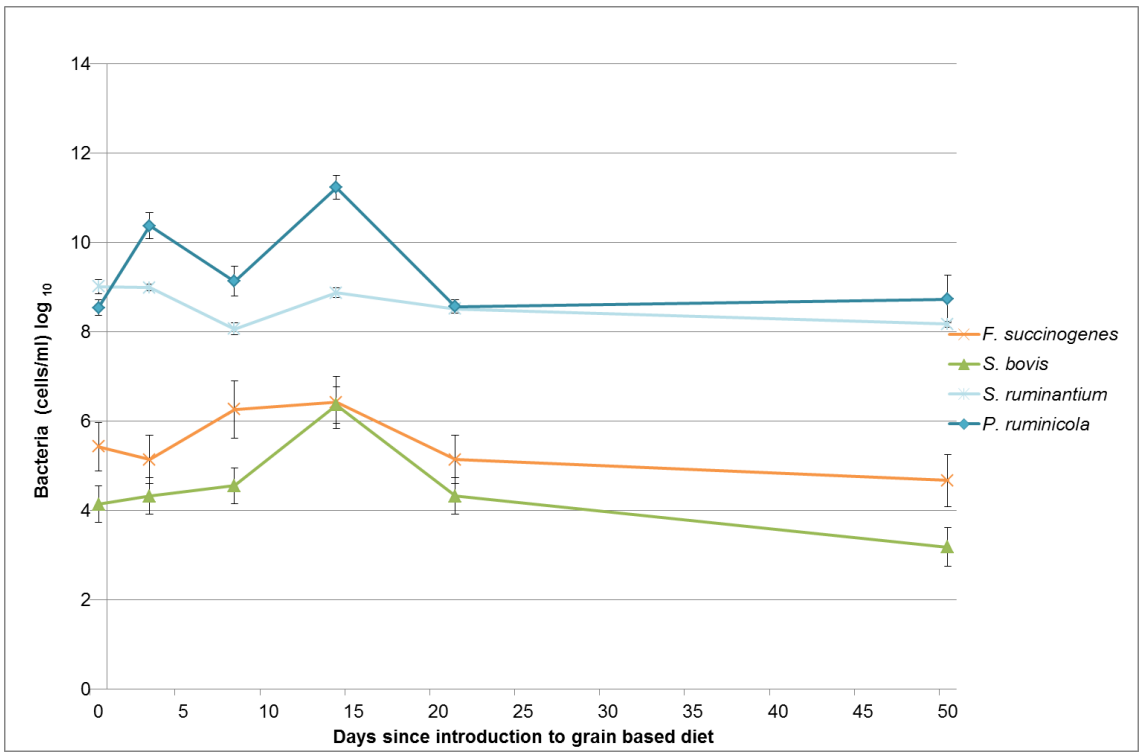


Figure 3.8 Changes in rumen populations of *F.succinogenes, P. ruminicola, S. ruminantium* and *S.bovis* cells/mL $\log_{10}$ (mean±SEM) of steers (n=8) from feedlot 1.

Protozoal counts were fairly constant (Figure 3.9) with a significant decrease at day 8 (LSD 5%), there were large SEM in the samples on day 21 and 50, there was no significant correlation between protozoa or other rumen parameters during sampling (P>0.05).

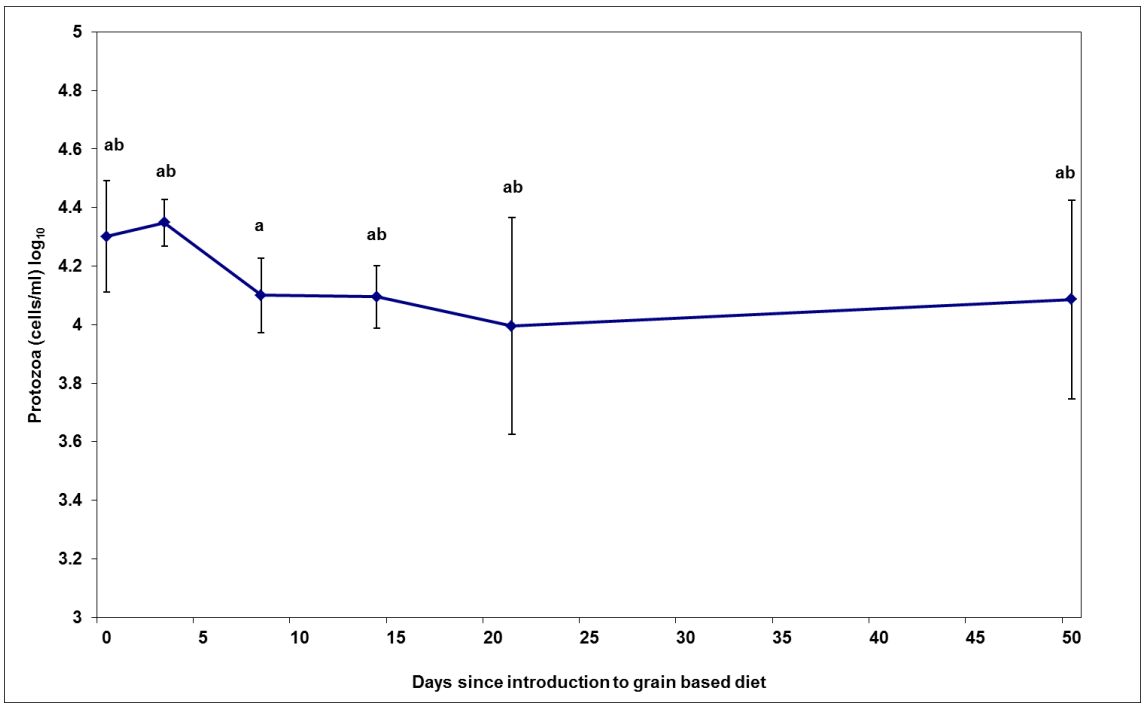


Figure 3.9 Protozoa populations (mean±SEM) of steers (n=8) from feedlot 1 with hay and grain fed separately. Means ±SEM with the same superscript are not significantly different ($P<0.05$) whereas values with different super scripts are significantly different ($P>0.05$).

Figure 3.10 is a bivariate plot that gives a graphical indication of the relationships between the bacterial populations over the sampling period, indicating that *P. ruminicola* and *S. bovis* were closely related to any changes in population numbers that occurred over the period of monitoring rumen microbial ecology while *F. succinogenes* and *S. ruminantium* were independent of other bacteria during the sampling period.

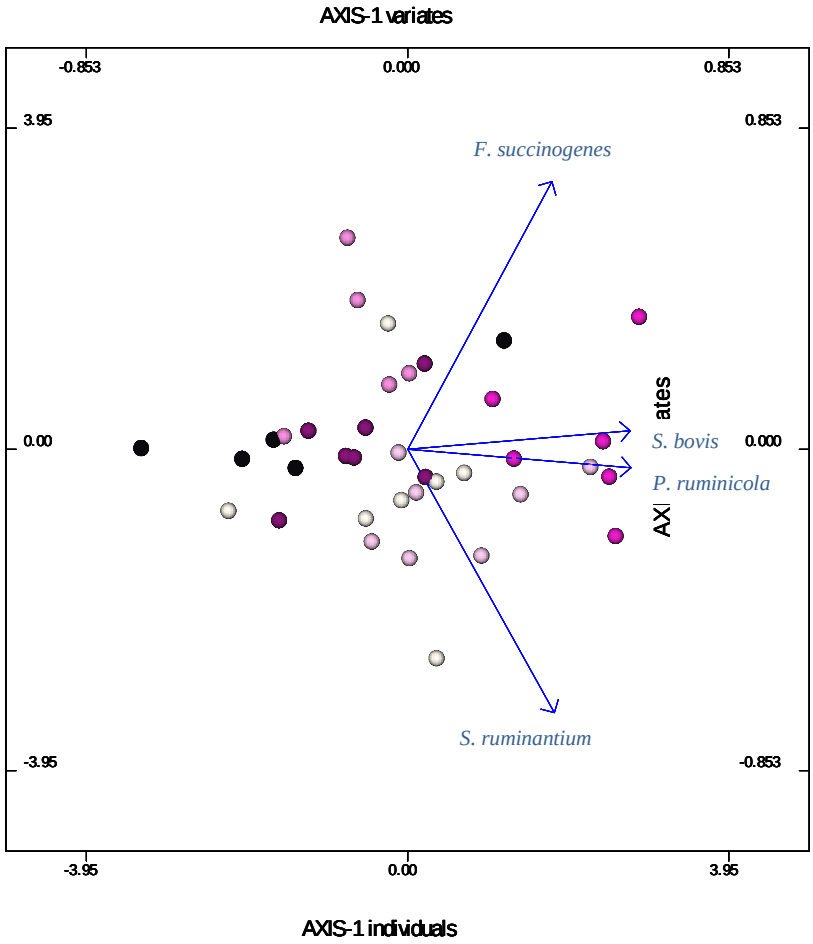


Figure 3.10 Bivariate plot based on the correlations between log bacterial counts for steers (n=8) from feedlot 1 with hay and grain fed separately. Day 0 (white), day 3 (pale pink), day 7 (pink), day 14 (medium pink), day 21 (deep pink) and day 54 (black).

3.3.2 **Feedlot Two**

There was no significant difference (P>0.05) between the continuously sampled and randomly sampled cattle being introduced onto the same feedlot diet, when analysed to determine the impact of the constantly sampled and randomly sampled group with regards to rumen parameters. Therefore, both the randomly sampled and continuously sampled values were combined as one group for analysis. Feedlot two had a growth rate of 2.14 kg/hd/day for cattle sampled throughout the 60-day period.

3.3.2.1 Rumen parameters

The rumen pH decreased between day 0 to 3 (LSD 5%) then decreased slightly ($P<0.05$) over the remaining 60 days (Table 3.4). Nevertheless, the mean rumen pH remained within the normal range over the period of the feedlot, with only one steer indicating a low pH of 5.3 on day 46. There were significant correlations between rumen pH and the following parameters: faecal field pH (R=0.72), ruminal ammonia (R=-0.6), ruminal L-lactate concentration (R=-0.56), rumen protozoa populations (R=0.38), total volatile fatty acid concentration (R=-0.57), concentration of the following VFAs; propionic (R=-0.64), butyric (R=-0.51), iso-butyric (R=-0.39) and iso-valeric (R=-0.37) and the ruminal populations of *F. succinogenes* (R=0.54) ($P>0.05$) during the grain introduction period.

Table 3.4 Rumen pH, field faecal pH and post fermentative faecal pH(mean±SEM) of steers (n=16) from feedlot 2, fed a total mixed ration with virginiamycin included in the ration until day 11. Means (±SEM) with the same superscript are not significantly different ($P<0.05$) whereas values with different super scripts are significantly different ($P>0.05$).

Day of sampling	**0**	**3**	**8**	**14**	**21**	**32**	**46**	**60**
rumen pH	6.95 (0.07)	6.75 (0.06)	6.62(0.12)	6.66 (0.05)	6.53 (0.07)	6.44 (0.07)	6.50 (0.12)	6.38 (0.09)
field faecal pH	6.94 (0.05)	6.97 (0.08)	6.58 (0.05)	6.79 (0.06)	6.56 (0.07)	6.60 (0.08)	6.59 (0.06)	6.56 (0.05)

post ferm faceal pH	4.89 (0.09)	5.59 (0.10)	5.14 (0.07)	5.21 (0.08)	5.20 (0.07)	4.93 (0.05)	5.21 (0.04)	5.35 (0.04)

The faecal pH taken did not change from day 0 to 3 (7.1) with a significant (LSD 5%) decrease in faecal pH at day 8 (6.5), increasing to 6.8 at day 14, then remaining relatively constant until day 60 with a very slow decline in pH. Faecal samples that were incubated with glucose as outlined in chapter 2.5.2 for 24 days showed a lower pH than the field pH (5.0) with a slight increase at day 3 (5.8) followed by a decrease (LSD 5%) to 5.3 at day 8 with a steady decline to 4.9 at day 32. The incubated faecal pH then increased from day 32, returning to 5.3 at day 60. There were correlations between post fermentative faecal pH and acetic acid ($R=-0.53$) ($P<0.05$) during the sampling period.

Rumen L-lactate concentrations increased from day 0 to 3 (LSD 5%) but then remained constant until the end of the feedlot period (Figure 3.11). importantly, all of the L-lactate concentrations were all within safe range during this time. There were correlations between L- lactate ($R=0.73$) and total VFA concentration, rumen pH ($R=-0.56$), valeric acid ($R=0.80$), propionic acid ($R=0.70$) as well as the populations of *F. succinogenes* ($R=-0.45$) and *S. ruminantium* ($R=0.46$) ($P<0.05$) during dietary transition of 60 days.

Rumen D-lactate concentrations remained constant through sampling (Figure 5.11). Measurements on day 32 and 60 were not available due to a laboratory error.

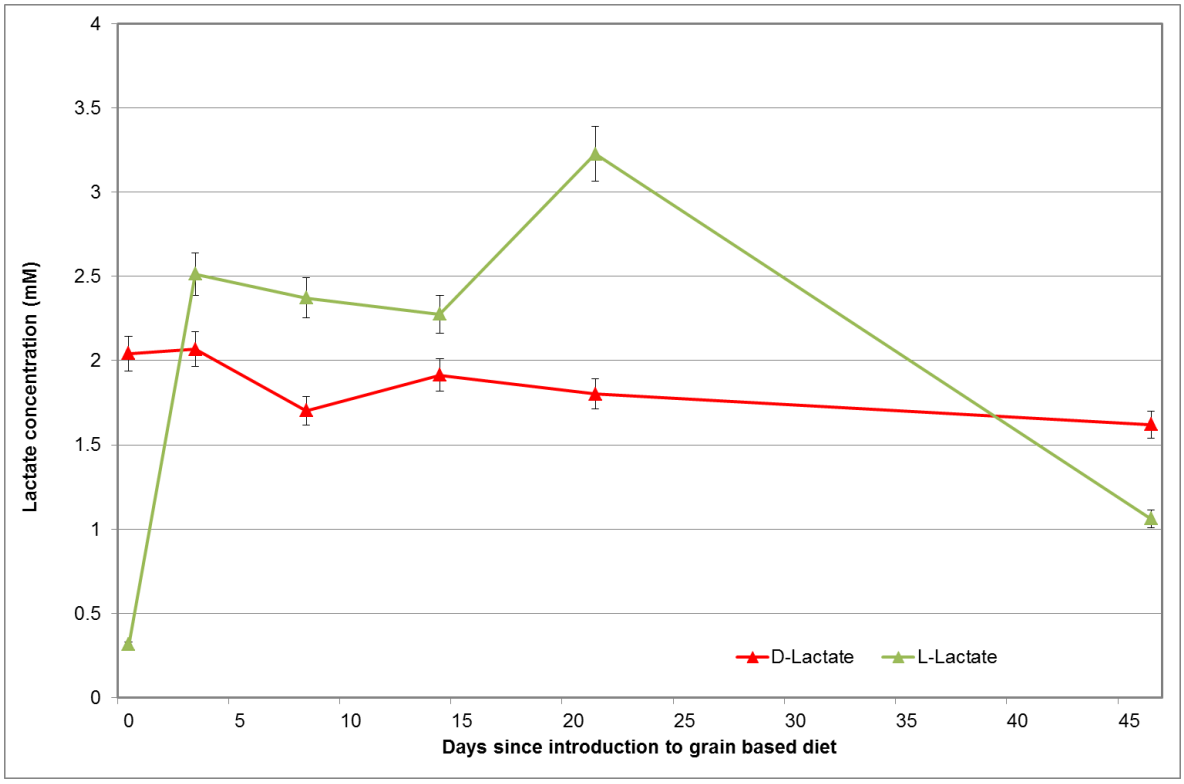


Figure 3.11 Rumen D and L-lactate concentrations (mean±SEM) of steers (n=16) from feedlot 2, fed a total mixed ration with virginiamycin included in the diet until day 11.

Total volatile fatty acid concentrations increased from day 0, peaking at day 21 and were significantly higher at day 8 and 60 (LSD 5%) (Figure 3.12). There was significant correlations between total VFA concentrations and field faecal pH (R=-0.55), rumen ammonia (R=0.65), L-lactate (R=0.65), protozoa (R=-0.53) and rumen pH (R=-0.57) ($P<0.05$).

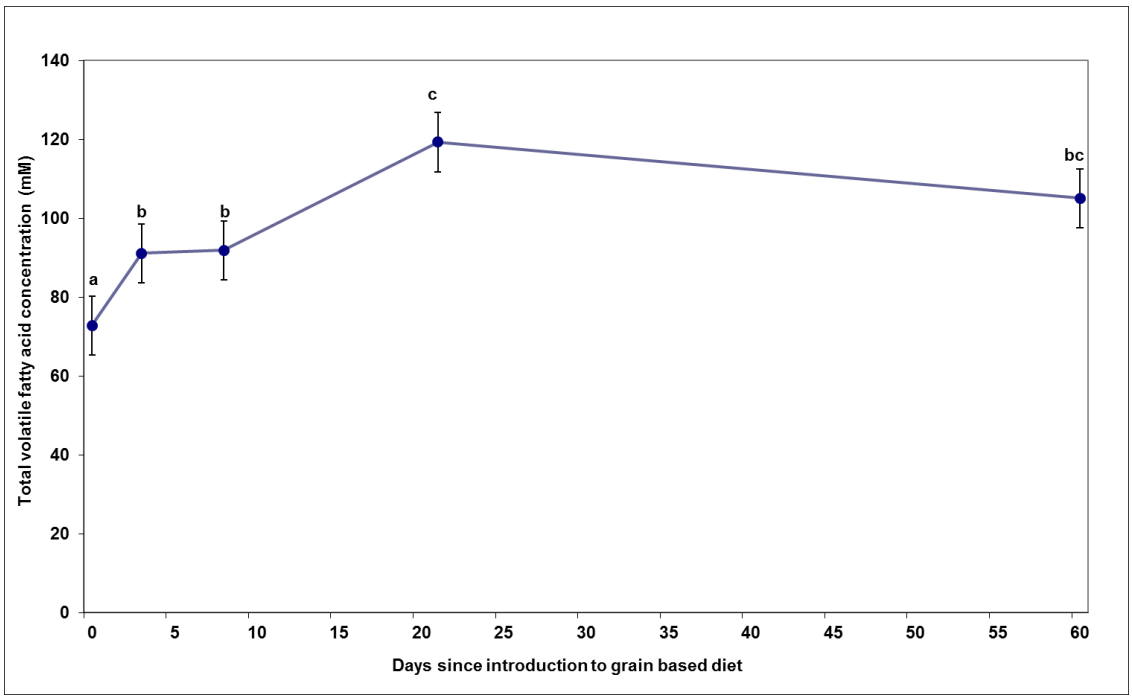


Figure 3.12 Total volatile fatty acid concentrations in the rumen (mean±SEM) of steers (n=16) from feedlot 2, fed a total mixed ration with virginiamycin included in the diet until day 11. Means (±SEM) with the same superscript are not significantly different (P<0.05) whereas values with different super scripts are significantly different (P>0.05).

Acetic acid concentration in the rumen decreased from day 0 (55.02 mM) to day 3 (51.24mM) then increased over the 60-day period by 12mM. There were significant correlations between acetic acid and the total VFAs (R=0.86), propionic acid (R=0.61) and butyric acid (R=0.56) as well as the *S. bovis* (P<0.05) populations (R=0.52) during grain introduction.

Propionic acid increased significantly from 9 mM to 25 mM (LSD 5%) and remained close to that level for the remainder of the sampling period. There were significant correlations between propionic acid and several rumen parameters measured including, total VFA (R=0.89), valeric (R=0.79), acetic (R=0.54), butyric (R=0.77), iso-butyric (R=0.66) and iso-valeric (R=0.69) as well as rumen pH (R=-0.64), field faecal pH

(R=-0.62), ammonia (R=0.75) and l- lactate (R=0.70) as well as *S. ruminantium* (R=0.5) populations.

Butyric acid concentration increased significantly (LSD 5%) from day 0 (6.7 mM) to day 3 (10 mM) peaking at day 21. There were significant correlations between butyric acid and field faecal pH (R=-0.52), rumen ammonia (R=0.56), L-lactate (R=0.67), rumen pH (R=-0.51) and the *S. bovis* (R=0.66) populations (P<0.05).

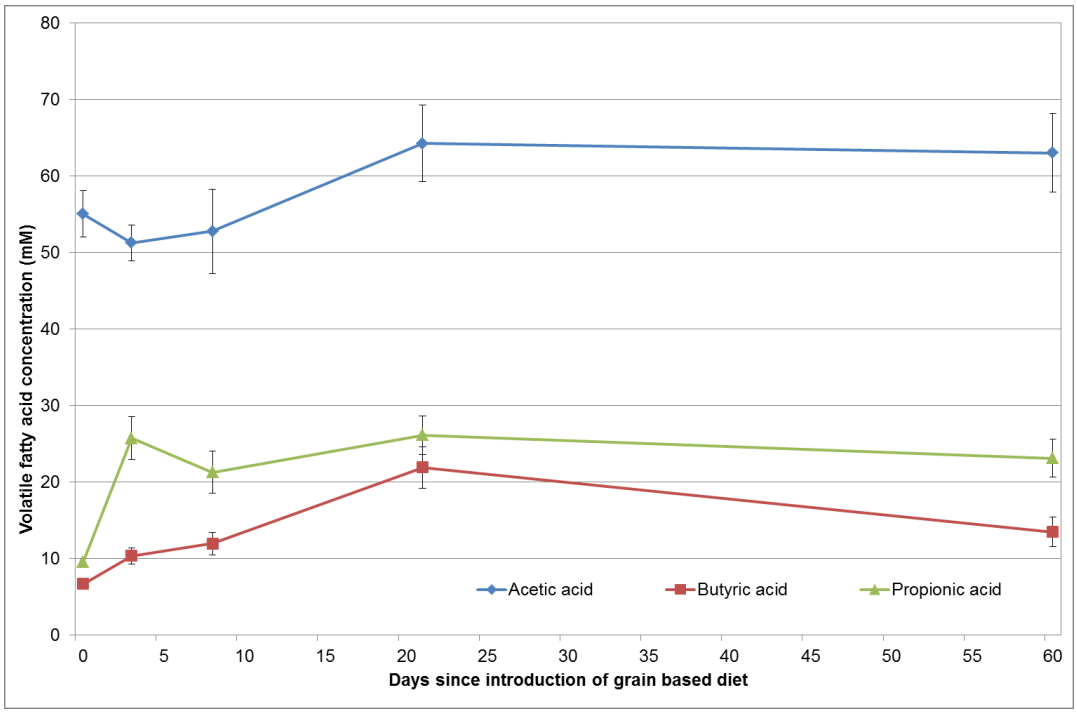


Figure 3.13 Changes in the rumen concentrations of acetic propionic and butyric acids (mean±SEM) of steers (n=16) from feedlot 2, fed a total mixed ration with virginiamycin included in the diet until day 11.

Iso-butyric acid concentrations increased (P<0.05) (LSD 5%) from days 0 and 3 to day 8, then continued to increase until day 21. There was significant correlations between iso-butyric and field faecal pH (R=-0.59), rumen ammonia (R=0.66), L-lactate (R=0.65) and *S. ruminantium* (R=0.59) populations (P<0.05) during the sampling period.

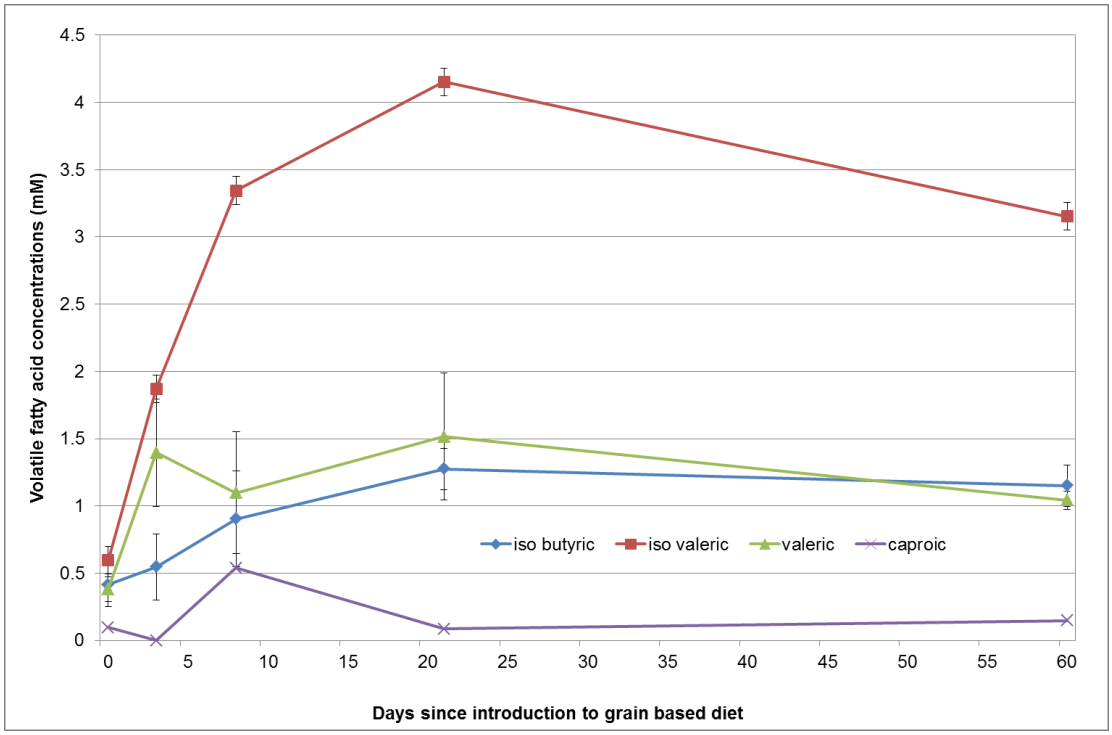


Figure 3.14 Concentrations of iso-butyric, iso-valeric, valeric and caproic acids (mean ± SEM) in the rumen of steers (n=16) from feedlot 2, fed a total mixed ration with virginiamycin included in the diet until day 11.

Caproic acid concentrations increased significantly (LSD 5%) at day 8 (5.4 mM) (Figure 3.14)). There were strong negative correlations between caproic acid and D-lactate (R=-0.45) as well as *F. succinogenes* (R=-0.58) populations (P<0.05) during the sampling period.

Valeric acid concentrations increased from day 0 to 3 (0.4mM to 1.4mM), then remained similar for the rest of the sampling period. There were significant correlations between valeric acid and rumen pH (R=-0.59), field faecal pH (R=-0.65), rumen ammonia (R=0.70), D-lactate (R=-0.32), L-lactate (R=0.80) and *F. succinogenes* (R=-0.47) (P<0.05) over the sampling period.

Iso-valeric acid concentrations increased significantly (LSD 5%) from day 0 to day 21. There were significant correlations between iso-valeric acid and the parameters of field faecal pH (R=-0.59), ammonia (R=0.67), rumen pH (R=-0.59), L-lactate (R=0.71) and *S. ruminantium* (R=0.58) populations as well as VFA's butyric (R=0.73), iso-butyric (R=0.72), valeric (R=0.64) and propionic (R=0.63) (P<0.05) during the sampling period.

3.3.2.2 Faecal parameters

The faecal scores indicated faecal matter was firm on day 0 and 3 (Figure 3.15) with some poorly formed faecal matter by day 8 (chapter 2.4.1). The steers had high concentrations of grain and watery faeces until day 21 with some incidence of loose faecal matter by day 32. The faeces then started to firm up more by day 60. There were significant correlations between faecal score and field faecal pH (R=-0.59) (P<0.05).

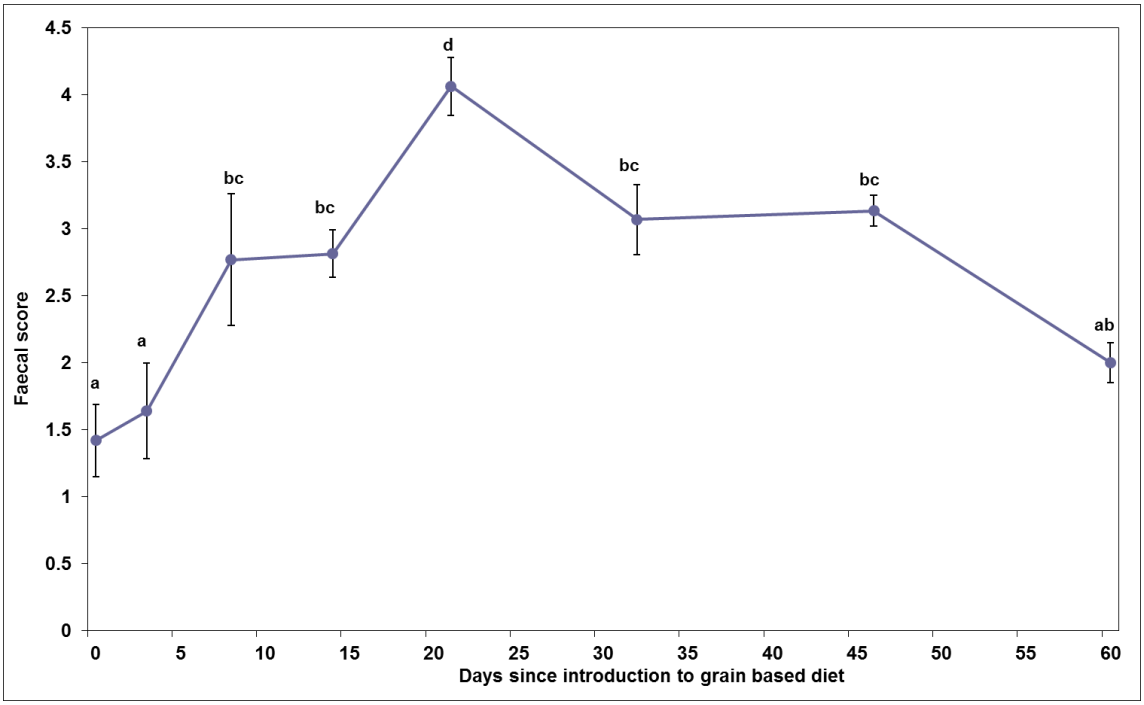


Figure 3.15 Changes in faecal scores (mean±SEM) of steers (n=16) from feedlot 2, fed a total mixed ration with virginiamycin included in the diet until day 11. Means ±SEM with the same superscript are not significantly different (P<0.05) whereas values with different super scripts are significantly different (P>0.05).

Rumen ammonia increased significantly from day 0 to 3 (LSD 5%) then concentrations remained constant until day 46 (Figure 3.16). There were significant correlations between rumen ammonia concentrations and field faecal pH (R=-0.76), rumen pH (R=-0.59), L-lactate (R=0.76), D-lactate (R=-0.41) and the populations of *S. ruminantium* (R=0.54) (P<0.05). There were also significant correlations with total VFA concentrations, butyric, valeric acid, propionic, iso-valeric and iso-butyric (P<0.05).

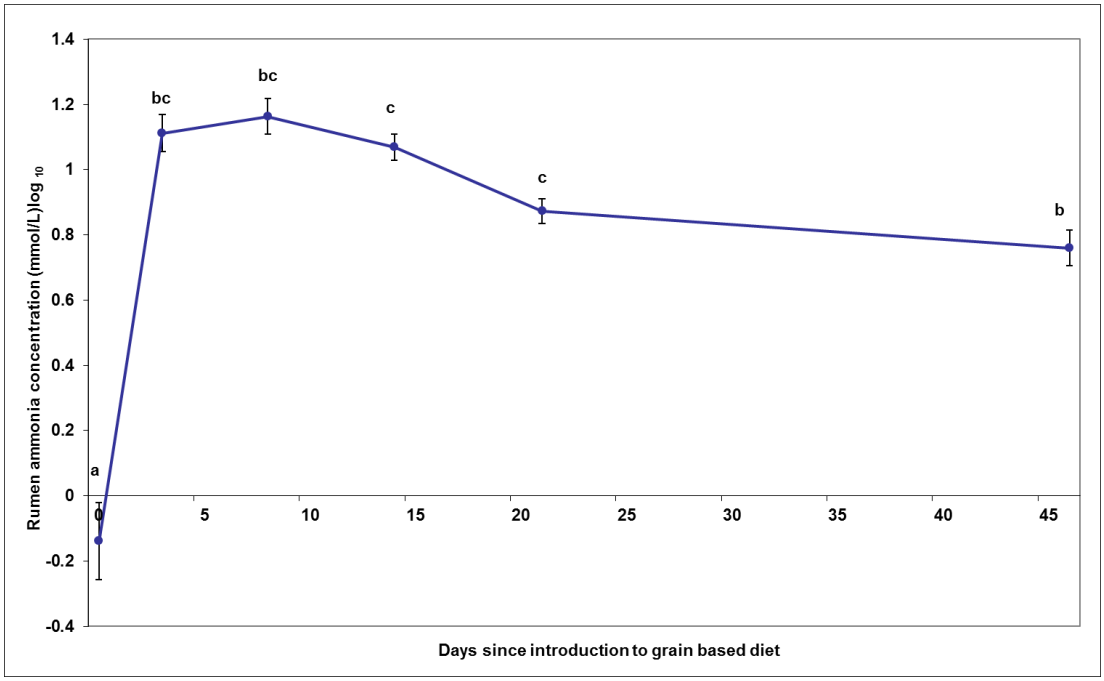


Figure 3.16 Changes in rumen ammonia concentration (mean±SEM) of steers (n=16) from feedlot 2, fed a total mixed ration with virginiamycin included in the diet until day 11. Means ±SEM with the same superscript are not significantly different (P<0.05) whereas values with different super scripts are significantly different (P>0.05).

3.3.2.3 Rumen bacterial parameters

The total bacterial population (Figure 3.17) increased (P<0.05) by day 14 (2.64 x 10^{10} cells/mL) with a fivefold increase in the total bacterial population from day 0 (3.03x10^{9} cells/mL). The population decreased significantly by day 21 (9.15x10^{9} cells/mL)

remaining reasonably constant by day 60 there was a significant decrease to concentrations of less than day 0. The total bacterial populations were significantly correlated with *F. succinogenes* (R=0.55) populations during the sampling period (P<0.05).

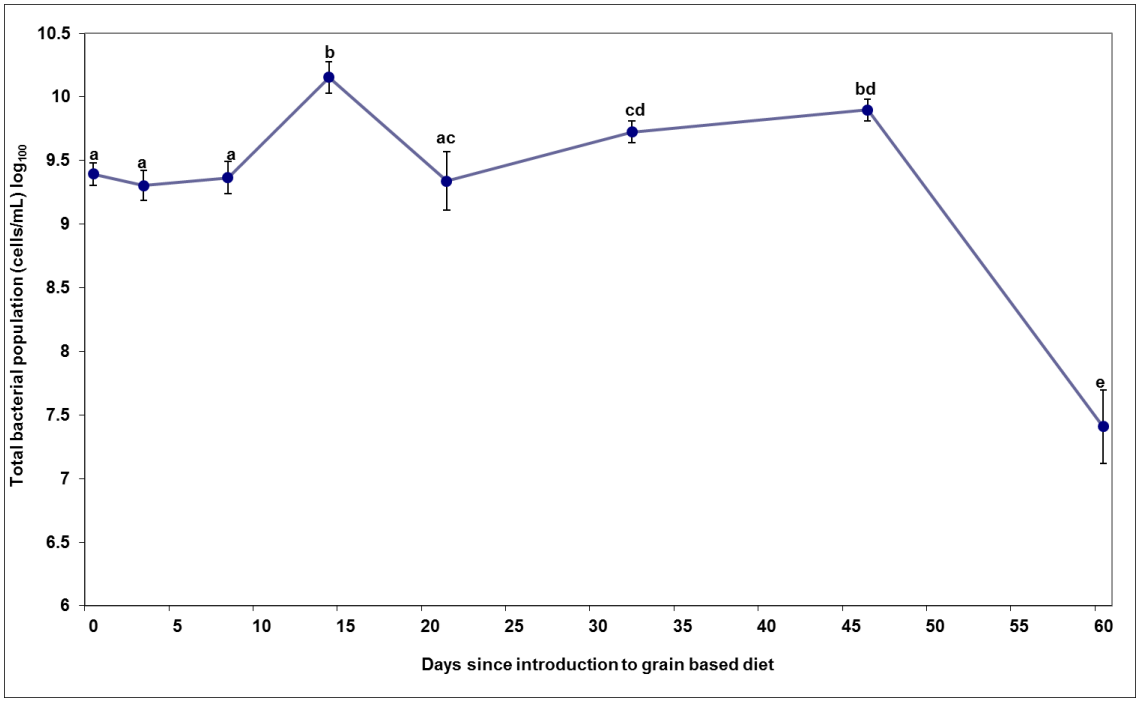


Figure 3.17 Changes in total bacterial populations (mean±SEM) in the rumen of steers (n=16) from feedlot 2, fed a total mixed ration with virginiamycin included in the diet until day 11. Means ±SEM with the same superscript are not significantly different (P<0.05) whereas values with different super scripts are significantly different (P>0.05).

The populations of *F. succinogenes* decreased significantly (LSD 5%) from days 0 to 3 (figure 3.18). There was a significant increase in the populations of *F. succinogenes* from days 3 to 8. The populations then decreased at day 60 (LSD 5%). *F. succinogenes* populations were significantly correlated with the field faecal pH (R=0.49), rumen pH (R=0.54), concentrations of L-lactate (R=-0.47), caproic acid (R=-0.53), and valeric acid (R=-0.47), and the total bacterial counts (R=0.55) (P<0.05) during the sampling period.

The populations of *P. ruminicola* (Figure 3.18) showed significant variation (LSD 5%) on alternating weeks, dropping to their lowest level at day 60. There were no

significant correlations between the populations of *P. ruminicola* and any other rumen parameters during the measurement period (P>0.05).

The population of *S. ruminantium* increased from days 0 then decreased slowly until day 21 (Figure 3.18). The population of *S. ruminantium* increased fourfold on day 32 (P<0.05) followed by a steady decline to day 46, returning to concentrations similar to feedlot entry (day 0) by day 60. The *S. ruminantium* populations were significantly correlated to the following parameters, field faecal pH (R=-0.55), D-lactate (R=-0.52), L-lactate (R=0.46), ammonia (R=0.54) and the VFAs; propionic (R=0.50), iso-butyric (R=0.64) and iso-valeric (R=0.64) (P<0.05).

The populations of *S. bovis* population increased (P<0.05) from day 0 ($3.06x10^4$ cells/mL) to 3 ($4.23x10^7$ cells/mL). Then the populations of *S. bovis* population decreased at day 8, remaining constant until day 21 with a higher degree of variation in the samples quantified. There was a significant correlation between the populations of *S. bovis* and the VFAs acetic (R=0.55) and butyric (R=0.56) (P<0.05) and the addition of virginiamycin to the diet (R=0.54)

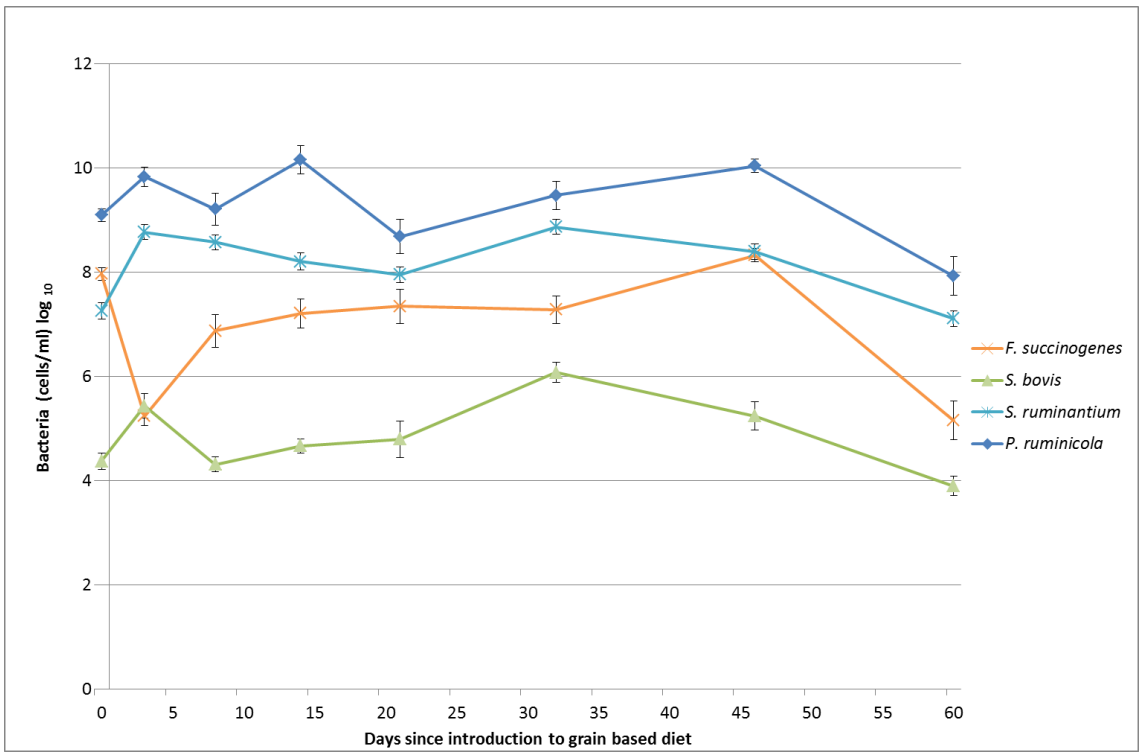


Figure 3.18 Changes in the populations of *F.succinogenes, P. ruminicola, S. ruminantium* and *S. bovis* (mean±SEM) of steers (n=16) from feedlot 2, fed a total mixed ration with virginiamycin included in the diet until day 11.

The interaction of the different rumen microbial populations monitored here (Figure 3.18) over the introduction period indicate that populations of *S. ruminantium* and *S. bovis* were very similar. While the populations of *F. succinogenes* were independent of the other quantified rumen populations. The changes in populations of *P. ruminicola* were closer to the changes that occurred over the sampling period with the populations of *S. bovis* and *S. ruminantium*.

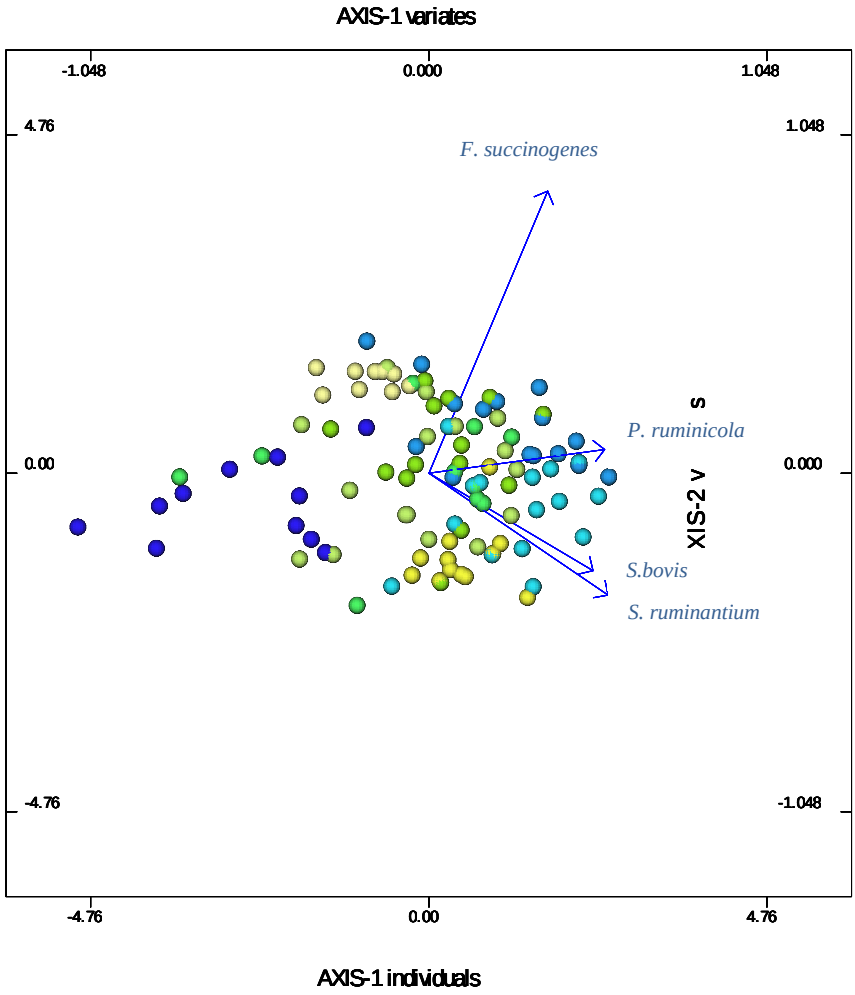


Figure 3.19 Biplot representing the correlations of log transformed bacterial populations during grain introduction in feedlot 2, fed a total mixed ration with virginiamycin included in the diet until day 11.. Day 0 (pale yellow), day 3 (yellow), day 8 (light green), day 14 (green), day 21 (dark green), day 28 (light blue), day 32 (medium blue), day 46 (blue) and day 60 (dark blue).

Protozoal numbers showed a slight decrease at day 3 followed by a constant increase in protozoa number until day 60 (Figure 3.20). Day one was only different ($P<0.05$) to the other sampling days at day 46 and 60 (LSD 5%), days 3 and 14 were significantly different to days 32, 46 and 60 (LSD 5%). Protozoal numbers were significantly correlated to the *P. ruminicola* population ($R=-0.34$) ($P<0.05$)

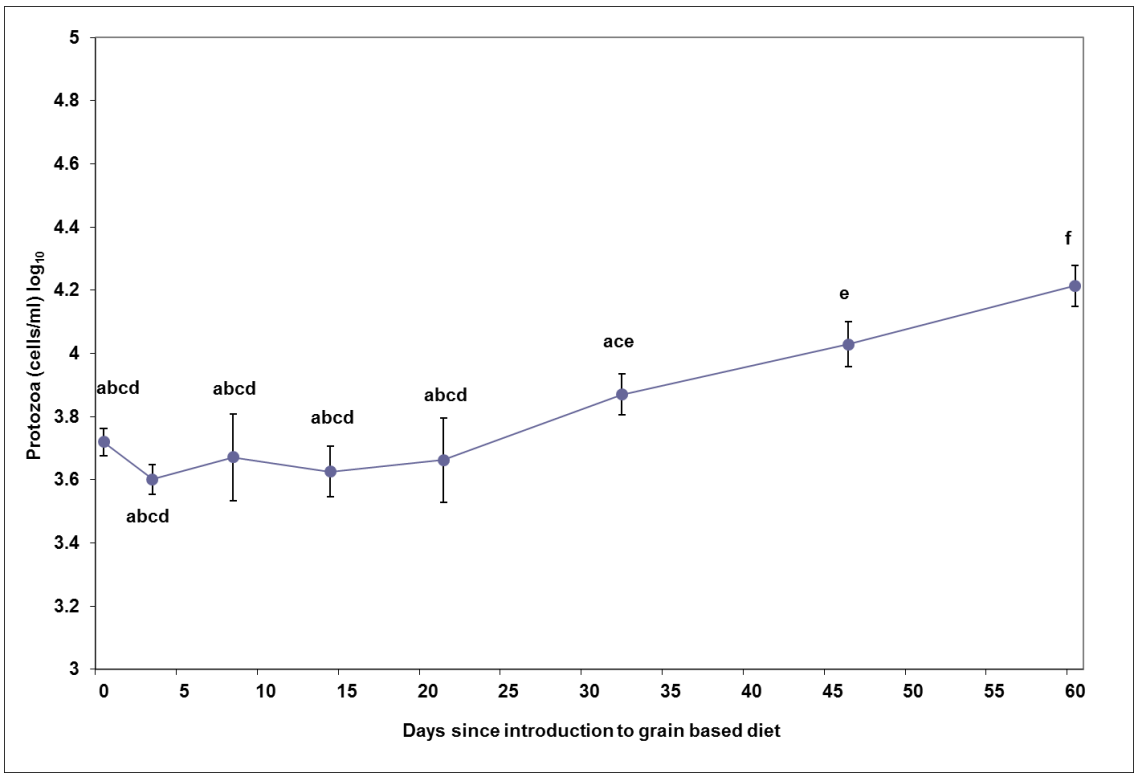


Figure 3.20 Changes in the population of rumen protozoa (mean±SEM) of steers (n=16) from feedlot 2, fed a total mixed ration with virginiamycin included in the diet until day 11. Means ±SEM with the same superscript are not significantly different (P<0.05) whereas values with different super scripts are significantly different (P>0.05).

3.4 **Discussion**

This study represents one of the first investigations into the changes in rumen microbial ecology in cattle fed grain under commercial feedlot conditions rather than under fully controlled experimental conditions. Cattle fed in commercial feedlots are usually introduced to large amounts of grain over a longer period compared to that of experimentally-induced acidosis where often there was a single large bolus challenge. This experimental approach diminishes the option for intake regulation by the ruminant to reduce rumen disturbance or commence adaptation. However, under commercial conditions such as in these two feedlots, there were no control groups since all of the animals receive the same treatment ration for commercial gain. As a consequence, this

study of cattle under commercial conditions provided descriptions of the ecological and metabolic changes in the rumen and host ruminant, but without controls. Therefore, analysis of, or consideration of the underlying mechanisms was difficult and possibly over-reliant on speculation. Just as importantly, this study was the first to monitor the changes in rumen microbial ecology under commercial conditions using molecular tools based on 16S rRNA gene sequences to assess the changes in populations of key species of bacteria. Previously studies using phenotypic subculture techniques to assess rumen ecological changes were tedious, and difficult to interpret in terms of a changing ecology.

This chapter explored the effect of grain feeding regimes on two commercial feedlots using two quite distinct approaches to feeding grain. In the first feedlot, cattle were introduced to grain over a 7-day period with grain and hay fed separately thereby allowing the cattle to self-select the proportions of hay and grain. On the other hand, in the second feedlot cattle were fed a total mixed ration incorporating the antibiotic, virginiamycin, until day 11 then the composition of the total mixed ration changed with gradual increases in grain and no virginiamycin until the end of feeding on day 54. Importantly each feedlot allowed the monitoring of changes in the rumen parameters over the introduction period. Although these two commercial feedlots were not directly comparable due to their different feedlot feeding regimes, nevertheless they provided clear indications of how different management practices impact on rumen microbial ecology and metabolism during the introduction period under realistic commercial feedlot systems rather than high grain induced acidosis under experimental conditions. The other important finding was that none of the cattle monitored in either feedlot or under the two feeding regimes developed clinical acidosis. In fact, the average daily gain (2.14 kg/day) of cattle in feedlot two was at near the best performance for commercial enterprises.

3.4.1 **Feedlot One**

In this feedlot cattle were fed the starting diet of roughage and grain provided in separate feeding bunks on day 1 of introduction transitioning to the full grain diet by day 7 (Table 3.1). However, cattle were bought in as feedlot stores and introduced to the diet on a continuous basis in this feedlot. Therefore, only those cattle in the feedlot from the first day of the initial introduction were sampled throughout the feedlot period. Moreover, these cattle that were continuously sampled from day 1 were significantly different to cattle sampled randomly (and therefore had potentially been at different stages of introduction given the continual introduction of stores) in terms of total bacterial populations, populations of *S. bovis*, *P. ruminicola*, faecal score and faecal pH ($P<0.05$). This difference could not be attributed directly to sampling or to the fact that they were cattle bought into the feedlot after the initial introduction period and therefore were at different stages of introduction. Hence the randomly sampled steers were removed from the analysis.

The decrease in rumen pH by day 3 in feedlot one is indicative of most feedlot introductions of grain (Slyter, 1976; Nagaraja and Nagamine, 2007; Krause and Oetzel, 2006; Owens *et al.*, 1998). In this case oats made up 70% of the diet until day 4 mainly because it was considered a "safer" grain (Walker, 2006) and consistent with this view was the finding that feeding oats did not lower the rumen pH to values outside the normal functioning range of rumen i.e. pH 6.0 to 7.0. In fact, the rumen pH remained within this normal range throughout the sampling period. Obtaining rumen samples via stomach tubing can impact pH values due to salivary contamination, and Duffield *et al.* (2003) showed that there can be as much as 0.44 units lower pH when samples are collected directly by rumencentesis compared to oral stomach tubes. This finding raises the possibility that the rumen pH measured here may be lower than actually measured.

Notwithstanding this, sampling by rumen tubing was the only alternative for continuous sampling of cattle being finished on a commercial property. The most important finding in feedlot one was the successful management of introduction of cattle onto the feedlot dietary regime. Thus the pattern of changes in rumen pH, rumen microbial ecology and rumen metabolism must be considered in the context of this successful management.

The concentration of the volatile fatty acids (VFAs), acetic, propionic acid and butyric acids all increased significantly between day 0 to 3 of this type of dietary introduction in feedlot one. The concentration of total VFA was greater than 100mM for the period of feed lotting, which was indicative of sufficient energy supply to support high growth rates in these cattle. This is also reflected in the high, commercially viable growth rate observed during the feedlot period (live weight gain was only measured in feedlot 2). During the introductory period when cattle could self-select between hay and grain, and even later when higher grain concentrations were fed through the feedlot, acetic acid production increased and remained the volatile fatty acid in greatest molar proportion indicative of fermentation of high concentrations of structural carbohydrates. While the propionic acid production also increased over these same periods, indicative of greater fermentation of soluble carbohydrates from grain, the molar proportion was never as great as that for acetic acid. Nevertheless, the concentration of propionic acid was always high enough to support high growth rates. This may indicate that the cattle self-selected sufficient amounts of hay to balance grain intake during the introduction period consistent with the findings of Dijkstra (1994); however this was not quantifiable without actual intake values. The high proportion of acetate in the VFAs was also indicative of successful cellulose fermentation which was not generally apparent from experimental challenges of high grain diets. Moreover, acetic acid concentrations and molar proportion were significantly correlated with populations of F. *succinogenes,* a cellulose utiliser, as well as

the values of rumen pH remaining in the normal range over the sampling period. This relationship with acetic acid is linked to the sensitivity of *F. succinogenes* to decreases in rumen pH. Each of these findings indicates that feedlot 1 had instigated a successful and safe feeding management for cattle during introduction to the feedlot which continued through to the period of feeding the higher grain ration.

The valeric acid concentration increased significantly during the first 8 days of dietary introduction. Valeric acid is an important metabolite for cellulytic bacteria (Cline *et al.*, 1958). Moreover, during starch digestion by amylolytic bacteria the amount of valeric acid formed increased. Iso-butyric acid is also a growth factor for cellulytic bacteria. Early work by (Bryant and Doetsch, 1955) showed that valeric, iso-valeric, iso-butyric and caproic acids or their amino acid precursors stimulated cellulose digestion and the conversion of urea nitrogen into protein by rumen microorganisms. The trend of higher VFAs and their branch-chain equivalents highlighted how successful the adaptation to consistent cellulytic digestion was in this feedlot.

Total VFA concentrations in the rumen increased with the increased dietary energy particularly from day 0 to 3; this is commonly found in cattle going onto grain diets. The total VFA concentrations were significantly linked to acetic acid concentrations over the sampling period, which was both the major VFA and exhibited the greatest increase in concentration over the sampling period.

Concentrations of L- lactate during the introduction period were low but variable, indicative that some animals adapted to a self-selection system for hay and grain better than others.

In feedlot one there were significant correlations between the populations of S. *bovis* and rumen concentrations of D-lactate as well as concentrations of propionic acid which all related to the increase in grain content. This type of relationship between S.

bovis populations, rumen pH and D- lactate has been shown previously by (Rowe, 1999; Hook *et al.*, 2011; Owens *et al.*, 1997; Al Jassim and Rowe, 1999) but these workers used experimentally induced acidosis rather than commercial feedlots with slower introduction. Therefore, benchmark concentrations of L and D-lactate were much higher in induced acidosis, indicating the trends of increasing concentrations of lactate and reducing pH was linked to increasing populations of *S. Bovis*. However, the extent to which this occurred and the ability to readjust the rumen may have been the reason for the difference.

Faecal scores were lowest (indicating good pat formation) at day 8, the day after the second increase in grain. However, a dramatic drop in post fermented faeces pH indicated that on day 7 the increase in grain content had potential to cause acidosis (Al Jassim, 2006) resulting from the amount of grain passing on to the caecum and lower gut. However, the values of field faecal pH were not particularly low and showed an increase rather than a decrease, indicating that there was little post rumen fermentation.

There was an increase in the total bacterial population during days 0-3, due to the readily available carbohydrate and showed a successful adaption of the total microflora in these cattle to the transition to the feedlot. There was a significant relationship between total bacterial populations and acetic acid as well as populations of *P. ruminicola*, which is one of the more predominant rumen bacteria (Stevenson and Weimer, 2007a) in the rumen. *Prevotella* was shown in the work by (Tajima *et al.*, 2001) to be in high proportions in the rumen as had been postulated by Hungate as the most commonly occurring rumen bacterial species. So the pattern of total bacterial populations and *P. ruminicola* quantified during the dietary transition in feedlot one was in accord with all previous studies. *Prevotella ruminicola* followed the same significant changes as seen in the total bacterial population, indicating that they did indeed constitute a high proportion of the bacterial population. However, since the number of copies the 16S rRNA gene in *P. ruminicola* is not certain,

then this did not permit absolute quantification of the relationship as a ratio to the total bacterial population.

During the dietary introduction, the population of *F. succinogenes* decreased initially then increased to a peak at day 8 and maintained a consistent proportion over the feedlot period. The consistent population of *F. succinogenes* indicated that cattle were selecting roughage from the hay bunk and not consuming as much of the grain initially. Therefore, a higher consumption of roughage leaves a lag time to increase the fibre utilising bacteria. The population then decreased at day 21 and stayed constant for the remainder of the feedlot period. However work done by (Fernando *et al.*, 2010) indicate that the *F. succinogenes* population gradually decreased to a 45 fold decrease in cannulated steers as they adapted to high concentrate diets.

Selenomonas ruminantium populations followed the same trend as those of *P. ruminicola* and *S. bovis* i.e. decreasing at day 8. Each of these species is characterised by their capacity to ferment starches, and soluble sugars and in the case of *P. ruminicola*, hemicelluloses as well. On the other hand, they have no cellulolytic capacity. As a consequence, this trend may indicate that the cattle were eating more roughage especially as the decrease in the populations of these species corresponded to the increase in populations of *F. succinogenes*, the fibre digesting bacteria. Also with *S. ruminantium* being a lactate utilising bacteria, there is an increase from day 8-14 which corresponds to an increase in the lactate producing *S. bovis* population.

Streptococcus bovis populations were significantly correlated with rumen pH and D-lactate concentrations which was consistent with reports from induced experimental acidosis (Asanuma and Hino, 2002; Russell and Hino, 1985; Coe *et al.*, 1999; Commun *et al.*, 2009; Goad *et al.*, 1998). It was interesting to note this relationship between *S. bovis*, D-lactate and rumen pH still held even at these relatively low concentrations of D-lactate

and relatively neutral rumen pH (>6.0). However it highlighted the potential for unproblematic self-correction under commercial feedlot introduction compared to experimentally induced acidosis.

There was an indication that *P. ruminicola* and *S. bovis* were similar in their trends for population shifts over time (Figure 3.7) with *P. ruminicola* role in the rumen being the degradation of protein, as well as the degradation and utilisation of starch while *S. bovis* is primarily a starch degrader (Stewart *et al.*, 1997). Over the introduction period, the populations of *S. ruminantium* and *F. succinogenes* were independent of the other rumen bacterial species monitored.

3.4.2 **Feedlot Two**

Feedlot two was different to feedlot one as the diet was fed as a total mixed ration with smaller increments in the grain content that were also slowly introduced over a 52-day period. Just as importantly, this feedlot also incorporated virginiamycin into the ration until day 11 to guard against acidosis. However, since there was no acidosis even or a total mixed ration control it is difficult to assert definitely whether the virginiamycin prevented acidosis or there was no acidosis under this feeding regime.

Coe, Nagaraja *et al* (1999) did not report any impact of virginiamycin on the molar proportions of volatile fatty acids. However, populations of *S. bovis* decreased in animals fed diets containing virginiamycin (Al Jassim and Rowe, 1999). The changes in molar proportions of the volatile fatty acids observed here is more likely to be the result of increasing the grain content of the diet and not directly related to the virginiamycin inclusion in the diet.

The rumen pH declined steadily over the 54-day period consistent with an early establishment of substrate fermentation capable of supporting higher production concentrations in the feedlot cattle. Interestingly, there were no severe changes or

decreases in rumen pH after removal of virginiamycin. On the other hand, L-lactate concentrations increased after the removal of virginiamycin significantly from day 14 to 21, indicating a slightly unstable period of carbohydrate fermentation before returning to an adaptive range. The significant correlation between increasing VFA concentrations and relatively stable normal rumen pH was indicative of adapted and productive rumen microbial ecology

The rumen ammonia concentrations increased significantly upon introduction of cattle to the feedlot peaking at day 21 which is the time of peak adaptation of nitrogen metabolism in most feedlots utilising high grain diets and aiming at optimal ruminal breakdown of the protein content of the diet. In fact, the pattern of carbohydrate fermentation indicated through the concentrations of the VFAs and acetic and propionic acids and the ammonia concentration all peaking and stabilising at that peak at day 21 accorded with the anecdotal notion that cattle take about three weeks to fully adapt to feedlot rations.

The faecal score on day 32 was indicative of the loosest faecal matter and also had visible grain in faeces indicating incomplete grain breakdown. This day and faecal scores also coincided with peak in populations of *S. bovis* and *S. ruminantium* populations. The lowest post fermentative faeces pH also occurred on day 32, indicating a higher potential for acidosis from the diet, possibly due to post-ruminal fermentation of increasing grain content.

The faecal field pH decreased significantly between days 3-8 when virginiamycin was being fed, and again after the removal of virginiamycin at day 11, decreasing significantly to day 21 but remaining relatively constant from day 21 until the end of feedlot. These changes in faecal pH may indicate that the virginiamycin may have been impacting on post rumen fermentation for a short period of time.

The populations of the fibre digesting bacteria *F. succinogenes* decreased from day 0 to 3 but increased and remained constant for the remainder of the feedlot period. This consistency of populations of *F. succinogenes* suggests that the cellulose fibre component of the diet was being digested even as the grain component of the diet was increasing and pH was remaining at a level at or above 6.0 which did not impact on the growth of this cellulolytic bacterium. In other words, the fibre and grain fermentation were fully adapted within the 21-day period even after removal of the virginiamycin at day 11.

The populations of *Prevotella ruminicola* were not significantly correlated with any other rumen parameters even though *P. ruminicola* was the predominant bacterial species in the rumen bacterial population in this and other studies (Griswold and Mackie, 1997; Avgustin *et al.*, 1997; Tajima *et al.*, 1999; Tajima *et al.*, 2001).

Selenomonas ruminantium which is linked to starch digestion increased significantly from day 0-3 showing an early adaptation to the TMR. Other parameters such as field faecal pH, D and L lactate, rumen ammonia and propionic acid were significantly correlated with increases in starch digestion.

Importantly, the population of *S. bovis* increased on day 14 after the removal of virginiamycin as well as increasing barley content of the diet. *S. bovis* populations peaked at day 32 when low concentrations of acidosis appeared to be present. Interestingly, *S. bovis* and *S. ruminantium* (Figure 3.18) were strongly linked indicating that the lactate utilisers *(S. ruminantium*) and lactate producers (*S. bovis*) were associated in this ecology under feedlot conditions just as they have been found to be associated under experimental conditions.

The protozoa populations in feedlot two showed a general increasing trend over period of feedlot. These increases in protozoa populations were correlated with propionic acid concentrations, total VFA and rumen pH all of which increased with higher carbohydrate

content in TMR diets. The inclusion of virginiamycin has been shown to inhibit the protozoal populations (Nagaraja *et al.*, 1995) so its removal may have led to the increase over time of the protozoal populations which was not evident in feedlot one with no additional virginiamycin in the ration.

Overall feedlot one which fed the hay and grain separately and introduced grain as oats relatively rapidly over seven days never showed any indication of acidosis and neither did cattle in feedlot two which were a total mixed ration with virginiamycin until day 14. In fact, the lactate concentrations in cattle from feedlot two were always lower than those in cattle from feedlot one. The incorporation of virginiamycin in the TMR diet in feedlot two did reduce *S. bovis* populations. However, when virginiamycin was removed from the ration, the rumen still was able to adapt to the TMR, with showing no decreases in productivity. If rumen samples could have been taken at day 16 or 18, these samples may have given a better indication of when *S. bovis* peaked after the removal of virginiamycin.

Overall in feedlot 2 only one animal with a rumen pH of 5.3 and all others remained at or above a rumen pH of >6.0. The growth rates of the cattle in feedlot 2 sustained a growth rate great then 2kg/hd/day which itself refutes a supposition of subclinical acidosis.

The differences in feeding practices between the two feedlots were confirmed in that feedlot one showed higher concentrations of total volatile fatty acids and acetate indicating very efficient fibre digestion. On the other hand, feedlot two had a higher level of propionate prevalent indicative of the higher grain diet. Notwithstanding the quite different dietary regimes, cattle in the two feedlots successfully negotiated the period of dietary introduction.

The use of virginiamycin was effective in keeping the rumen pH within the normal range, although the increase in S. *bovis* after virginiamycin removal was indicative of its effect on the microbial population. However it is also interesting that when virginiamycin

was withdrawn from the dietary regime it did not appear to be long acting in terms of carryover effect. This is contrary to what is advised in terms of its short term use under grain feeding scenarios.

When you look at the whole picture of how commercial feedlots are introduced slowly over a longer period in which hypothesis one states that cattle introduced gradually under commercial feedlot conditions will have a reduced incidence of ruminal acidosis compared to previous work done on grain loading under experimental conditions. Both feedlots showed no signs of clinical acidosis over the sampling period indicating a successful transition under both feeding regimes.

The hypothesis that cattle fed a feeding a total mixed ration containing virginiamycin will have a reduced incidence of ruminal acidosis compared to those fed grain and hay separately was not supported in this case. Thus both feeding regimes may have their merits and that leaving cattle to select dietary components between hay and grain to adjust the rumen environment can be as successful as feeding a total mixed ration.

The hypothesis that feeding virginiamycin in the ration under feedlot conditions reduces the indicators of ruminal acidosis under a commercial grain feeding regime was supported. However it should also be noted that neither feedlot showed any indications of acidosis. The virginiamycin did not appear to have any long term effect on rumen physiology and metabolism.

It was also hypothesised that the presence of cellulytic bacteria, *F. succinogenes* was sustained during a successful dietary transition under commercial feedlot conditions. This hypothesis was supported in both feedlots, which is very different to the findings under induced experimental conditions.

3.5 **Conclusions**

The two different feeding management regimes for these feedlots were both successful with evidence that they adapted over a 14 to 21-day period after grain introduction. Feedlot one fed the hay and grain separately over a reasonably rapid period of seven days using oats as a safe 'buffer' grain. Feedlot two incorporated virginiamycin in a total mixed ration until day 14 and then fed the TMR until the end of the feedlot. This feedlot's effective introduction of grain from the outset and feeding of high grain rations was reflected in high liveweight gain during the feedlot period. Unfortunately, feedlot one did not record weights over the feedlot period, with cattle showing no physical signs of acidosis. Therefore hypothesis one that cattle introduced under commercial feedlot conditions would have a higher incidence of acidosis could not be evaluated from these observations with the bacterial populations monitored remaining steady without cattle developing acidosis. This highlighted that the crucial role of management practices by the feedlotters in reducing the impact of acidosis under commercial feedlot conditions. Feedlot management therefore should be considered one of the main factors impacting the incidence of clinical and subclinical acidosis.

The feeding of total mixed ration incorporating virginiamycin for the first 11 days in feedlot two was successful in keeping the rumen pH within a normal range. The increase in the populations of S. *bovis* after virginiamycin removal on day 11 suggests that it was having an effect on the microbial population ecology. Nevertheless the microbial ecology in the rumen was still able to adapt with no decrease in productivity. Therefore, hypothesis 2 testing if the addition of a feed additive such as virginiamycin reduced the incidence of acidosis through changes in bacterial ecology changes could not be supported or refuted as no incidence of acidosis was evident nor was the effect of virginiamycin controlled in the design of this experiment. The work done by Rowe et al in which they looked at a gradual

and fast adaption to high grain based diets found that with faster introduction there was higher levels of rumen pH variation and therefore a greater opportunity for acidosis to occur in these animals.

The hypothesis that a feeding a total mixed ration to cattle will have a reduced incidence of ruminal acidosis compared to those fed grain and hay separately could not be evaluated since neither feeding regime resulted in acidosis. Both feeding regimes had their merits and that the ability to select dietary components to adjust the rumen environment can be as successful as feeding a total mixed ration. This again highlighted the importance of animal husbandry during dietary transitions.

Hypothesis four tested whether cellulytic bacteria *F. succinogenes* will decrease during grain introduction. This hypothesis was supported in both feedlots during the initial introductory period of three days to, but the cellulytic species within the microbial population recovered throughout both commercial feedlots by day 21, which was very different the findings under induced experimental conditions.

Hypothesis five that lactic acid utilising bacteria such as *S. ruminantium* would increase with an increase in the starch component of the diet was supported under both feeding regimes. However, by the end of the sampling period the populations of *Selenomonas ruminantium* had returned to concentrations similar to or lower than on introduction to the feedlot.

The hypothesis six that *Prevotella ruminicola* was the most prevalent bacteria in the rumen during dietary transition was supported in both feedlot 1 and 2. However more recent metagenomic and molecular studies (Petri *et al.*, 2013a; Stevenson and Weimer, 2007a) report vast projected numbers of rumen bacteria which were not represented in this study. So this hypothesis was only supported in comparison of the monitored rumen

bacterial species and by earlier phenotypic studies of (Hungate, 1966; Bryant, 1970; Hungate, 1950).

Hypothesis seven that the populations of *Streptococcus bovis* will increase significantly and possibly pathogenically during introduction to grain based diets was supported in the sense that these populations increased significantly but did not correspond to any decrease in ruminal pH or indicators of lactic acidosis. Therefore, the correlations that were found under induced acidosis may in fact be normal population changes with the substrates that are made available to the rumen microbial population.

In summary, this study of rumen microbial ecology and metabolism in cattle managed under commercial feedlot conditions was the first to monitor changes in rumen microbial ecology using molecular technologies. Moreover, the changes in rumen microbial ecology were significantly correlated to metabolic changes in the rumen (e.g. concentration of VFAs and BCVFAs) that underpinned the production measures such as liveweight gain in these feedlots. The concentrations of volatile fatty acids in the rumen for both feedlots were all above 100mM which is indicative of good growth levels under a grain feeding scenario. From the perspective of this study, it was disappointing that the incidence of clinical or subclinical acidosis was not evident. However, this study was undertaken under commercial constraints and confirmed the effective management and husbandry practices of both feedlot programs through positive indicators in the metabolic and microbial changes quantified.

4 How variation in calving time impacts on rumen parameters during introduction to grain based diets.

4.1 Introduction

In the studies on commercial feedlots, the origin of birth or other factors were unknown, whereas the aim of this chapter was to sample cattle from the same known birthplace but of different season (or time) of calving i.e. autumn vs winter and then grazed on the same property before entering feedlots. The cattle for this study were obtained through another project that provided the reproductive and nutritional background information and known genetics of cattle that were placed into a feedlot at the same time but with the major difference of varied time of calving. The main aim here was to determine if the time of calving onto pastures of different quality had long term impacts on the development and retention of the rumen microbial population.

The Beef CRC II regional combinations project assessed the long-term impact and growth path of cattle calved into different seasons, i.e. they were either calved in March during autumn (early calvers) and needing supplementary feeding which is traditional in south west of Western Australia or June during winter (later calvers) to match feed supply with animal nutritional demand. This project was outlined in McIntyre *et al.* (2009), the calves were all weaned in January 2004 (2003 drop calves), making them approximately 10 months for early calved (EC) and 7 months for late calved (LC) and placed onto a fast growth diet (>1kg/hd/day). During this trial the cattle were placed onto various feeding regimes, but the study reported here analysed bacterial changes only in those cattle maintained on the fast growth path (>1kg/hd/day) which meant that all of these animals were placed onto a feedlot ration.

Since the growth path on the feedlot ration was similar for all calves, this study focused on the impact that calving onto an actively growing green pasture or a dry pasture

may have had on the rumen microbial population, if any. Work done by Al Jassim *et al.* (2003) indicated that when sheep were backgrounded on hay or pasture, the sheep that were adapted to green pasture initially had higher ruminal pH than those adapted to hay alone. Those adapted to green pasture did not develop lactic acidosis when challenged with grain. Their work suggested that previous nutritional history influenced the onset of acidosis.

The aim of this experiment was to determine if cattle of varying age not born onto the same pastures (i.e. dry autumn pasture or fresh growing winter/spring pasture) but raised under the same conditions (i.e. location and feeding regime prior to weaning) had any variation in their rumen metabolism and rumen microbial populations. It was hypothesised that calves born onto green pastures (LC) would have lower incidence of ruminal acidosis compared to those calves born onto dry pasture (EC) when both groups of calves are provided with the same grain-based diet.

1. The time of calving has a long-term influence on rumen microbial ecology subsequently established in the new born cattle.
2. Fibre utilising rumen bacteria (*Fibrobacter succinogenes*) populations will decrease during grain feeding or any associated reduction in rumen pH.
3. Lactic acid utilising rumen bacteria (*Selenomonas ruminantium*) populations will increase with an increase in the grain component of the diet.
4. *Prevotella ruminicola* will be the most prevalent bacteria in the rumen during dietary transition.
5. *Streptococcus bovis* will increase significantly and possibly pathologically, during introduction to grain-based diets.
6. If increases in *Streptococcus bovis* are linked with a decrease in ruminal pH, then *Lactobacillus spp.* will also increase significantly.

7. Metabolic changes in the rumen can be related to changes in the molecular ecology during dietary transitions in cattle and sheep.

4.2 **Materials and methods**

Cattle were weaned at Alcoa Farmlands, Pinjarra in January 2004 and bought to Vasse Research Centre, Busselton where they were designated on stratified weights to the fast growth ration group (>1.0kg/hd/day). When these weaners came through for their first weighing, rumen samples were also collected (as per chapter 2) randomly from 8 animals in both the EC and LC group. Since the cattle were quite young and small, no brass attachment used [as shown in the photograph (chapter 2.3)] when these weaners were sampled; they were only sampled with the tubing containing a smoothed ending. The final diet consisted of 38% hay, 45% barley, 15% lupins and 2% mineral mix with no rumen modifiers incorporated into the diet. The cattle were on a full access (*ad libitum*) ration by day 8.

Rumen samples were collected from these cattle on day 0 (just prior to feedlot entry), days 3, 7, 14, 21, 28 and 64. Live weights were also measured over at these times of sampling. Samples were analysed for rumen pH, rumen key bacterial species, rumen lactate (L and D), and protozoal counts.

4.2.1 **Statistics**

All data from this trial, except pH values and live weight, displayed lognormal distributions and were log transformed (log_{10}) prior to statistical analysis, with total bacterial counts log transformed to base 100 (log_{100}). A linear mixed model which included a fixed effect comparing the two times of calving, a fixed effect for sample date and an interaction between sample type and date was fitted to each variate using the REML procedure in GenStat (edition 14). The model also included an autoregressive covariance

structure between sample dates. All fixed effects were tested using F-statistics or Wald statistics. If there was no significant difference between sample types ($P<0.05$) all samples were used to calculate averages at each sample date which were compared using 5% least significant differences (5% LSD).

Correlations between variates were compared to zero using a two-sided test. The matrix of correlations between logarithms of the counts of individual bacteria was used to construct a biplot which showed the relationships between protozoa and bacterial counts and how sample counts varied across sample dates

4.3 **Results**

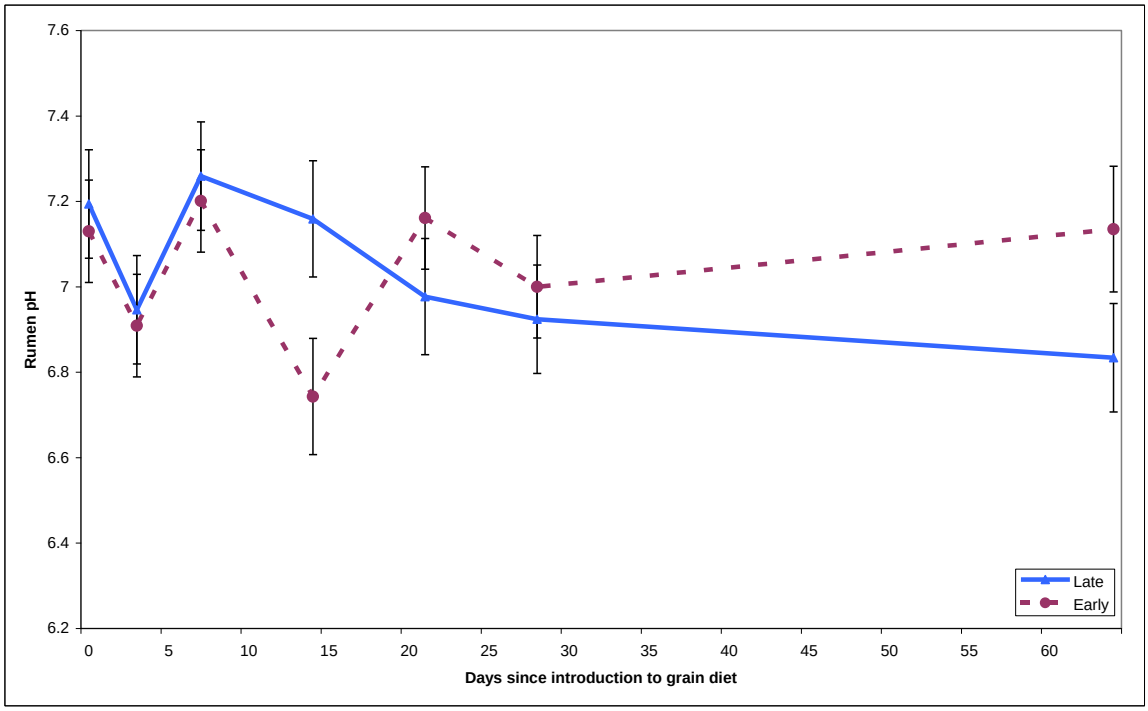


Figure 4.1 Rumen pH (mean ±SEM) in late and early calved cattle introduction to grain during feedlot at Vasse Research Centre.

The rumen pH (Figure 4.1) for both the EC weaners (6.7-7.2) and the LC weaners (>6.6) were within a normal range throughout the introduction period, although rumen pH decreased in all cattle on day 3. It should be noted that these pH values are on the higher

side and possibly may in fact have resulted from saliva contamination. The brass attachment as outlined in chapter 2.4 was not used for this project. There were no significant differences between the two calving groups, date of sampling or the interaction of group and date sampled (P>0.05). The rumen pH in the early calving animals was significantly correlated with *P. ruminicola* populations (P<0.05), during the introduction period. Rumen pH in the late calved weaners was not significantly correlated to any of the other rumen parameters. However, the rumen pH on days 0 and 7 during the introduction period was significantly higher that the final measured pH on day 64 (LSD 5%) but all pH values were within the normal range.

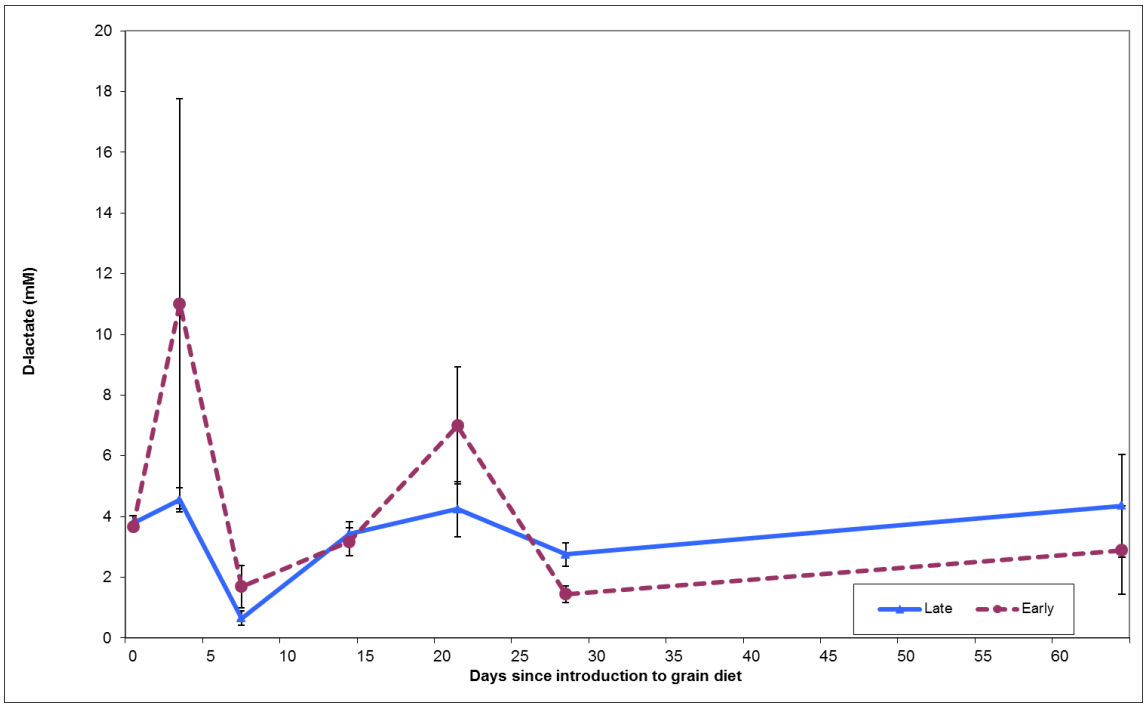


Figure 4.2 D-lactate concentrations (mean ±SEM) in the rumen of late and early calved cattle after introduction to grain during feedlot at Vasse Research Centre.

The rumen D-lactate concentrations (Figure 4.2) followed a similar trend over time to rumen pH. There were no significance differences between the calving groups (P>0.05). However, there is a significant difference in D-lactate concentrations between the dates sampled (P<0.05). Overall there was not a significant difference between the

interaction of group or date sampled (P>0.05). However, the D-lactate concentrations increased on day 3 and had returned to day 0 concentrations when sampled at day 14.

The early calved weaners D-lactate concentrations were significantly correlated to total lactate (R=0.87) and *S. ruminantium* populations (R=0.33) (P<0.05). In the late calved weaners, ruminal D-lactate concentrations were significantly correlated to the populations of *P. ruminicola* (R=-0.36) (P<0.05).

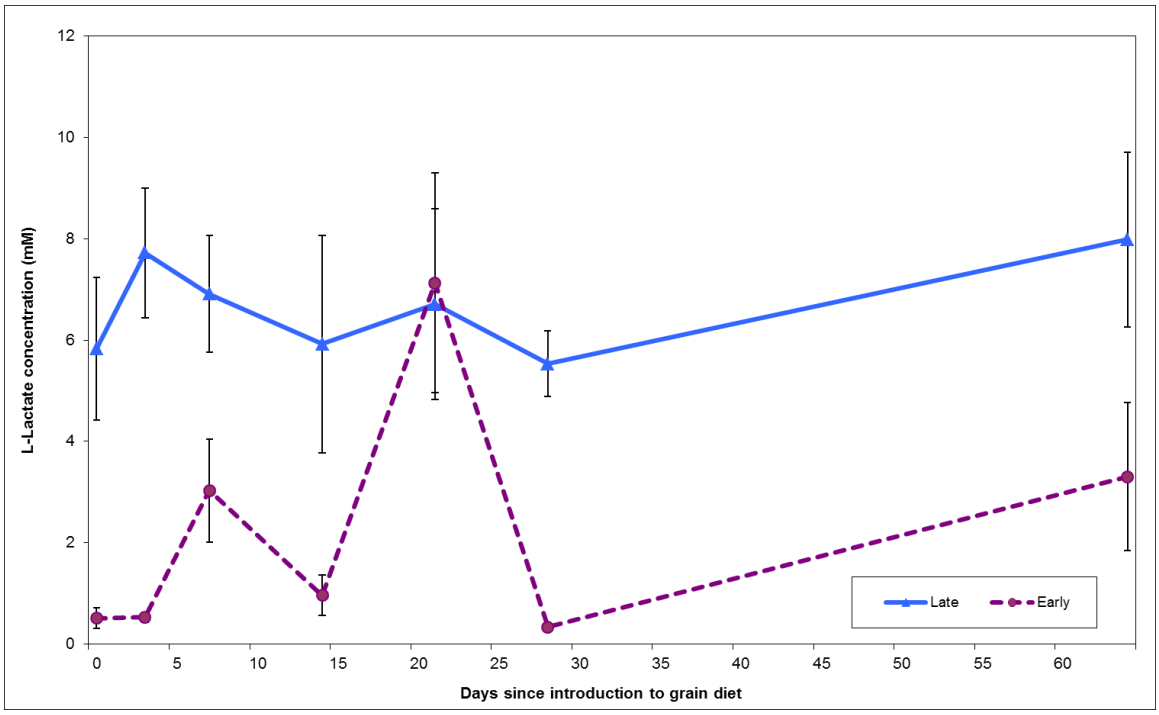


Figure 4.3 L-lactate concentrations (mean ±SEM) in the rumen of late and early calved cattle after introduction to grain during feedlot at Vasse Research Centre.

The L-lactate concentrations (Figure 4.3) in the rumen of late calved weaners were relatively constant throughout the sampling period while the early calved cattle had large fluctuations in the L-lactate concentrations. There was a significant difference between the groups with cattle from the late calving group always higher in L-lactate concentrations, and on the date sampled and the interaction between the calving groups and date sampled (P<0.05).

L-lactate concentrations were significantly correlated to protozoa numbers (R=0.21) (Figure 4.10) and D-lactate concentrations (R=0.22) (Figure 4.2) (P<0.05) in the cattle from the early calving group. During the sampling period there was significant differences in the sampling days (P<0.05). The LC cattle L-lactate concentrations (Figure 4.3) were significantly correlated to D-lactate concentration (R=0.36) (P<0.05), with no significant differences between sampling days.

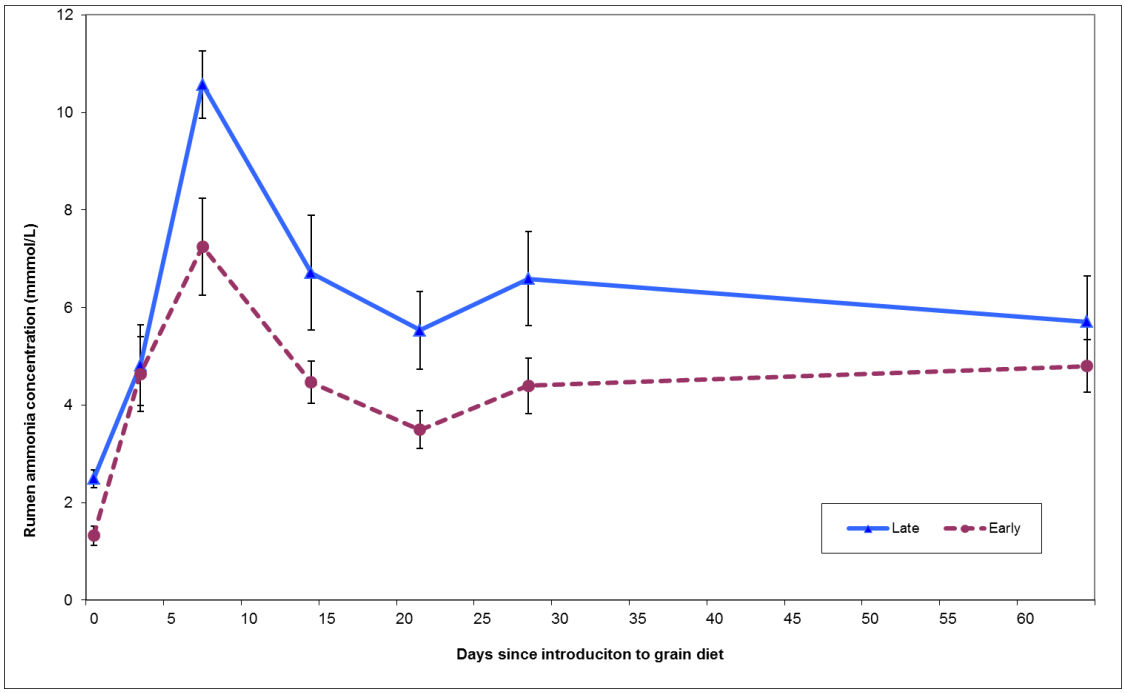


Figure 4.4 Rumen ammonia concentrations (mean ± SEM) in the rumen of late and early calved cattle after introduction to grain during feedlot at Vasse Research Centre.

The rumen ammonia concentrations (Figure 4.4) increased sharply until day 8, after which there were significant differences between the groups in rumen ammonia concentrations as well as differences in the dates the samples were collected (P<0.05). However, there were no significant differences between the interaction of the group and date sampled (P>0.05)

The rumen ammonia concentrations during the grain introduction for the EC weaners showed that day 0 was significantly lower than the remainder of the sampling

period (LSD 5%).There was a significantly correlation between the rumen ammonia concentrations and protozoa populations($P<0.05$). In the late calved weaners, rumen ammonia concentrations on day 0 were significantly lower than the remainder of the sampling period (LSD 5%). The rumen ammonia (Figure 4.4) concentrations were significantly correlated to rumen protozoa with the EC weaners.

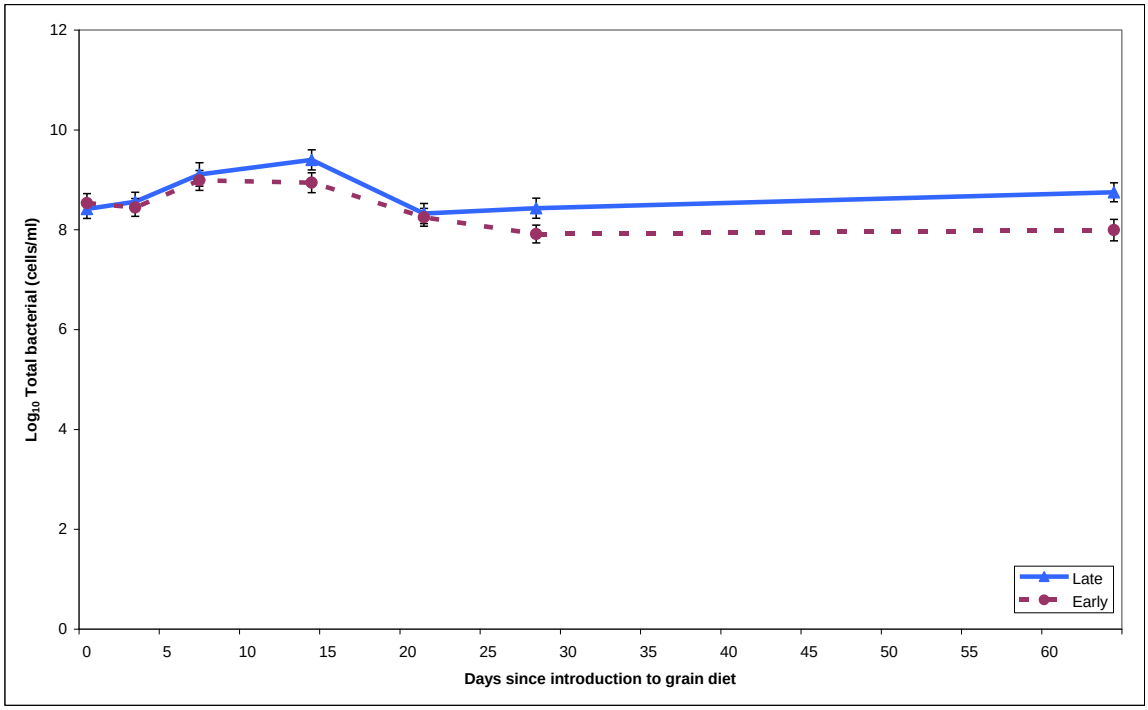


Figure 4.5 Total bacterial cells/mL (mean ±SEM) in the rumen of late and early calved cattle after introduction to grain during feedlot at Vasse Research Centre.

There are no significant differences in total bacterial cell numbers (Figure 4.5) between the 2 calving groups ($P>0.05$). The total bacterial populations in the EC weaners increased from day 0 to 4 ($P<0.05$) then remained constant.

The total rumen bacterial populations in the LC weaners showed that day 0 was lower than day 7 and 14 (LSD 5%) while day 3 was lower ($P<0.05$) than day 7.

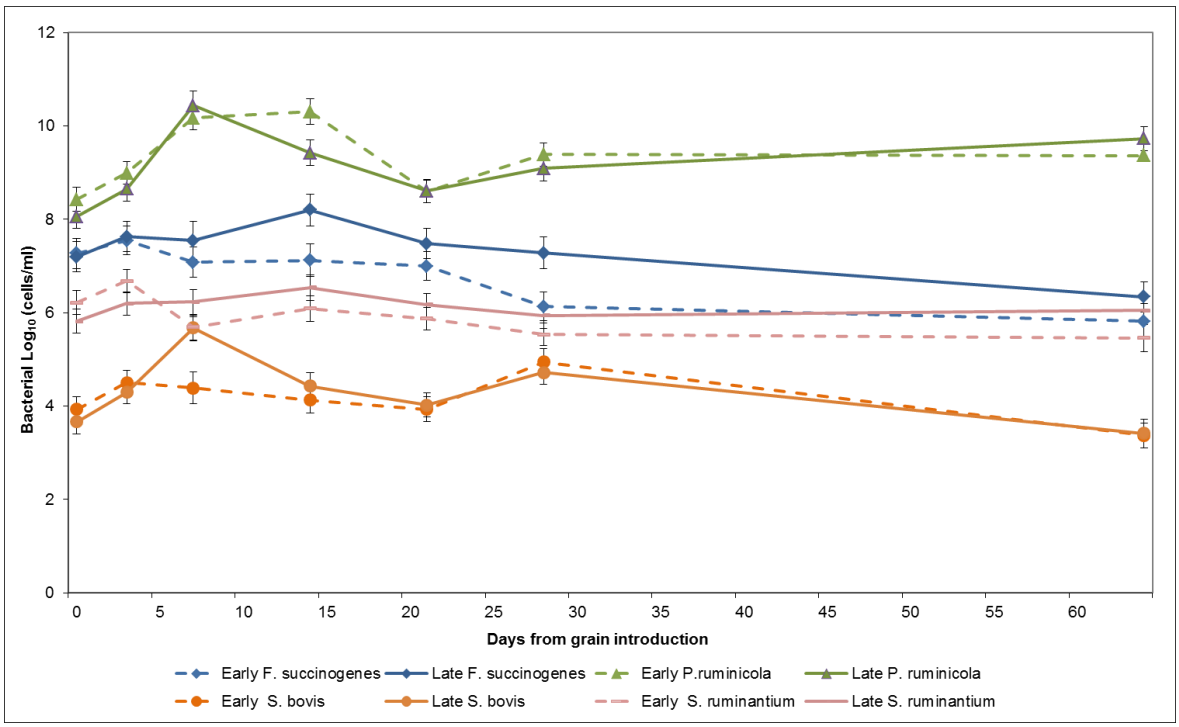


Figure 4.6 The populations of *Fibrobacter succinogenes*, *Selenomonas ruminantium*, *Streptococcus bovis*, *Prevotella ruminantium* cells/mL (mean ±SEM) in the rumen of late and early calved cattle after introduction to grain during feedlot at Vasse Research Centre.

The *F. succinogenes* population cells/mL (Figure 4.6) showed a steady decline over time, with a difference between the two calving groups and the dates samples were collected ($P<0.05$). However, there were no significance differences between the interaction of the group and the date sampled ($P>0.05$).

The populations of *F. succinogenes* (Figure 4.6) taken during the sampling on the grain based diet showed the LC steers were different and greater in population than the EC steers on days 14 and 28 ($P<0.05$). During the sampling period, the *F. succinogenes* rumen populations in the early weaned group were lower at day 0 to 3. In the EC weaners group, *F. succinogenes* cells/mL (Figure 4.6) populations were correlated to the *P. ruminicola* cells/mL ($R=0.32$) (Figure 4.6), *S. bovis* cells/mL ($R=0.46$) (Figure 4.6) and *S. ruminantium* cells/mL ($R=0.73$) (Figure4.6) populations ($P<0.05$).

The LC weaners samples indicated that *F. succinogenes* populations (Figure 4.6) were on day 0 lower to the population at day 14 (LSD 5%), while days 3, 7, 14, 21 and 28 were higher to day 64 (LSD 5%). In cattle from the late calved group, the *F. succinogenes* population was significantly correlated to *S. bovis* cells/mL (R=0.60) (Figure 4.6) and *S. ruminantium* cells/mL (R=0.76) (Figure 4.6) ($P<0.05$).

The *S. ruminantium* population (Figure 4.6) remained constant over the sampling period, with no significant differences between the two calving groups ($P>0.05$). However, there is a significant difference between the dates samples were taken ($P<0.05$). The interaction of calving group and date sampled was also not different ($P>0.05$).

The *S. ruminantium* population was higher (LSD 5%) for the LC weaners on day 64. The LC weaners on day 0 had y lower *S. ruminantium* populations than on day 14 (LSD 5%). In the LC weaners *S. ruminantium* population was significantly correlated to other bacterial populations *F. succinogenes* (R=0.76), *S. bovis* (R=0.53) ($P<0.05$).

The EC weaners *S. ruminantium* population indicated that day 0 was significantly lower to days 28 and 64. The *S. ruminantium* population was significantly correlated to *F. succinogenes* (R=0.77) ($P<0.05$).

The *P. ruminicola* populations (Figure 4.6) were not different between the calving groups and there was no significant the interaction of the groups and the date sampled ($P>0.05$). The *P. ruminicola* bacterial populations were similar for the two calving periods, with only a small significant difference at day 14 of sampling (LSD 5%).

In the EC weaners *P. ruminicola* populations on day 0 were lower than days 7, 14, 28 and 64 ($P<0.05$).

The LC weaners showed that during the introduction *P. ruminicola* population increased then decreased at day 28 then remained constant. The LC *P. ruminicola* population was correlated to rumen ammonia *S. bovis* (R=0.51) (Figure 4.6) ($P<0.05$).

The *S. bovis* population (Figure 4.6) was not significantly different between the two calving groups, the date sampled, nor was there any the interaction of the calving group (P>0.05). The only significantly difference between the two times of calving was *S. bovis* population cells/mL at day 7 (LSD 5%).

The EC weaners *S. bovis* at day 0, 14 and 21 were lower than day 28 (LSD 5%) while days 3, 7 and 28 were significantly different to day 64 (LSD 5%) the EC weaners *S. bovis* population was significantly different to the total bacterial population, *F. succinogenes, S. ruminantium* and *P. ruminicola* (P<0.05).

The LC weaners *S. bovis* population at day 0 was lower than (P<0.05) day 7 and 28, while day 3 was significantly higher than day 64 (LSD 5%). Day 7 was significantly different to days 14, 21, 28 and 64 (LSD 5%) while days 14 and 28 were both significantly different to day 64 (LSD 5%). The LC weaners *S. bovis* population was also correlated to the *F. succinogenes* cells/mL (R=0.60)

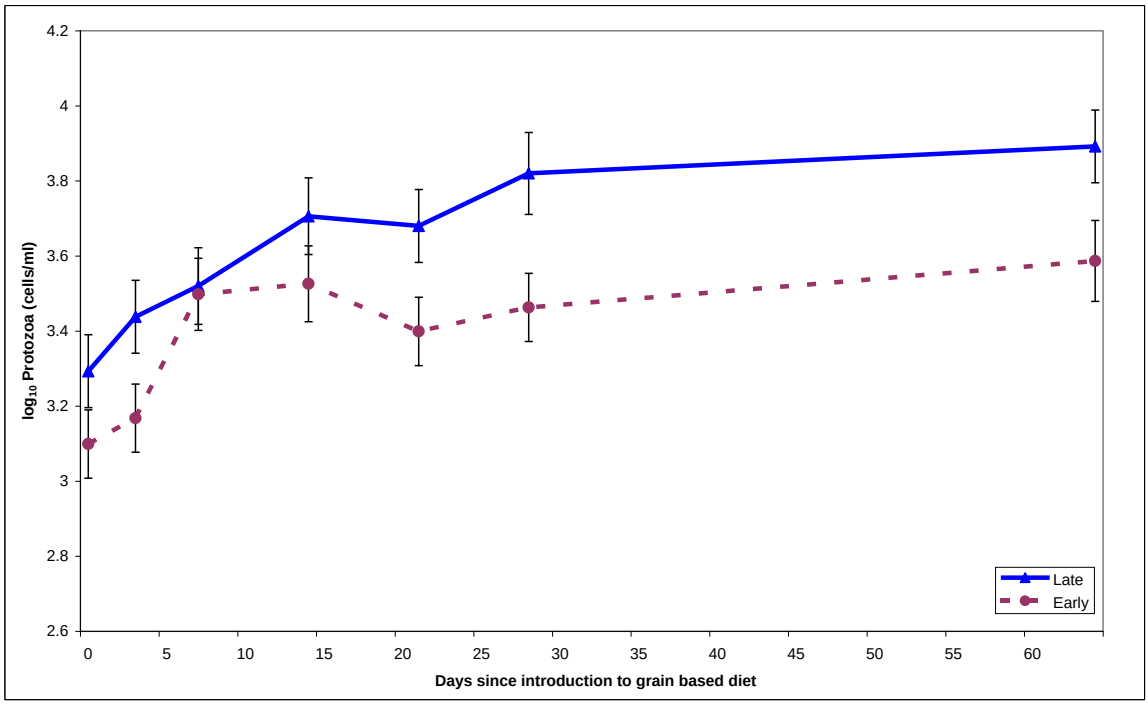


Figure 4.7 Protozoa populations in cells/mL (mean ± SEM) during grain introduction for late and EC cattle after introduction to grain during feedlot at Vasse Research Centre.

The protozoa concentrations (Figure 6.7) increased over the sampling period and were significantly different between the two calving groups as well as the dates they were sampled ($P<0.05$), however there was no significant difference between the calving group and the sampling date ($P>0.05$).

The protozoa counts from the rumen samples taken from the weaners of the two different times of calving that all days apart from days 7 and 14 were different between the two groups (LSD 5%). The EC weaners' protozoa samples from days 0 and 3 were significantly different to all other samples taken during the adaptation period (LSD 5%). The EC protozoa populations also had a significant correlation to the rumen ammonia ($P<0.05$).

The LC weaners protozoa populations at days 0 and 3 was lower than days 14, 21, 28 and 64 (LSD 5%) while day 7 was lower to day 64 (LSD 5%). The LC weaners protozoa numbers over the adaptation period were correlated to the rumen ammonia concentrations ($R=0.41$) ($P<0.05$).

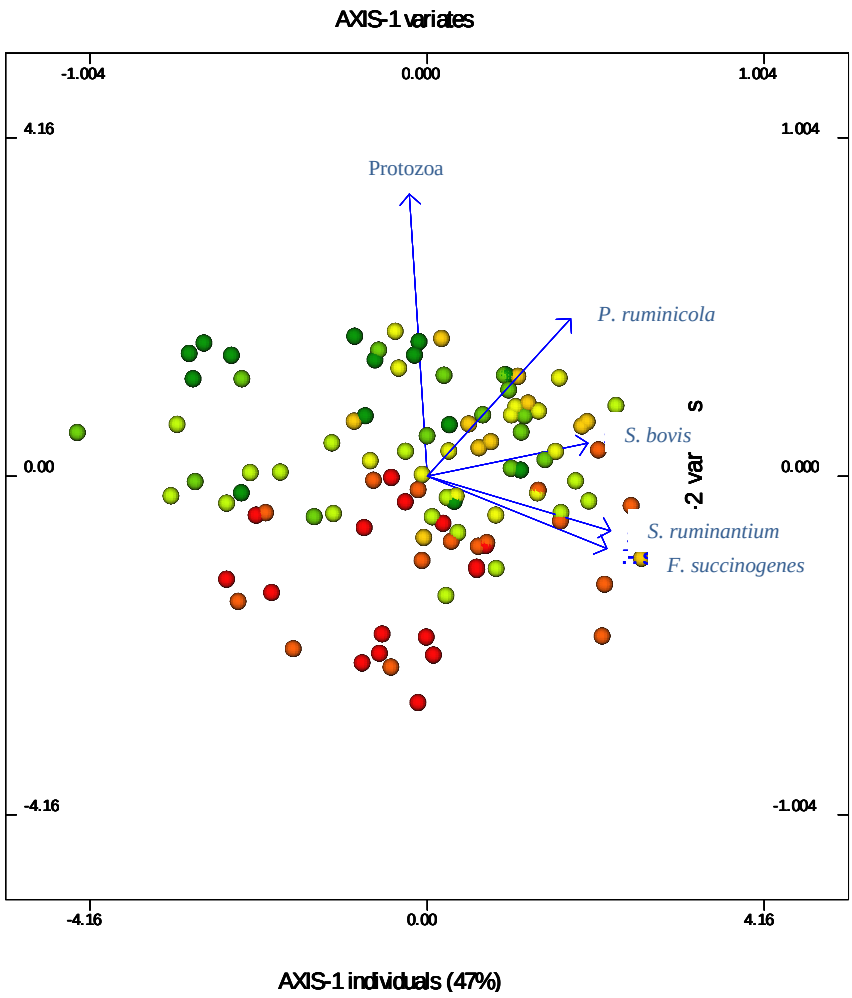


Figure 4.8 Biplot representing the 70% of correlations of log transformed bacterial populations in cattle after introduction to grain during feedlot at Vasse Research Centre. Red – samples day 0 to dark green samples at day 64.

The biplot (Figure 4.8) indicates that the *S. ruminantium* and *F. succinogenes* populations were associated. *S. bovis* populations were more closely associated to *S. ruminantium* and *F. succinogenes* populations than the *P. ruminicola* populations. Protozoa populations were independent of the bacterial populations assessed.

4.4 **Discussion**

The interesting features from this study were the sustained differences observed between cattle in the late weaned group as distinct from the early weaned group in rumen

microbial ecology of *Fibrobacter succinogenes* and the protozoa populations and rumen parameters of. L-lactate and ammonia. On the other hand, there were no differences in rumen pH, D-lactate, and total rumen bacterial populations. Thus the pre-weaning dietary environment where the early weaned group was supplemented with hay and the later weaned group was weaned onto green pastures had a lingering effect on rumen microbial ecology and physiology in the weaned animals fed grain diets.

The work done by Li *et al.* (2012) indicates that in pre-ruminant calves of varied age their microbiota was of considerable heterogeneity during early development with all functional classes of bacteria between the two groups being markedly similar. This lays a solid foundation for the transition of pre-ruminant to ruminant. The development of the rumen of newborn calves is influenced by the consumption of dry feed (Anderson *et al.*, 1987), therefore the impact of what may have only been a few months of difference in available roughage only of varied quality was not enough to have a long term impact on the rumen populations of these two times of calving. The diets may slightly impact on this but the bacterial populations in essence are set and just change with the introduction of different dietary components. Therefore, it indicates here that although the cattle were calving onto different diets there appears to be no long term effect on the ability for these weaners to go onto a grain based diet. This may however be different if the early weaners had stayed on a dry lower plane of nutrition and the late on a higher plane of nutrition prior to the introduction of a grain based diet. This trial importantly highlighted that an introduction onto a grain based diets does not require feed additives provided care is taken with the ration formulation and the roughage content is kept high in the ration.

The rumen pH values were all at the upper end of the normal range (> pH 6.7) throughout the sampling, possibly because there was no brass attachment used on the end of the sampling tube to ensure that the end of the tube dropped to the bottom of the rumen.

Thus there may well have been higher concentrations of saliva than in the other trials, although saliva contamination was checked. Since this technique was consistent over the sampling period, all samples would still be analysed to assess the linkages between rumen pH and other rumen parameters.

The D-lactate concentrations decreased in both groups on day 8 and returned to the original concentrations for the remainder of the sampling period. Overall the D-lactate concentrations were low and not indicative of acidosis. The L-lactate concentrations were constant for the late-calved weaners. On the other hand, L-lactate concentrations in EC weaners although lower showed large variation in concentrations with fluctuations throughout the sampling.

The rumen ammonia (Figure 4.4) concentrations increased as the grain content in the diet increased; although the groups were different in absolute concentrations the trends were the same for the two groups. Rumen ammonia concentrations were correlated to an increase in the *P. ruminicola* and *S. bovis* populations and importantly the more frequently observed correlation of an increase in protozoa populations concentrations (Dehority, 2003) when ruminants have increased protein availability in the diet. Throughout the grain feeding period that samples were taken, there was no significant difference for the total bacterial population for the two calving times with some differences between the days sampled. This observation did not support the hypothesis that time of calving in this instance impacted on the rumen microbial ecology. In fact, good management practices during grain introduction led to no major increases or decreases in the rumen bacterial population. The *F. succinogenes* populations decreased during the feeding period for both groups with the early calvers decreasing at a faster rate after day 3. The decrease in the fibre degrading bacteria supported the hypothesis that *F.* succinogenes decreases with increases in grain content but in this case was not associated with a reduction in rumen pH.

The *S. ruminantium* populations were not significantly different between the two groups but the biplot (Figure 4.11) data indicated a strong relationship with the *F. succinogenes* populations. There is some evidence to suggest that some isolates of *S. ruminantium* can cooperate with *F. succinogenes* in fibre digestion (Sawanon *et al.*, 2011) which may be evident in these samples.

The *P. ruminicola* populations were not significantly different between the two times of calving groups. Populations in both groups increased until day 8 then returned to the same concentrations as the first day of grain feeding. The *Prevotella* populations were the most abundant in the rumen and were significantly correlated to the rumen populations of the other bacteria and rumen parameters. This was suggestive of consistent changes with minimal deleterious changes in the rumen. Moreover, in the case of these two groups of cattle, the slow introduction of grain without any rumen modifiers was indicative of a very successful introduction of grain in the high energy diets.

The *S. bovis* populations increased for the late time of calving group at day 8 but there were no significant differences between the calving groups. Again this finding indicated that there was not excessive growth of this lactic acid producer. Moreover, these population changes in *S. bovis* were linked to low lactate concentrations and maintenance of the rumen pH in a normal range.

The protozoal numbers in these samples indicated that LC animals that were born onto high quality pastures had a higher level of protozoal populations during grain feeding. Overall it has been noted in other studies that protozoal populations increased with an increase in the proportion of concentrate within a ration (Leng *et al.*, 1980; Dehority, 2003). Work done by Belanche *et al.* (2011) indicated that as in this study protozoal increases are associated with increase in rumen ammonia concentrations. In accord with this finding, the later calved cattle demonstrated a higher protozoa cells/mL and a

significantly higher rumen ammonia concentration. Studies (Purser and Moir, 1966) have proposed that calves born on to readily fermentable high quality pastures also had higher protozoal densities. It is interesting to note that even when on the same diet in feedlot protozoal numbers in the EC cattle never reached those of the later calved cattle throughout the feedlot period It is documented that calves acquire rumen protozoa from adults during grazing (Coleman, 1980) therefore impacting on the abundance of rumen protozoa at early stages of production prior to these weaners being placed onto a high concentrate feedlot ration. Monitoring of the bacterial and protozoal populations under the pasture grazing system would have given a better overall picture of how the rumen microbial population was set up over time.

Overall cattle from the two calving times showed very successful adaptation to grain introduction without any obvious signs of rumen dysfunction. The measured metabolic indicators show that the total energy values of volatile fatty acids and rumen ammonia were ideal for cattle in this growth phase. The other important finding from this data showed that even when there is a decrease in cellulytic bacteria such as the decreasing populations of *F.succinogenes* or an increase in lactate producing bacteria such as population of *S. bovis,* this is not always indicative of subclinical or clinical acidosis. The rumen pH remained at what could be classified as safe normal concentrations throughout the introduction and transition to grain diets for both calving groups and D- and L-lactate concentrations remained low throughout the grain feeding period. In particular the data here does not support the time of lambing study of Al Jassim *et al.* (2003) in sheep on dry versus green pastures. Therefore, from this study, the time of calving onto either dry pasture or green pasture did not have sustained or deleterious impacts on rumen microbial ecology or metabolism when the cattle were subsequently introduced to high grain diets in feedlots, if reasonable care was taken during that introductory period.

5 Changes in the rumen microbial population of dairy cattle sampled in Australian herds.

5.1 Introduction

Previous chapters have used quantitative RT-PCR to show the relationships between parameters of rumen physiology and metabolism and changes in rumen microbial ecology during dietary transition in commercial beef feedlots, the feeding of lupins and soybeans to sheep and the effect of time of calving. In this chapter, rumen samples were obtained from a study by (Bramley *et al.*, 2008) which monitored commercial dairy herds under various nutritional regimes and tested the relationships between various indicators of acidosis such as low rumen pH, elevated D and L - lactate concentrations and other parameters of rumen metabolism such as the concentration and profile of volatile fatty acids. However changes in rumen microbial ecology had not been assessed in these dairy cattle. Therefore, these samples were analysed using molecular techniques to determine if these traditional metabolic indicators of acidosis were associated with impacts on the rumen bacterial populations of *S. bovis* and *Lactobacillus spp. S. ruminantium, P. ruminicola* or *F. succinogenes.*

The aims of this chapter were to use qRT-PCR to assess these rumen samples and determine changes in the ecology or populations of key indicator bacterial populations represented in a dairy herd. Ecological changes were then linked to various indicators of physiological and biochemical (e.g. D-lactate) acidosis.

It is hypothesised that:

1. The addition of any feed additive such as antibiotics or ionophores will reduce the incidence of acidosis through changes in the bacterial ecology established in the rumen.

2. Fibre utilising cellulolytic rumen bacteria (*Fibrobacter succinogenes*) populations are lower during grain feeding or low rumen pH.
3. Lactic acid utilising rumen bacteria (*Selenomonas ruminantium*) populations increase in herds with a higher grain component of the diet.
4. *Prevotella ruminicola* will be the most prevalent bacteria in the rumen samples of those screened.
5. *Streptococcus bovis* will be higher (cells/mL) in cattle with subclinical or clinical acidosis in dairy cattle fed grain-based diets.
6. Higher levels of *S. bovis* are linked with low ruminal pH, and high *Lactobacillus spp.*
7. Metabolic changes in the rumen can be related to changes in rumen molecular ecology in cattle.

5.2 **Materials and Methods**

Full sampling procedures of the dairy herds are outlined in (Bramley *et al.*, 2008). A brief outline is listed below: One hundred commercial herds were selected from 5 areas in NSW and Victoria, from these herds a subsample of herds (n=12) were selected for analysis of the rumen microbial populations. Eight animals were randomly sampled from each of these twelve herds using a random number chart according to these two criteria: They were lactating cows, in the first 100 days of lactation the total sample consisted of three primiparus and five multiparus animals.

Animals were sampled 2-4 days after milking, if they were fed on concentrate in bail or 4-6 days after feeding a total mixed ration outlines in table 7.1. They were given access to water if possible during this time. (Bramley *et al.*, 2008).

5.2.1 **Rumen parameters**

All parameters and dietary analysis information are outlined in (Bramley *et al.*, 2008) including volatile fatty acid analysis, D and L-lactate and rumen ammonia concentrations analysed in the collected rumen samples.

5.2.2 **DNA extraction and quantitative Real Time PCR (qRT- PCR)**

Extraction of DNA was undertaken as outlined in chapter three and the qRT-PCR was undertaken as outlined in chapter four.

5.2.3 **Statistical analysis**

It must be noted and acknowledged that all biochemical and physiological data except bacterial populations were sourced from the Bramley *et al.* (2008) data set.

Residual plots were examined to ensure that statistical tests complied with assumptions of normality and homogeneity of variance. Where necessary data was transformed to ensure that this was the case. The bacterial populations for each species quantified were log-transformed (log_{10}) prior to statistical analysis, with total bacterial population also log-transformed (log_{100}).

- Firstly, the data was analysed in herd categories (n=12) to determine what were the main factors that differentiated these herds (Table 5.1).
- Secondly the data was analysed as one full data set (n=95) irrespective of herds to determine if there were any linkages between measurements and also the impact of addition of feed additives.
- Thirdly the data was analysed based on a cluster analysis as undertaken by (Bramley *et al.*, 2008) for the sub samples to determine if the selection based on the physiological measures was linked to variations in bacterial populations. Please note that the results in this Masters are only a subsample of those represented in Bramley's paper.

- Finally, the data was then analysed based on rumen pH categories to determine if the linkage of pH was related to variations in rumen bacterial populations.

Correlations between variates were compared to zero using a two-sided test. The matrix of correlations between logarithms of the counts of individual bacteria was used to construct a biplot which showed the variation in the relationships between bacterial counts in samples collected.

Herd and pH category means were compared using ANOVA for all comparisons apart from the cluster analysis which due to its uneven sample numbers was analysed using a REML analysis with herd as the random effect. Samples were used to calculate averages at each sample date which were compared using 5% least significant differences (5% LSD).

5.2.4 **Herd feed rations**

Table 5.1 Outline of feed rations for each of the 12 herds sampled subsample (Bramley *et al.*, 2008) ranking from increasing forage and decreasing concentrate in rations. Shading indicates no ionophores or antibiotics in the ration.

Herd	***Forage %***	***Conc %***	***Total diet energy MJ ME/kg DM***	***Total diet CP%***	***Pasture CP%***	***Total diet NDF%***	***Pasture diet NDF%***	***% sugar in pasture***	***Ration Components***	***Ration Additives***
74	30.5	69.5	11.4	18.3	20.8	23.5	46.3	10.2	5.2kg DM pasture, 9.08kg wheat and 1.75kg of cotton seed meal	Monensin (315mg), bicarbonate (126mg) and limestone (378g)
83	46.6	53.4	10.8	18.1	N/A	42.7	N/A	N/A	9.29kg DM silage, 5.05kg triticale, 0.08 urea, 2.74kg brewers grain	illrun(1.71), monensin (250mg), tylon (150mg) and limestone (200g)
9	48.5	51.5	11.7	19.1	25.2	26.6	31.5	23.6	0.92kg straw, 7.71kg DM pasture and 1.62kg DM silage, 9.35kg wheat	0.03% oil/fat and monensin (200mg)
61	53.9	46.1	10.4	18.2	18	38.8	52.7	9.1	5.93kg DM pasture, 1.33kg DM silage, 3.54kg hay, 5.22kg triticale, 0.04kg wheat and 0.02kg cotton seed, 0.08kg safflower, 3.29kg barley	Illrun (0.46) and 0.01% of molasses and oil/fat
8	58.0	42.0	9.2	12.5	26.2	38.9	33.7	32	3kg DM pasture, 1.9kg straw and 2.4kg hay, 1.73kg barley, 0.53kg corn, 0.63kg triticale and 1.75kg wheat	oil and fat (0.04%) and addition of monensin (156mg), limestone (38.4g), Agox (14.4g) and bentonite (120g)

89	58.7	41.3	10.7	19.2	23.7	35.9	43.1	14.4	4.55kg DM pasture, 3kg DM silage, 4kg DM hay, 6.75kg triticale, 0.63kg canola meal, 0.71kg faba bean	Monensin (163mg), limestone (40g) and Agox (15mg)
16	58.8	41.2	10.4	15.2	16.7	28.5	36	30.6	10.99kg DM pasture, 6.64kg barley and 0.63kg canola	Monensin (240mg), virginiamycin (200mg), limestone (59.5g), acid buffer (28g) and Agox (22g)
5	62.3	37.7	11.3	20.6	29.4	28.1	40.7	14.9	9.28kg pasture, 5.02kg DM hay, 0.46kg DM straw and 8.6kg wheat	Monensin (450mg), virginiamycin (250mg) and bicarbonate (70)
38	73.8	26.2	10.3	22.5	24.2	36.7	44.6	11	15.7kg DM pasture, 4.27kg wheat, 0.54kg canola and 0.56kg of lupins	Monensin (200mg), virginiamycin (250mg) and bicarbonate (27g)
6	76.7	23.3	10.8	19.5	32.2	34	32.1	14.2	8.15kg DM pasture, 3.17 kg barley, 1.07 kg wheat	Bicarbonate (70g), limestone (285g) and Agox (19.3g)
98	77.0	23.0	10.7	21.2	24	36.2	42.3	16.6	10.23kg DM pasture 0.45kg barley, 0.6kg corn, 0.15kg rice, 0.45kg sorghum, 0.43 triticale, 0.15 faba bean, 0.24kg safflower and 0.16kg corn-starch	molasses, monensin (170mg), limestone (57.8g), acid buffer (17g) and Agox (17g)
12	90.3	9.7	10.7	23.8	26.2	29.2	29.3	8.5	14.28kg pasture, 2.37kg hay and 1.78kgWheat	No additives

5.3 **Results**

All samples were analysed originally as herds (n=12) then as a single data collection irrespective of herd and dietary intake (n=95). This was undertaken to increase the size of the database as samples were only taken at one point in time rather than over a period of time. Pooling the data should enable a better indication of relationships that may be linked to the rumen bacterial population and to determine if rumen pH or other physiological parameters impacted on the rumen bacterial population. Thirdly all samples were analysed as categories 1, 2 or 3, based on the cluster analysis from Bramley *et al.* (2008) (Table 7.5), then finally they were analysed based on 3 pH categories (low, medium and high; Table 7.6).

5.3.1 **Herd analysis**

A subsample of 12 herds as outlined in (Bramley *et al.*, 2008) were analysed, herds outlined below are listed from lowest to highest forage percentage in the ration.

5.3.1.1 Herd 74

Cattle in Herd 74 were fed the lowest forage component in their diet consisting of 30.5% forage consisting of 5.2kg of pasture of the following analysis: 18.3% crude protein, 11.4 MJ ME/kg DM and 23.5% NDF and 69.5% concentrate consisting of 9.08kg wheat and 1.75 of cotton seed meal with the addition of monensin (315mg), bicarbonate (126mg) and limestone (378g) the highest of any herd. Herd 74 had the highest *P. ruminicola* population and second highest populations of *F. succinogenes* (even though it was one of the lowest forage contacting rations) and *S. ruminicola* of the herds analysed at this one point in time. The *S. bovis* population was significantly correlated to the butyric acid concentration (R =0.96) (Table. *P. ruminicola* was correlated to the iso-butyric acid (R=0.96) and *S.*

ruminantium to the D- lactate concentration in the rumen (R = -0.97). There were no correlations between the key bacterial populations monitored in this herd.

5.3.1.2 Herd 83

Herd 83 was fed a diet consisting of 46.5% forage which constituted 9.29kg DM of silage; 18.1% crude protein, 10.8 MJ ME/kg DM and 42.7% NDF with 53% concentrate including 5.05kg triticale, 0.08 urea, 2.74kg brewers grain and 1.71 illrun, monensin (250mg), tylon (150mg) and limestone (200g) added into the diet ($P<0.05$).

The rumen pH was significantly correlated to the bacterial populations of *S. bovis* ($R=0.76$) ($P<0.05$) and *S. ruminantium* ($R=0.73$).

The *F. succinogenes* population was significantly correlated to the acetic ($R=0.86$), propionic ($R=0.85$) and valeric acid ($R=0.84$) and also the *S. ruminantium* population ($R=0.89$) ($P<0.05$).

The *S. bovis* population was significantly correlated to the iso-butyric ($R=0.78$), butyric concentrations ($R=0.85$), rumen pH ($R=0.76$) and D-lactate concentrations ($R=0.77$) ($P<0.05$). The *S. ruminantium* population was significantly correlated to the acetic ($R=0.93$), iso-butyric ($R=0.86$), butyric ($R=0.83$), valeric ($R=0.87$) and total volatile fatty acid concentrations ($R=0.95$) ($P<0.05$) as well as the *F. succinogenes* population ($R=0.89$) ($P<0.05$).

The *P. ruminicola* population was significantly correlated to the rumen acetic ($R=0.80$), propionic ($R=0.79$), iso-valeric ($R=0.73$), valeric ($R=0.84$) while the *Lactobacillus spp.* were correlated to the rumen ammonia concentration ($R=0.72$) ($P<0.05$).

Overall this herd had bacterial populations which were average for all bacterial populations analysed.

5.3.1.3 Herd 9

Herd nine was fed a diet of 48.5% forage (0.92kg straw, 7.71kg DM pasture: 19.1% crude protein, 11.7 MJ ME/kg DM and 25.65% NDF and 1.62kg DM of silage) and 51.5% concentrate, consisting of 9.35kg wheat, 1.35kg canola meal and 0.03 oil/fat and monensin (200mg/cow/day)

The bacterial populations of *F. succinogenes* in cows on this ration were significantly correlated to the total bacterial population (R=0.98) ($P<0.05$). The *S. bovis* bacterial populations were not correlated to the other rumen bacterial populations. The *P. ruminicola* populations were significantly correlated to iso-valeric acid (R=-0.82) concentrations and total bacterial population (R=0.96) ($P<0.05$). The *Lactobacillus spp.* were significantly correlated to the *S. ruminantium* ($R^{=}$-0.99) and total bacterial population (R=-0.95).

This herd had a low rumen pH of 5.48 one of the lowest total rumen bacterial populations as well as the lowest *F. succinogenes, P. ruminicola* and *S. bovis* population levels.

5.3.1.4 Herd 61

Herd 61 was consuming a diet of 54% roughage (5.93kg DM of pasture: 18.2% crude protein, 38.8% NDF 1.33kgDM silage and 3.54kg DM of hay) with 46% concentrate consisting of 5.22kg triticale, 0.04kg wheat and 0.02kg cotton seed, 0.08kg safflower, 3.29kg barley and 0.46 illrun and 0.01 of molasses and oil/fat per cow.

The *F. succinogenes* population was significantly correlated to the total bacterial population (R=0.88) as well as the *S. ruminantium* population (R=0.97) ($P<0.05$). The *P. ruminicola* population was significantly correlated to the D- lactate concentration (R=0.88) while the *Lactobacillus spp.* population was significantly correlated to the *S. ruminantium* population (R=0.90) ($P<0.05$).

Herd 61 had the lowest total bacterial population levels of all the herds analysed and one of the lowest *P. ruminicola* populations.

5.3.1.5 Herd 8

Herd eight was consuming 52% forage (3kg DM pasture: 12.5% crude protein, 9.3 MJ ME /kg DM and 8.9% NDF 1.9kg DM straw and 2.4kg DM hay. The 48% concentrate consisted of barley (1.73 kg), corn (0.53 kg), triticale (0.64 kg) and wheat (1.75 kg) with the addition of some oil and fat and addition of monensin (156mg/cow/day), limestone (38.4g), Agox (14.4g) and bentonite (120g) to the ration.

The *P. ruminicola* bacterial populations were significantly correlated to butyric acid (R= -0.85), acetic acid (R= -0.85) and D-lactate concentrations (R= -0.88), while *S. ruminantium* populations were correlated to the *S. bovis* populations (R=0.95) ($P<0.05$). The *Lactobacillus spp.* population was correlated to the iso-butyric acid concentrations (R=-0.82)

This herd had the lowest pH value of all herds in this analysis with the highest *S. bovis* population and *S. ruminantium* population of all herds with a high *F. succinogenes* and average *Lactobacillus spp.* population levels.

5.3.1.6 Herd 89

Herd 89 consumed a diet of 58.8% forage (4.55kg DM pasture: 19.2% crude protein, 10.7 MJ ME /kg DM and 35.9% NDF 3kg DM silage and 4kg DM day) and 41.2% concentrate which consisted of 6.75kg triticale, 0.63kg canola meal, 0.71kg faba bean and the additives of monensin (163mg), limestone (40g) and Agox (15g).

The D and L-lactate concentrations were significantly correlated (R=0.98) ($P<0.05$), while the rumen pH was significantly correlated to the acidosis status (R=0.95)

and the bacterial populations of *F. succinogenes* (R=0.75) and *P. ruminicola* (R=0.81) (P<0.05).

The *F. succinogenes* population was significantly correlated to propionic acid concentrations (R=0.91) and the bacterial populations of *P. ruminicola, S. ruminantium* and the total bacterial population (P<0.05). The total bacterial population was significantly correlated to the acetic (R=-0.75), propionic (R=-0.92), caproic (R= -0.85) acid concentrations (P<0.05). The total bacterial population was also correlated to the bacterial populations of *F. succinogenes* (R=0.77), *Lactobacillus spp.* (R=0.74), *P. ruminicola* (R=0.96) and *S. ruminantium* (R=0.84).

The *S. ruminantium* population in herd 89 was correlated to the total bacterial population, *F. succinogenes* (R=0.87), *P. ruminicola* (R=0.91) and the concentrations of caproic ($R^{=}$0.89), propionic (R=0.86) and valeric acids (R=-0.77).

Herd 89 had one of the lowest rumen pH (5.74) the highest *S. bovis* and lowest *Lactobacillus spp.* populations with average population levels of other rumen bacterial species monitored.

5.3.1.7 Herd 16

Herd sixteen was fed a ration consisting of 59% forage (10.99kg DM pasture: 15.2% crude protein, 10.4 MJ ME/kg DM and 28.5% NDF) and 41% concentrate consisting of 6.64kg barley and 0.63kg canola with the addition of monensin (240 mg), virginiamycin (200mg), limestone (59.5g), acid buffer (28g) and Agox (22g).

The *F. succinogenes* populations in this herd were significantly correlated to the rumen pH (R=0.82), butyric acid concentration (R=0.81) and bacterial populations of *S. ruminantium* (R=0.92) and *P. ruminicola.* (R=0.94) (P<0.05). The *S. bovis* populations were not correlated to any other parameters. The *S. ruminantium* populations were

significantly correlated to butyric acid (R=0.87) as well as the bacterial populations of *F. succinogenes* (R=0.92) (P<0.05). The *P. ruminicola* populations were significantly correlated to the rumen pH (R=0.82) while the *Lactobacillus spp.* populations were significantly correlated to the rumen pH (R=0.78) and iso-butyric acid (R=0.75

Herd 16 had one of the lowest *F. succinogenes* populations and average *P. ruminicola* and *S. ruminantium* and an average *S. bovis* population in comparison to the other herds analysed.

5.3.1.8 Herd 5

Herd five was consuming a diet of 62% forage (9.28kg DM pasture; 20.6% crude protein, 11.3 MJ ME/kg DM and 28.1% NDF5.02kg DM hay and 0.46kg DM straw) and 38% wheat with the incorporation of monensin (450mg/cow/day), virginiamycin (50mg/cow/day) and bicarbonate (70g/cow/day) into the diet. The rumen samples analysed for this herd indicate that the bacterial populations of *F. succinogenes* were significantly correlated to rumen acetic acid (R=0.89), butyric acid (R=0.82), iso-butyric acid (R=0.80), iso-valeric (R=0.94) and the bacterial populations of *P.ruminicola* (R=0.95), *S. ruminicola* (R=0.92), *Lactobacillus spp*. (R=0.84) and total bacterial population (R=0.97) (P<0.05).

The *P. ruminicola* population was correlated to acetic acid (R=0.77), iso-butyric (R=0.75), total bacterial populations (R=0.97) and the other bacterial populations of *F. succinogenes* (R=0.96), *S. ruminantium* (R=0.92) and *Lactobacillus spp.* (R=0.84) (P<0.05).

The *S. ruminantium* population was significantly correlated to acetic (R=0.87), butyric (R=0.92), iso-butyric (R=0.86), iso-valeric (R=0.76), *F. succinogenes* (R=0.92) *and* total bacterial populations (R=0.89) (P<0.05).

The *S. bovis* population was significantly correlation the *Lactobacillus spp.* (R=0.79) (P<0.05) population. The *Lactobacillus spp.* was correlated to acetic acid

(R=076), butyric (R=0.83), iso-butyric acid (R=0.83), iso-valeric (R=0.72), F. *succinogenes* (R=0.86), *S. bovis* (R=0.80), *S. ruminantium* (R=0.85), *P. ruminicola* (R=0.84) and the total bacterial population (R=0.76).

The total bacterial population was significantly correlated to the acetic acid, butyric and iso-valeric acid concentrations and the bacterial populations of *F.succinogenes, Lactobacillus spp., P. ruminicola* and *S. ruminantium*.

Herd 5 had one of the lowest rumen pH values (5.4) and highest average total volatile fatty acids of all the herds, their bacterial population levels were however average in comparison to the other herds.

5.3.1.9 Herd 38

Herd 38 was fed a diet consisting of 73% forage (15.7kg DM pasture: 22.5% crude protein, 10.3 MJ ME /kg DM and 36.7% NDF) and 20% concentrate which consisted of 4.27kg wheat, 0.54kg canola and 0.56kg of lupins with the addition of monensin (200mg), virginiamycin (250mg) and bicarbonate (27g).

The *F. succinogenes* populations were significantly correlated to the bacterial populations of *S. bovis* (R=0.94), *P. ruminicola* (R=0.81) and *Lactobacillus spp.* populations (R=0.91) ($P<0.05$). The total bacterial population was significantly correlated with *S. ruminantium* (R= 0.82) The *S. ruminantium* bacterial population was significantly correlated to the total bacteria population (R=0.82), iso-butyric acid (R=0.85) and *P. ruminicola (*R=0.82) ($P<0.05$). The *P. ruminicola* population was significantly correlated to iso-butyric acid (R= 0.83) and valeric acid concentrations (R=0.82), the populations of *F. succinogenes* (R=0.78) and total bacterial population (R=0.66) ($P<0.05$) while the *Lactobacillus spp.* was significantly correlated to rumen pH (R=0.901 ($P<0.05$).

Herd 38 had the lowest *S. bovis* population and lowest total volatile fatty acid concentrations with one of the lowest *F. succinogenes* and *P.* ruminicola populations of all herds analysed.

5.3.1.10 Herd 6

Herd six was a diet consisting of 77% forage (8.15kg DM pasture; 19.5% crude protein, 10.8 MJ ME/kg DM and 34% NDF and 6.54kg DM silage) with 23% concentrate consisting of barley (3.17kg), wheat (1.07kg) and diet additives of bicarbonate (60g/cow/day), limestone (29mg/cow/day) and Agox (19.3g/cow/day). The bacterial populations of *P. ruminicola* were significantly correlated to the *S. ruminantium* populations (R=0.78) while the *S. bovis* populations were significantly correlated to the *Lactobacillus spp*. populations (R=0.81) ($P<0.05$).

Herd six had the highest rumen pH and also highest *F. succinogenes* population of all herds.

5.3.1.11 Herd 98

Herd 98 was consuming a diet consisting of 77% forage (10.23kg DM pasture: 21.6% crude protein, 10.7 MJ ME/kg DM and 36.25% NDF) with 23% concentrate. The concentrate component consisted of 0.45kg barley, 0.6kg corn, 0.15kg rice, 0.45kg sorghum, 0.43 triticale, 0.15 faba bean, 0.24kg safflower and 0.16kg corn-starch with the additives of molasses (0.08%), monensin (170mg), limestone (57.8g), acid buffer (17g) and Agox (17g).

The *F. succinogenes* population was significantly correlated to the *S. ruminantium* population (R=0.90) ($P<0.05$). The *S. bovis* population was significantly correlated to butyric (R=0.81), valeric (R=0.81) and concentrations and L-lactate (R=0.84) ($P<0.05$)

while the total bacterial population is significantly correlated to the *P. ruminicola* (R=0.94) and *S. ruminantium* populations (R=0.83) (P<0.05).

Herd 98 had one of the highest *S. bovis* and lowest *Lactobacillus spp.* populations at a pH of 6.34 and average populations of the other key bacterial species monitored.

5.3.1.12 Herd 12

Herd 12 was consuming the diet that consisting of the highest roughage component at 90.4% (14.23kg DM of pasture: 23.8% crude protein, 10.7 MJ ME/kg DM and 29.2% NDF and 2.37kg DM hay). The concentrate component comprising 9.6% of the diet consisted of 1.78kg of wheat with no other additives in the diet. Herd 12 had the lowest *S.ruminantium* populations, one of the highest *P. ruminicola* populations and the highest *Lactobacillus spp.* populations of the herds analysed.

Table 5.2 Average values for rumen parameters of dairy herds for samples taken at one point in time and 5% least significant differences (5%LSD).

Retransformed means are shown in brackets. Averages with the same subscript are not significantly different (Fisher's protected 5%LSD).

Herd ID %Forage	*74* **30.5**	*83* **46.6**	*9* **48.5**	*61* **53.9**	*8* **58.0**	*89* **58.7**	*16* **58.8**	*5* **62.3**	*38* **73.8**	*6* **76.7**	*98* **77.0**	*12* **90.3**	**5%LSD**
Rumen pH *	6.11 cd	6.67 f	5.53 ab	6.14 de	5.07 a	5.74 bc	6.47 def	5.43 Ab	6.34 def	6.62 f	6.36 def	6.53 ef	0.39
F. succinogenes (log_{10})	8.28 (1.89E+08) fg	7.62 (4.14E+07) def	5.80 (6.36E+05) a	6.34 (2.20E+06) ab	8.01 (1.02E+08) efg	7.93 (8.60E+07) efg	6.87 (7.47E+06) bcd	7.32 (2.08E+07) cde	6.69 (4.95E+06) abc	7.83 (6.74E+07) efg	7.44 (2.73E+07) cdef	8.70 (5.05E+08) g	0.89
P. ruminicola (log_{10})	7.89 (7.80E+07) d	7.34 (2.17E+07) bcd	6.09 (1.24E+06) a	6.19 (1.54E+06) a	7.46 (2.90E+07) cd	6.76 (5.76E+06) abc	7.15 (1.41E+07) bcd	7.22 (1.66E+07) bcd	6.63 (4.25E+06) ab	7.21 (1.64E+07) bcd	7.31 (2.03E+07) bcd	7.52 (3.28E+07) cd	0.78
S. ruminantium (log_{10})	8.10 (1.25E+08) ef	7.53 (3.42E+07) cde	6.31 (2.03E+06) a	6.92 (8.35E+06) abc	8.37 (2.33E+08) f	6.96 (9.03E+06) abc	7.22 (1.65E+07) bcd	7.54 (3.46E+07) cde	6.52 (3.29E+06) ab	7.75 (5.63E+07) def	7.71 (5.11E+07) def	8.33 (2.15E+08) f	0.76
S. bovis (log_{10})	4.07 (1.19E+04) bcd	4.02 (1.06E+04) bcd	3.34 (2.18E+03) a	3.65 (4.43E+03) abc	4.76 (5.75E+04) f	4.60 (4.02E+04) ef	4.20 (1.59E+04) de	3.59 (3.88E+03) ab	3.33 (2.15E+03) a	3.95 (8.90E+03) bcd	4.62 (4.13E+04) ef	4.11 (1.29E+04) cd	0.49
Lactobacillus spp (log_{10})	4.79 (6.15E+04) cdefg	4.72 (5.22E+04) cdefg	4.48 (2.99E+04) bcde	4.96 (9.22E+04) defg	4.79 (6.10E+04) cdefg	3.88 (7.67E+03) ab	4.81 (6.47E+04) cdefg	4.24 (1.73E+04) bc	4.49 (3.12E+04) bcdef	4.28 (1.90E+04) bcd	3.34 (2.17E+03) a	5.17 (1.49E+05) eg	0.70
Total Bacterial (log_{100})	9.42 (2.60E+09) cde	8.91 (8.20E+08) ab	8.95 (8.97E+08) abc	8.64 (4.41E+08) a	9.52 (3.32E+09) de	9.08 (1.21E+09) abcd	9.47 (2.95E+09) de	9.23 (1.70E+09) bcd	9.25 (1.77E+09) bcd	9.13 (1.34E+09) bcd	8.96 (9.07E+08) abc	9.74 (5.44E+09) e	0.47
Rumen ammonia *	7.18 e	4.98 bcde	1.27 A	4.78 bcde	6.71 de	6.10 cde	2.96 ab	4.11 bcd	2.82 ab	2.94 ab	3.97 abc	3.31 ab	2.71
Acetic acid*	50.9 cde	34.1 a	33.6 a	46.8 bcd	61.9 e	51.7 de	39.7 abc	56.2 de	29.9 a	35.6 a	37.9 ab	40.2 abc	11.2
Butyric Acid*	10.25 ef	7.12 bcd	4.57 ab	9.62 de	13.07 f	9.82 de	7.08 bcd	9.46 de	4.05 a	7.84 cde	6.42 abc	8.72 cde	2.97
Propionic acid*	18.7 bc	9.1 a	25.8 de	14.0 abc	19.4 cd	32.2 e	18.8 bcd	31.6 e	15.4 abc	9.7 a	13.7 abc	12.2 ab	6.8

Total VFA*	83.0 cd	52.7 a	67.1 abc	73.0 bc	97.7 d	97.9 d	68.5 abc	101.1 d	51.5 a	55.3 ab	60.2 ab	63.7 abc	19.6
Valeric acid*	1.16 cd	0.62 a	1.42 d	0.91 abc	1.17 cd	2.29 e	1.14 bcd	1.56 d	0.86 abc	0.63 a	0.65 ab	0.81 abc	0.49
Iso-butyric acid*	0.70 cde	0.56 abcd	0.53 abc	0.63 abcde	0.73 de	0.53 ab	0.58 abcd	0.76 e	0.47 a	0.61 abcde	0.54 abc	0.66 bcde	0.17
Iso-valeric acid*	1.29	1.09	0.91	0.96	1.16	0.97	0.90	1.16	0.78	0.86	0.96	0.92	0.31
Caproic Acid *	0.036 a	0.071 ab	0.212 bc	0.148 abc	0.244 c	0.437 d	0.235 bc	0.252 c	N/A	0.156 abc	N/A	0.147 abc	0.165
D-lactate*	0.042 a	0.100 abc	0.134 bc	0.050 ab	0.066 abc	0.122 abc	0.147 c	0.257 d	0.062 abc	0.051 ab	0.079 abc	0.075 abc	0.087
L- lactate*	0.080 a	0.071 a	0.126 a	0.081 a	0.065 a	0.138 a	0.114 a	0.259 b	0.081 a	0.065 a	0.086 a	0.081 a	0.078
Acidosis Status*	2.37	3.0	2.57	2.75	1.75	1.63	2.75	1.85	3	2.86	2.88	3	

N/A indicates not enough measurements of this parameter for statistical analysis within that herd.

*Data sourced from (Bramley *et al.*, 2008)

The rumen pH of the herds over sampling period (Table 5.2) indicated that four of the twelve herds had an average a pH of less than 6 which was the threshold set for what was classed as acidosis in previous chapters. There were four herds with a rumen pH in the medium low range of 6.01-6.45 and four in the high rumen pH range >6.46.

5.3.2 **Analysis of all samples irrespective of herds**

The analysis of all data points was undertaken to determine if irrespective of herds, that specific changes in the bacterial populations could be associated with changes in the rumen parameters. It can be noted that in the various bacterial populations that there is a large variation of the key bacterial populations monitored (Figure 5.1) over the 95 samples analysed.

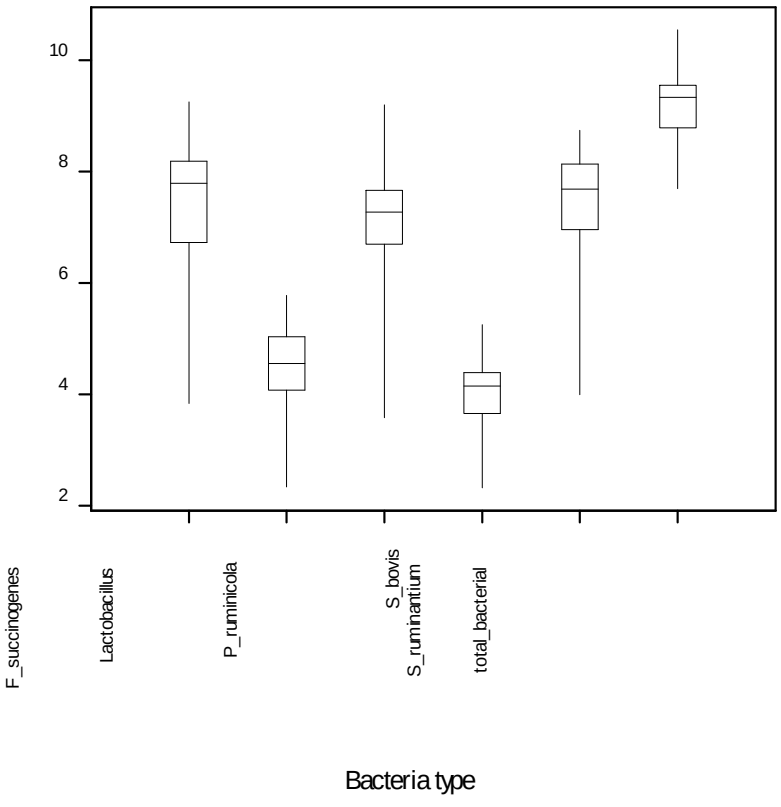


Figure 5.1 Box and whisker plot of bacterial populations (cells/mL log_{10}) (n=95) analysed using qRT-PCR in the rumen of dairy cattle on various diets, total bacterial log_{100} all other bacterial populations log_{10}.

Table 5.3 Significant correlations (P<0.05) between bacterial populations and rumen parameters over all animals. All bacterial counts were log transformed prior to calculation of correlations. Shaded areas are redundant.

Variable	***p_ruminicola***	***s_bovis***	***s_ruminantium***	**Total_bacterial**	**Rumen pH**	**Acetic**	**Butyric**	**Caproic**	**D-lactate**	**iso-butyric**	**iso-valeric**	**L_lactate**	**Rumen ammonia**	**Propionic**	**Valeric**
F_succinogenes	0.658	0.536	0.767	0.620		0.315	0.412			0.301			0.293		
Lactobacillus spp.				0.322											
P_ruminicola		0.326	0.779	0.714	0.23			-0.319		0.263	0.323				-0.274
S_bovis			0.432	0.253		0.251	0.345						0.334		
S_ruminantium				0.596		0.305	0.460			0.405	0.323		0.227	-0.234	-0.293
Total_bacterial						0.271	0.229			0.300					

The analysis of the data set irrespective of their herd indicates that there is a minimal relationship between the other key bacterial populations and the *Lactobacillus spp.* This is visually evident in figure 5.2 with the only correlation being with the total bacterial populations. The relationship of all other monitored bacterial populations was evident with the strongest being that of *P. ruminicola* and *S. ruminantium*.

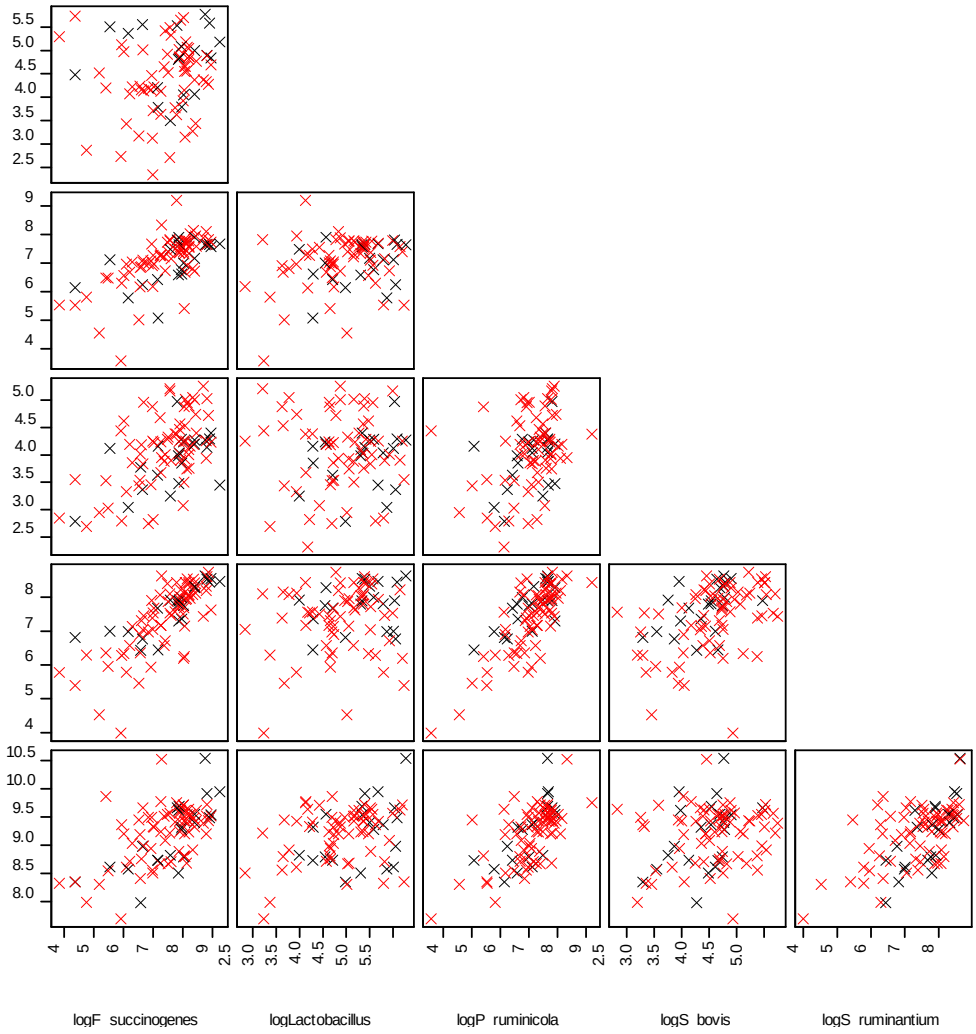


Figure 5.2 Correlations between the key rumen bacterial populations when analysed on a collective basis (n=95).

5.3.2.1 Rumen pH

From analysis of the subsample pooled data irrespective of herd, rumen pH was significantly correlated to the volatile fatty acids concentrations of acetic (R=-0.50), butyric (R=-0.33) and valeric acids (R=-0.51) ($P<0.05$). Rumen pH was also significantly correlated to the D-lactate concentrations (R=-0.39), acidosis status (R=0.64) and the *P. ruminicola* population (R=0.23) analysed by qRT-PCR.

5.3.2.2 General trends for bacterial species

All bacterial populations were significantly correlated to the total bacterial populations with the populations of *P. ruminicola, F. succinogenes* and *S. ruminantium* having the strongest relationship (P<0.05). (Table 5.4)

5.3.2.3 *Fibrobacter succinogenes* population

From analysis of the pooled data, the populations of the cellulolytic bacteria, *F. succinogenes* was significantly correlated to the volatile fatty acid concentrations of acetic (R=0.32), butyric (R=0.41) and iso-butyric acids (R=0.30) and rumen ammonia (R=0.29) (P<0.05). There was also a strong correlation between the populations of *F. succinogenes* and those of *S. bovis* (R=0.54), *P. ruminicola* (R=0.66) and *S. ruminantium* (R=0.76) and total bacterial populations (R=0.62) (P<0.05).

5.3.2.4 *Streptococcus bovis* populations

The *S. bovis* populations were significantly correlated to the volatile fatty concentrations, acetic acid (R=0.25) and butyric acid (R=0.34) and the rumen ammonia concentrations (R=0.33) (P<0.05). The populations of *Streptococcus bovis* were also significantly correlated to the populations of *F. succinogenes* (R=0.54), *S. ruminantium* (R=0.43), *P. ruminicola* (R=0.32) and the total bacterial population (R=0.25), (P<0.05)

5.3.2.5 Total bacterial populations

The total bacterial populations were correlated to several factors including the volatile fatty acid concentrations of acetic (R=0.27), butyric (R=0.23) and iso-butyric (R=0.30) (P<0.05). The total bacterial populations also showed a strong correlation to the bacterial populations of *Lactobacillus spp.* (R=0.32), *P. ruminicola* (R=0.71), *S. bovis* (R=0.26), *F. succinogenes* (R=0.62) and *S. ruminantium* (R=0.59) (P<0.05).

5.3.2.6 *Prevotella ruminicola* populations

The *P. ruminicola* populations were significantly correlated to rumen pH (R=0.23), butyric acid (R=0.22), valeric acid (R=-0.27), iso-butyric (R=0.26) and iso-valeric acid (R=0.32). The populations of *P. ruminicola* were also significantly correlated to the populations of *F. succinogenes* (R=0.66), *S. bovis* (R=0.33), *S. ruminantium* (R=0.78) and the total bacterial populations (R=0.71) ($P<0.05$).

5.3.2.7 *Selenomonas ruminantium* population

The *S. ruminantium* populations were strongly correlated to the concentrations of acetic acid (R=0.35), butyric acid (R=0.46), iso-butyric (R=0.41) and iso-valeric acids (R=0.32) ($P<0.05$). There was a strong correlation between the populations of *S. ruminantium* and ammonia concentrations (R=0.23) ($P<0.05$) as well as the other bacterial populations of *F. succinogenes* (R=0.76), *P. ruminicola* (R=0.78), *S. bovis* (R=0.43) and the total bacterial population (R=0.60) ($P<0.05$).

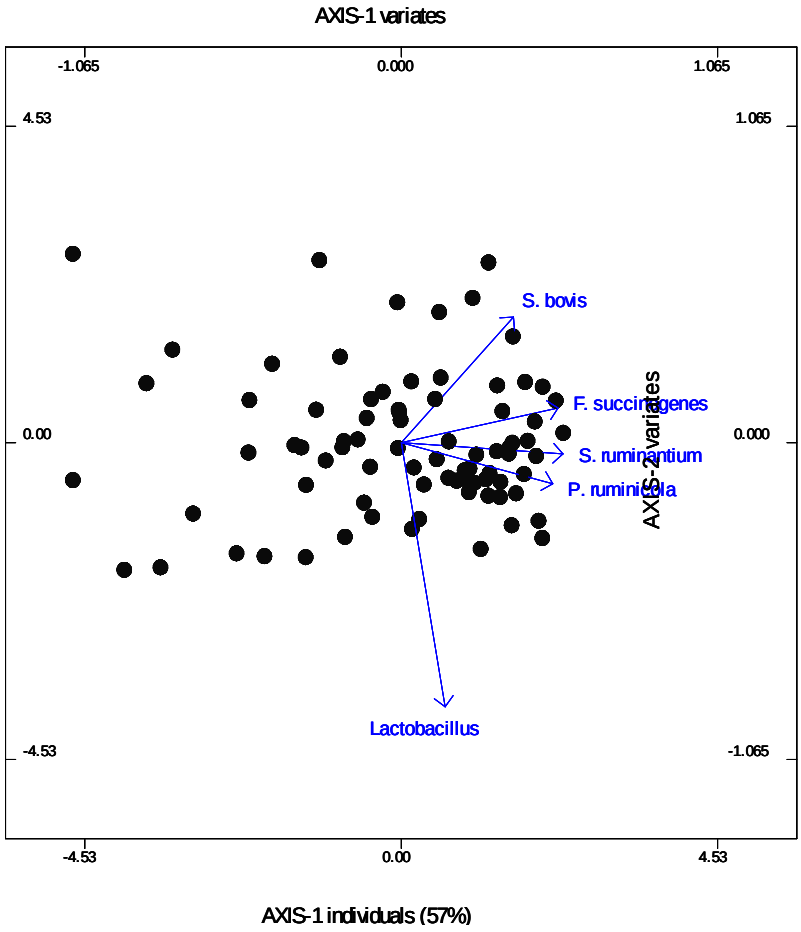


Figure 5.3 Biplot representing 78% of correlations of log transformed bacterial populations of dairy cows under various feeding regimes and indicators of ruminal acidosis.

The rumen bacterial populations as represented in the biplot (Figure 5.3) showed that the *Lactobacillus spp*. populations of the samples analysed collectively were not strongly correlated to any of the other bacterial populations in the samples. On the other hand, the *P. ruminicola*, *S. ruminantium* and the *F. succinogenes* population concentrations were all correlated to each other in the samples analysed. Moreover, the *S. bovis* populations were strongly correlated to the *S. ruminantium* (R=0.43) and the *F. succinogenes* populations (R=0.54) (P<0.05).

5.3.3 **Impact of ionophores or antibiotics on rumen parameters**

Cattle fed monensin and/or virginiamycin had significantly (<0.05) higher concentrations of propionic, valeric and total volatile fatty acid as well as L and D-lactate concentrations and

rumen ammonia concentrations. There were no significant differences in the bacterial populations monitored.

Table 5.4 The rumen parameters (means ± SEM) for cattle that had not been supplemented with monensin or virginiamycin in their ration (n=24) in comparison to those that had (n=71). All data sourced from **(Bramley *et al.*, 2008)**.

	No feed additives (n=24)	Feed additives (n=71)
Rumen pH	6.42 ± 0.09	5.99 ± 0.076
Acidosis status	2.87 ± 0.091	2.42 ± 0.99
Propionic acid	11.98 ± 0.94	20.40 ± 1.22
Butyric acid	8.73 ±0.74	8.03 ± 0.44
Acetic acid	40.86 ± 2.30	44.15 ±1.80
Valeric acid	0.78 ± 0.06	1.21 ± 0.084
Caproic acid	0.15 ± 0.03	0.165 ± 0.25
Total Volatile fatty acid	64.0 ± 3.70	75.6 ± 3.19
L-lactate concentration	76.0 ± 7.20	111.0 ± 12.0
D-lactate concentration	59.0 ± 5.80	111.0 ± 13.0
Rumen ammonia	3.67 ± 0.39	4.50 ± 0.39

On the other hand, cattle that were not fed feed additives showed no correlation to any of these rumen metabolites ($P>0.05$).

The *F. succinogenes* populations were significantly correlated with the populations of *P. ruminicola* ($R=0.66$), *S. bovis* ($R=0.54$), *S. ruminantium* ($R=0.77$) and total bacterial populations ($R=0.62$) ($P<0.05$). In cows where feed additives were fed, the *F. succinogenes* populations were significantly correlated to the concentrations of the volatile fatty acids: acetic ($R=0.32$), butyric ($R=0.45$), iso-butyric ($R=0.32$), iso-valeric acids ($R=0.31$) as well as rumen ammonia ($R=0.38$) concentrations ($P<0.05$).

The populations of *Lactobacillus spp.* are not correlated to any other rumen metabolic and physiological parameters in cows where feed additives were not included in the ration. However, with the addition of feed additives the populations of *Lactobacillus spp.* were significant correlated to the total bacterial population (R=0.37) as well as the iso-butyric concentration (R=0.38) in the rumen (P<0.05).

The *P. ruminicola* populations in cows without additives were significantly correlated to the populations of *S. ruminantium* (R=0.78), total bacterial populations (R=0.71), and rumen pH (R=0.33) (P<0.05). Cows with additives included in the diet, the *P. ruminicola* populations were significantly correlated to the populations of *S. bovis* (R=0.33), *S. ruminantium* (R=0.78), and total bacterial populations (R=0.71) as well as butyric (R=0.27), caproic (R=-0.61), iso-butyric (R=0.29) and iso-valeric acid (R=0.34) concentrations (P<0.05).

The *S. bovis* populations were significantly correlated to the populations of *F. succinogenes* (R=0.54), the total bacterial population (R=0.25) as well as *S. ruminantium* populations (R=0.78) in cows not fed feed additives (P<0.05). In cows with additives included in the diet, the *S. bovis* populations were correlated to the populations of *F. succinogenes* (R=0.54), *P. ruminicola* (R=0.33), *S. ruminantium* (R=0.43) as well as acetic acid (R=0.25), and butyric acid concentrations (R=0.35) and rumen ammonia concentration (R=0.37) (P<0.05).

The *S. ruminantium* populations were significantly correlated to the populations of *F. succinogenes* (R=0.76), *P. ruminicola* (R=0.76), *S. bovis* (R=0.78), the total bacterial population (R=0.59) (P<0.05) in all cows irrespective of feed additives in the ration. The *S. ruminantium* populations were significantly correlated to the rumen parameter of D-lactate concentration (R=-0.31) (P<0.05) in cows not fed additives. In contrast in cows fed feed additives the *S. ruminantium* populations were significantly correlated to the acetic (R=0.32), butyric (R=0.51), iso-butyric (R=0.43), iso-valeric concentrations (R=0.33) and rumen ammonia concentrations (R=0.25) (P<0.05).

The total bacterial population in cows without feed additives were significantly correlated to the populations of *F. succinogenes* (R=0.70), *P. ruminicola* (R=0.71), *S. bovis* (R=0.25), *S. ruminantium* (R=0.59) and the D-lactate concentrations (R=0.29) in the rumen (P<0.05). In samples from cows with the addition of rumen additives the total bacterial populations were significantly correlated to the populations of *F. succinogenes* (R=0.62), *P. ruminicola* (R=0.70), *Lactobacillus spp.* (R=0.32), *S. ruminantium* (R=0.59) and rumen acetic (R=0.30), butyric (R=0.25) and iso-butyric acid concentrations (R=0.32) (P<0.05).

The rumen pH in cows not fed feed additives was significantly correlated to the bacterial populations of *P. ruminicola* (R=0.51) and rumen parameters of acetic (R=-0.54), propionic acid (R=-0.53) and total volatile fatty acid concentrations (R=-0.56) (P<0.05). In cows fed feed additives the rumen pH was significantly correlated to the acidosis status (R=0.64), negatively correlated with propionic (R=-0.61), acetic (R=-0.43), butyric (R=-023) and L-lactate concentrations (R=-0.33) (P<0.05).

5.3.4 **Bacterial changes based on cluster analysis by (Bramley *et al.*, 2008)**

The data was re-analysed based on the same cluster analysis of the data outlined in (Bramley *et al.*, 2008) for a selected 12 herds and quantified for key bacterial species. A REML analysis with herd as random effect and cluster categories as fixed effect was used to compare the three clusters. Cluster one was consistent with an acidosis model with high rates of carbohydrate fermentation resulting in high volatile fatty acid concentrations with high valerate and propionate. The cluster had cows with normal to high levels of D-lactate and normal rumen ammonia concentrations. Cluster two include cows that had possible mismatched energy and protein concentration rates in their rations and rumen parameters which indicated slow rumen fermentation and lower milk production and were classified as having suboptimal rumen function. Cluster three had a similar diet to cluster one but lower volatile fatty acid

concentrations and better milk production and were classed as normal rumen function (Bramley *et al.*, 2008).

Table 5.5 Rumen metabolic indicators (mean ± SEM) categorised into the cluster analysis as undertaken by (Bramley *et al.*, 2008) different subscripts indicate values are significantly different.

Rumen parameter	1 (n=12)	2 (n= 21)	3 (n=63)
Rumen pH *	5.37 ± 0.06^{a}	5.72 ± 0.13^{b}	6.39 ± 0.06^{c}
F. succinogenes (log_{10})	6.95 ± 0.44^{a}	8.00 ± 0.14^{b}	7.3 ± 0.14^{ab}
P. ruminicola (log_{10})	6.45 ± 0.36^{a}	7.65 ± 0.092^{b}	7.03 ± 0.10^{a}
S. ruminantium (log_{10})	6.81 ± 0.37^{a}	8.10 ± 0.12^{b}	7.37 ± 0.11^{ab}
S. bovis (log_{10})	3.90 ± 0.23^{a}	4.38 ± 0.12^{b}	3.96 ± 0.08^{a}
Lactobacillus spp. (log_{10})	4.25 ± 0.25^{a}	4.55 ± 0.19^{a}	4.51 ± 0.10^{a}
Total Bacterial (log_{100})	9.01 ± 0.18^{a}	9.35 ± 0.12^{b}	9.18 ± 0.064^{c}
Rumen ammonia *	3.86 ± 1.10^{a}	7.21 ± 0.85^{b}	3.39 ± 0.22^{a}
Acetic acid*	53.66 ± 3.29^{a}	59.81 ± 2.54^{a}	35.73 ± 1.10^{b}
Butyric Acid*	8.95 ± 0.83^{a}	13.16 ± 0.63^{b}	6.39 ± 0.28^{c}
Propionic acid*	36.35 ± 2.20^{a}	20.65 ± 1.91^{b}	14.04 ± 0.70^{c}
Total VFA*	103.25 ± 5.36^{a}	97.21 ± 4.56^{a}	58.51 ± 1.76^{b}
Valeric acid*	2.21 ± 0.27^{a}	1.28 ± 0.09^{b}	0.82 ± 0.043^{c}
Iso-butyric acid*	0.61 ± 0.043^{a}	0.80 ± 0.039^{b}	0.54 ± 0.018^{c}
Iso-valeric acid*	1.04 ± 0.064^{a}	1.31 ± 0.071^{b}	0.88 ± 0.033^{a}
Caproic Acid*	0.19 ± 0.05^{a}	0.092 ± 0.03^{ab}	0.082 ± 0.006^{b}
L-lactate*	0.19 ± 0.045^{a}	0.12 ± 0.027^{b}	0.085 ± 0.0046^{c}
D-lactate*	0.19 ± 0.048^{a}	0.10 ± 0.029^{b}	0.08 ± 0.0065^{b}
Acidosis Status*	1.0 ± 0.0^{a}	1.97 ± 0.048^{b}	3.05 ± 0.39^{c}

*Data sourced a subsample of data from (Bramley *et al.*, 2008)

5.3.4.1 Correlations within cluster one

Within cluster one the *F. succinogenes* populations were significantly correlated to the concentrations of butyric acid and the bacterial populations of *S. ruminantium*, *P. ruminicola* and total bacterial population ($P<0.05$).

The *Lactobacillus spp.* populations were significantly correlated to the caproic acid concentration ($P<0.05$). The *P. ruminicola* populations were significantly correlated to the valeric acid concentrations and the rumen bacterial populations of *F. succinogenes* and *S. ruminantium* ($P<0.05$). The *S. bovis* populations were significantly correlated to the concentrations of D and L-lactate, butyric acid and rumen ammonia concentrations ($P<0.05$). Finally, the total bacterial populations in this cluster one category were significantly correlated to the *S. ruminantium* populations ($P<0.05$). The rumen pH in this category was significantly correlated to the propionic acid concentration within these rumen samples ($P<0.05$).

5.3.4.2 Correlations within cluster two

The *F. succinogenes* populations were not correlated to any other rumen bacterial populations. The *Lactobacillus spp.* populations were significantly correlated to the *S. bovis* ($P<0.05$) populations. The *P. ruminicola* populations were correlated to the rumen parameters of butyric acid and rumen pH ($P<0.05$). The *S. bovis* populations were significantly correlated to the rumen pH of the samples tested ($P<0.05$). The *S. ruminantium* populations were significantly correlated to the total bacterial populations ($P<0.05$) while the total bacterial populations were significantly correlated to the *Lactobacillus spp*. populations ($P<0.05$).

5.3.4.3 Correlations within cluster three

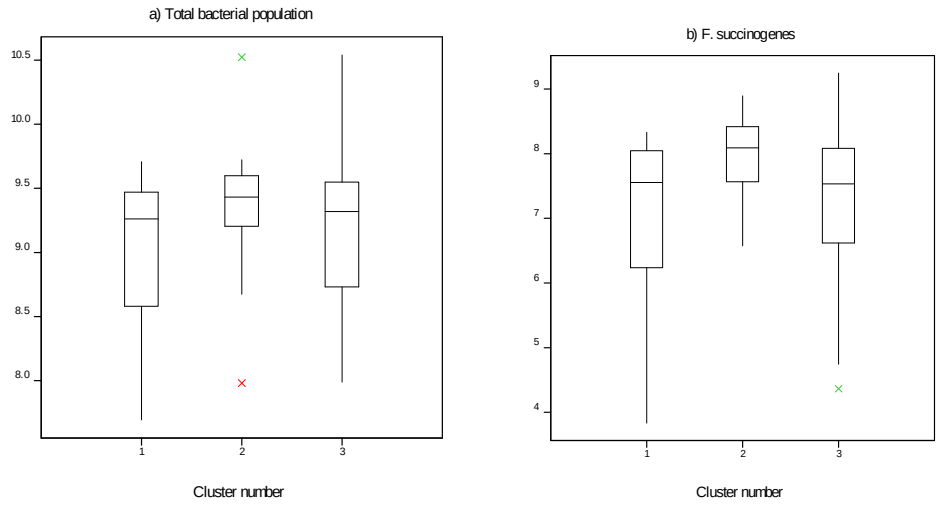
The *F. succinogenes* populations were significantly correlated to the concentrations of butyric acid, and iso-butyric and rumen pH as well as the bacterial populations of *P. ruminicola*, *S. bovis*, *S. ruminicola* and the total bacterial populations ($P<0.05$).

The *Lactobacillus spp.* populations within the rumen samples collected were significantly correlated to the rumen pH and total bacterial populations (P<0.05). The *S. bovis* populations were significantly correlated to rumen pH and bacterial populations of *F. succinogenes, P. ruminicola* and *S. ruminantium* (P<0.05).

The *P. ruminicola* populations were significantly correlated to the rumen concentrations of acetic, butyric, and iso-butyric acids and the rumen pH (P<0.05) as well as the bacterial populations of *F. succinogenes, S. bovis* and *S. ruminantium* (P<0.05).

The *S. ruminantium* populations were significantly correlated to the rumen concentrations of acetic, butyric, and iso-butyric acids, rumen pH and feed additives (P<0.05). The *S. ruminantium* populations were also significantly correlated to the bacterial populations of *F. succinogenes, P. ruminicola, S. bovis* and the total bacterial populations (P<0.05).

The total bacterial populations were significantly correlated to the rumen concentrations of acetic, iso-butyric, propionic, and valeric acids and total volatile fatty acid concentrations (P<0.05) and the rumen bacterial populations of *F. succinogenes, Lactobacillus spp.* and *P. ruminicola* (P<0.05).



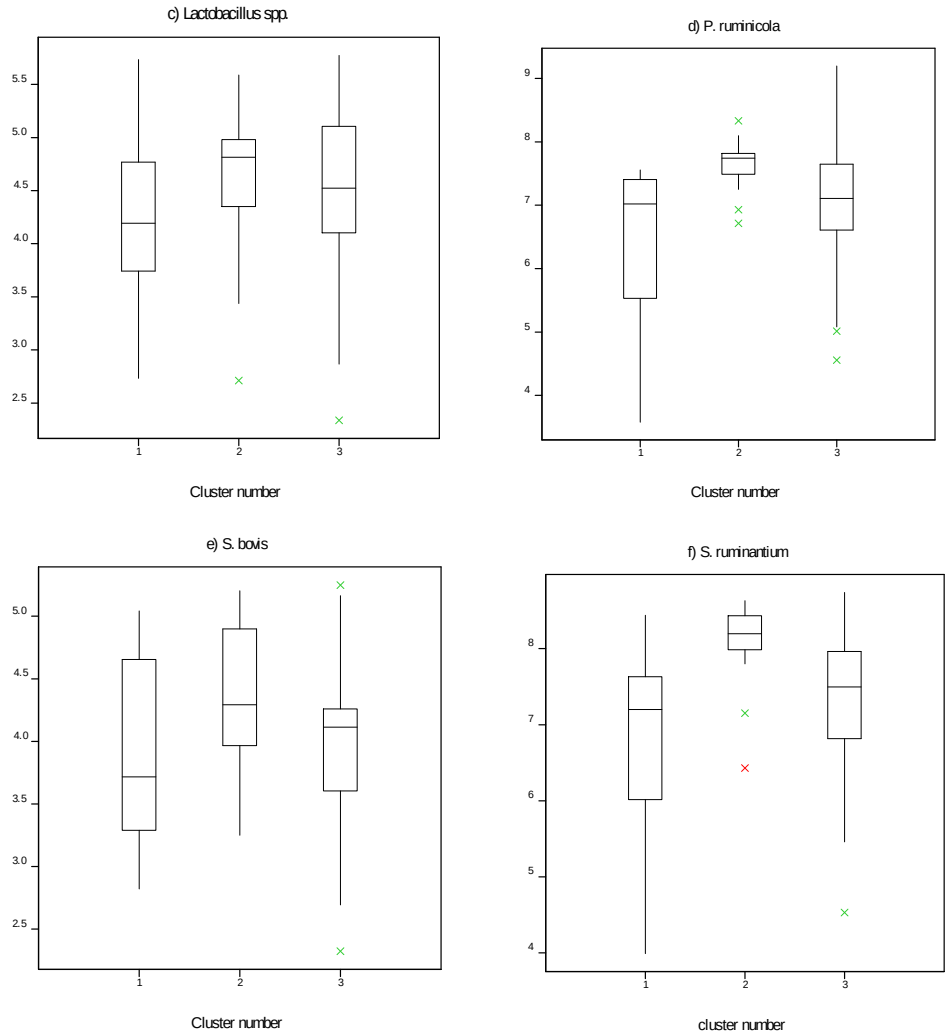


Figure 5.4 Boxplot for rumen bacterial populations in cluster 1, 2 or 3 for dairy cows sampled by rumen centesis on twelve properties and varied diets. Data sourced a subsample of data from (Bramley *et al.*, 2008).

When analysing the key bacterial populations' variations in figure 7.4, the transitional group (category 2) had the lowest variation in the bacterial population numbers particularly for the most prevalent bacterial populations of *P. ruminicola* and *S. ruminantium.*

5.3.5 **Analysis of data categorised into pH categories.**

The data was categorised into three rumen pH variable as outlines in Table 7.6

Table 5.6 Ranking of rumen pH of all samples based on a high, medium or low pH used to classify and compare bacterial populations.

Category	Rumen pH range (rumen centesis)
High	6.48-7.15
Medium	6.01-6.45
Low	4.82-5.98

On analysis of the pH categories as outlined in table 8.6 the pH categories were significantly correlated to the concentrations of caproic acid, D- & L-lactate, propionic acid, valeric acid and acidosis status ($P<0.05$). The pH categories were also significantly correlated to the bacterial populations of *Lactobacillus spp*., *F. succinogenes*, *P. ruminicola* and *S. ruminantium* ($P<0.05$). The pH categories were almost correlated to the use of feed additives ($P=0.0561$).

Table 5.7 Rumen parameters (mean ± SEM) in cattle from Bramley pH categories.

Category	Low (pH 4.2-5.8) (n= 35)	Medium (pH 6.01-6.45) (n= 23)	High (pH 6.48-7.15) (n=33)
Rumen pH*	5.44 ± 0.058^{a}	6.25 ± 0.034^{b}	6.71 ± 0.034^{c}
F. succinogenes (log_{10})	7.22 ± 0.21^{a}	7.37 ± 0.27^{ab}	7.72 ± 0.16^{b}
P. ruminicola (log_{10})	6.89 ± 0.17^{a}	7.15 ± 0.18^{b}	7.29 ± 0.11^{b}
S. ruminantium (log_{10})	7.29 ± 0.19^{a}	$7.31 + 0.19^{a}$	7.77 ± 0.89^{b}
S. bovis (log_{10})	4.06 ± 0.13^{a}	3.97 ± 0.13^{a}	4.10 ± 0.11^{a}
Lactobacillus spp. (log_{10})	4.21 ± 0.15^{a}	4.63 ± 0.15^{b}	4.67 ± 0.13^{b}
Total Bacterial (log_{100})	9.11 ± 0.098	9.11 ± 0.11	9.30 ± 0.072
Rumen ammonia *	4.75 ± 0.68^{a}	4.77 ± 0.62^{a}	3.85 ± 0.27^{b}

Acetic acid*	51.08 ± 2.73[a]	42.24 ± 2.79[b]	37.28 ± 1.52[c]
Butyric Acid*	9.34 ± 0.68[a]	8.40 ± 0.85[b]	7.31 ± 0.47[b]
Propionic acid*	25.35 ± 1.95[a]	15.55 ± 1.13[b]	12.47 ± 0.92[b]
Total VFA*	89.22 ± 4.7[a]	68.9 ± 4.38[b]	59.42 ± 2.67[c]
Valeric acid*	1.51 ± 0.14[a]	0.95 ± 0.07[b]	0.75 ± 0.059[b]
Iso-butyric acid*	0.64 ± 0.34[a]	0.61 ± 0.043[a]	0.58 ± 0.25[a]
Iso-valeric acid*	1.07 ± 0.054[a]	1.05 ± 0.05[a]	0.90 ± 0.041[a]
Caproic Acid*	0.23 ± 0.043[a]	0.10 ± 0.032[b]	0.13 ± 0.025[b]
L-lactate*	0.14 ± 0.023[a]	0.082 ± 0.0063[a]	0.084 ± 0.0072[a]
D-lactate*	0.13 ± 0.025[a]	0.065 ± 0.007[a]	0.091 ± 0.012[a]
Acidosis Status*	1.94 ± 0.14[a]	2.65 ± 0.10[b]	2.91 ± 0.051[b]

*Data sourced from (Bramley *et al.*, 2008)

5.3.5.1 Rumen pH

The key bacterial populations of *F. succinogenes, P. ruminicola, S. ruminantium and Lactobacillus spp.* were significantly correlated to the rumen pH. It allowed some explanation of magnitude of changes indicating that for every one unit of increase in the rumen pH, there was a 4.51-fold increase in *F. succinogenes,* 3.1 fold increase in *P.* ruminicola and a 3.8 fold increase in *S. ruminantium* but what was unexpected was that the *Lactobacillus spp.* populations showed a 2.87 fold increase with each one unit of increase in rumen pH.

5.3.5.2 Within low rumen pH category

Within the low rumen pH category (i.e. category one), rumen pH was significantly ($P<0.05$) correlated to the concentrations of acetic acid, and butyric acid and acidosis status ($P=0.056$) in the cattle sampled in this category.

The *F. succinogenes* populations were significantly correlated to the populations of *P. ruminicola* (R=0.62), *S. ruminantium* (R=0.76), *S. bovis* (R=0.63) and the total bacterial populations (R=0.78) ($P<0.05$). The *F. succinogenes* populations were also correlated to the rumen concentrations of ammonia (R=0.46), butyric acid (R=0.66), iso-butyric acid (R=0.37)

and total volatile fatty acids (R=0.42) concentrations. The *P. ruminicola* populations were significantly correlated to the populations of *S. ruminantium* (R=0.78) and total bacterial (R=0.84) populations as well as valeric (R=-0.32) and iso-valeric (R=0.38) acid concentrations and the addition or rumen modifiers in the diet (R=0.36) (P<0.05).

The *S. ruminantium* populations were significantly correlated to the acetic (R=0.44), butyric (R=0.53), valeric (R=0.32), iso-butyric (R=0.38), iso-valeric (R=0.39) and caproic acid concentrations (R=0.31) (P<0.05) and the rumen bacterial populations of *S. bovis* (R=0.46) and the total bacterial populations (R=0.81) (P<0.05). The *S. bovis* populations were significantly correlated to the rumen ammonia (R=0.46) and acetic (R=0.47) and butyric (R=0.66) acid concentrations (P<0.05). The *Lactobacillus spp.* populations were correlated to iso-butyric concentration (R=0.38) (P<0.05) and iso-valeric acid (R=0.35) (P=0.054). The total bacterial populations were significantly correlated to acetic (R=0.42), butyric (R=0.40) and iso-butyric (R=0.40) and iso-valeric (R=0.43) concentrations (P<0.05).

5.3.5.3 Within medium rumen pH category

Rumen pH in this category was correlated to the concentrations of propionic (R=-0.24) and valeric acid (R=-0.34) (P<0.05). The acidosis status was significantly correlated to the *P. ruminicola* (R=0.73) and *S. ruminantium* (R=0.98) bacterial populations (P<0.05).

The *F. succinogenes* populations were significantly correlated to the rumen bacterial populations of *P. ruminicola* (R=0.81), *S. ruminantium* (R=0.79) *and* the total bacterial populations (R=0.33) (P<0.05) as well as the rumen concentrations of ammonia (R=0.55), butyric acid (R=0.58), iso-butyric acid (R=0.65) and total volatile fatty acids (R=0.61) concentrations and acidosis status (R=-0.51) (P<0.05).

The *P. ruminicola* populations were significantly correlated to the *S. ruminantium* (R=0.86) and *S. bovis* populations (R=0.49) (P<0.05) and the rumen concentrations of ammonia (R=0.56), acetic acid (R=0.72), propionic acid (R=0.34), valeric acid (R=0.81), iso-butyric acid

(R=0.67), iso-valeric acid (R=0.60) and total volatile fatty acid (R=0.71) concentration (P<0.05) as well as the acidosis status of the cows in the category (R=-0.51) (P<0.05).

The *S. ruminantium* populations were significantly correlated to the *S. bovis* (R=0.47) bacterial populations (P<0.05) and rumen concentrations of ammonia (R=0.67), acetic (R=0.75), butyric (R=0.74), valeric (R=0.33), iso-butyric (R=0.80) and iso-valeric acids (R=0.59) as well as the acidosis status (R=-0.54) (P<0.05).

The *S. bovis* populations were significantly correlated to the rumen ammonia (R=0.32), butyric (R=0.29) and acetic acid (R=0.41) concentrations (P<0.05).

5.3.5.4 The high rumen pH category

The rumen pH of high pH category cows was significantly correlated to the total bacterial populations (R=-0.49) and rumen concentrations of ammonia (R=0.57) and acetic acid (R=-0.37) (P<0.05). The total bacterial populations were significantly correlated to the rumen pH (R=-0.49), *F. succinogenes* (R=0.59), *P. ruminicola* (R=0.68) and *S. ruminantium* (R=0.53) populations (P<0.05). The *F. succinogenes* populations were significantly correlated to the populations of *P. ruminicola* (R=0.52), *S. bovis* (R=0.45) and *S. ruminantium* (R=0.71) (P<0.05). The *P. ruminicola* populations were significantly correlated to the *S. ruminantium* (R=0.60) *and S. bovis* populations (R=0.50) (P<0.05). The *S. bovis* populations were significantly correlated to the concentrations of iso-butyric (R=-0.38), and D-lactate (R=0.53) (P<0.05). The *Lactobacillus spp.* populations were not correlated to any of the measured rumen parameters.

5.4 **Discussion**

This chapter presents the first integrated findings relating the molecular ecology of the rumen to metabolism in the rumen of dairy cows under practical farming environments where the risk and prevalence of acidosis was being monitored and categorised. There are some

general observations that can be made from the results. Using rumen pH as the benchmark, the populations of the key main cellulytic bacteria, *Fibrobacter succinogenes* were linked to the pH of the rumen since this bacterial species is known to be sensitive to pH, with population decreasing when rumen pH decreased below 6.0. Whether dairy cattle were classified according to herd or pH category, the populations of *F. succinogenes* were in fact highest in those cattle with the highest pH values in the rumen as expected and confirmed in this study. However, the populations of *Streptococcus bovis* which were expected to show the opposite trend to *F. succinogenes,* given its presumed association with rumen acidosis, did not show the clear relationship either with pH category or in herds with a relatively high incidence of acidosis. On the other hand, the populations of *Prevotella ruminicola* were present in the greatest relative abundance as expected from phenotypic observations of rumen populations and observed in previous chapters with some exceptions where the populations of *S. ruminantium* were in greatest abundance. Moreover, populations of *P. ruminicola* were correlated to concentrations of the volatile fatty acids, and to rumen ammonia which again accorded with expectations given the significant role of this species not only in carbohydrate fermentation but also in proteolysis and deamination. The *Selenomonas ruminantium* populations as expected increased in concentrate diets and were in greater abundance than the populations of *P. ruminicola* in some cases

Moreover, the populations of *F. succinogenes* were correlated to ruminal iso-butyric acid and iso-valeric concentrations as well as ruminal ammonia concentrations. Each of these metabolites is an essential growth factor for *F. succinogenes* and therefore these associations are not unexpected and in fact reassuring. Thus the metabolism in the rumen of these dairy cows is closely linked to the rumen microbial ecology of *F. succinogenes*, the major cellulolytic species monitored in this study.

5.4.1 **Herd analysis**

The herds analysed were correlated to rumen pH, rumen ammonia, total bacterial and *Lactobacillus spp*. populations. When data was analysed on a herd basis, this allowed assessment of the potential of the diets and therefore management practices in place, to impact on the rumen parameters and microbial populations. The quality of roughage that was fed to the cows was a key to some of the metabolic and bacterial indicators. Also herds which were fed a more balanced quality of crude protein (%) and NDF between the forage and the concentrate components showed lower indicators of acidosis based on these one-off samples.

Cows in herd 8 that had the lowest rumen pH were fed straw (low quality roughage source indicated by the high NDF and lowest energy compared to the other herd diets) as roughage within the ration. This herd had highest rumen ammonia levels and nearly the highest *Fibrobacter succinogenes* populations. Thus the populations of *F. succinogenes* were more strongly associated with the substrate availability in this herd than the presence of lower rumen pH (i.e. < pH 6.0). *Fibrobacter succinogenes* requires rumen ammonia for growth and ammonia is essentially the sole source of nitrogen for most strains (Dehority *et al.*, 1967). Herds within this analysis with elevated rumen ammonia concentrations also had quantified higher *F. succinogenes* populations (herds 12, 74 and 89).

The very high ruminal ammonia concentrations in the cows from Herd 8 may have been related to the low overall dietary energy component of the diet (9.34 MJ ME/kg DM). In addition, the overall dietary crude protein in the shed ration was the lowest at 12.5% in these cows and compared to the other monitored herds, the pasture component of the ration for cows in herd 8 was one of the highest at 26.2% CP which may have impacted on the ability to utilise the available dietary protein. The most interesting component is the pasture in the diet had the highest percentage sugar in which may have contributed to the drop in ruminal pH. These variables indicate that the pasture component of the ration was having the largest impact on the

acidotic rumen parameters contributing to the low ruminal pH and high valeric and caproic acid. Herd 8 also had the highest *S. ruminantium* populations (which are lactic acid utilisers) and interestingly they had some of the lowest lactate levels indicating that lactate may have been successfully removed from the rumen even with the highest *S. bovis* population (lactate producer) rumen ammonia, butyric and total VFA concentrations compared to the other herds.

A study by Bhat *et al.* (1990) showed that *F. succinogenes* had maximum adhesion at rumen pH 6 during the mid to late growth phase and with the collection of rumen samples from non-attached rumen fluid there may have been more free bacteria for the herds with lower ruminal pH but higher *F. succinogenes* populations. The attachment of *F. succinogenes* is imperative for their ability to digest cellulose as the cellulose enzymes of *F. succinogenes* are cell bound requiring an intimate association between the cell envelope and substrate for cellulose digestion to occur (Groleau and Forsberg, 1981).

Herds 5 and 9 which had a lower ruminal pH (pH; 5.43 and 5.53 respectively) were interesting in that their concentrate component was solely wheat which is documented to rapidly ferment due to readily available carbohydrates. Herd 9 also had a diet in which the pasture component constituted a high % sugar content (23.6%) and a lower *F. succinogenes* population compared to that of herd 5 with a lower overall dietary NDF. In combination the overall low dietary NDF indicates a more highly digestible ration (lower dietary fibre) potentially impacting a lower ruminal pH, although there was the inclusion of straw in the ration feeding. This low quality straw component may not be enough to balance the diet. Both these diets had higher lactate levels than most rations (Herd 5 the highest); indicating that the cereal grain component was a major contributor to the acidotic state. It is interesting to note that herd 5 had both monensin and virginiamycin at the recommended rate while herd 9 had monensin at the recommended dose. Noteworthy is the fact that herds with higher lactate levels are also in fact the ones that incorporate both virginiamycin and monesin into their dietary regime.

Cows in Herd 16 showed a good pH range (6.47) but had one of the highest percentage sugar content in the pasture component of the diet and lower NDF. The concentrate component of the ration supplying some of the protein contained a reasonable percentage of bypass protein in the form of canola meal. This herd exhibits one of the lower *F. succinogenes* populations of all herds with higher corresponding ruminal D- and L lactate concentrations compared to other herds. Possibly the pasture component fed to cows in Herd 16 was having a greater impact on the rumen metabolites than the in-shed ration. Cows in Herd 89 had one of the lower mean ruminal pH (5.47) and higher rumen ammonia concentrations at 6.10mM with a high component of roughage added in the form of silage and hay. The timing of the rumen sampling may have impacted the rumen pH in this herd that was fed a triticale diet with additional canola meal and faba beans. Interestingly, cows in this herd had one of the highest valeric acid concentrations which has been shown by Bramley *et al.* (2008) to be a major predictor of acidosis with a corresponding high ruminal lactate level in this case and a lower than recommended dosage of monensin.

Cows in Herd 12 had the highest forage component of all of the herds with an associated normal pH range possibly since the pasture component with a lower percentage of soluble sugars (8.5%), and highest crude protein (23.77%). This diet also contained only wheat as the concentrate and no added rumen modifiers yet the rumen parameters were indicative of good rumen function as shown by the highest total bacterial populations and *F. succinogenes* populations as well as populations of *Lactobacillus spp.*.. The additional crude protein in the pasture was not associated with excessively high ruminal ammonia concentrations.

Other herds monitored were within the normal ranges of ruminal pH and the pasture components contained lower soluble sugar percentages and higher NDF percentages in the pasture components of the rations. The pasture or forage component did not seem to contributing to the indicators of acidosis. In fact, the quality variations between the forage and the concentrate

balance in the ration seem to be a major determining factor in the incidence of acidosis in the herd.

Overall the ruminal acidosis metabolic indicators were not always aligned with the expected bacterial population differences, for instance cows in the herds with the highest concentrate proportions in the diet did not necessarily have the lowest rumen pH, or the highest populations of *S. ruminantium* or the lowest *F. succinogenes* populations.

Consequently, feeding management type may have been a major contributing factor to the measures of rumen metabolism related to acidosis or general rumen function. To examine this further the herd data for all cows was collated irrespective of the herds and their rumen pH was related to their lactate concentrations in the rumen. Using this grouping, the *S. bovis* populations were strongly correlated positively to acetic, butyric and high rumen ammonia concentrations. The populations of *S. ruminantium* were also strongly correlated to high rumen ammonia concentrations. T On the other hand, the *Lactobacillus spp.* populations did not show any relationship with the populations of other rumen bacterial species monitored. One possible explanation for the fact that there was no correlation between *Lactobacillus spp*. and the other bacterial species may be that the management practices in place in each herd were the key factor in bacterial population relationships. In fact, from the biplot data the weakest bacterial relationship was with *S. bovis* populations. Tajima *et al.* (2001) postulated that populations of *S. bovis* may possess other fundamentally important characteristics for rumen fermentation of plant polysaccharides than fermentation of starch and moreover in this study the populations of *S. bovis* increased during periods of high fibre availability in diets.

In the analysis using the collated data, the populations of *P. ruminicola* were correlated to rumen pH and also to the concentrations of the volatile fatty acids; acetic, valeric and butyric acid. The strongest relationships were between the populations of the most predominant

bacterial species, *P. ruminicola* and all of other bacterial species but *Lactobacillus* with the strongest relationships between *P. ruminicola* and *S. ruminantium* populations.

The cellulytic bacterial populations of *F. succinogenes* were correlated to all other rumen bacterial populations as well as rumen concentrations of ammonia and the volatile fatty acids; acetic, butyric and iso-butyric acids. Again this finding is consistent with the growth requirements for *F. succinogenes.*

Prevotella ruminicola was the only bacterial population that was correlated negatively to caproic acid concentrations (-0.32). The populations of *P. ruminicola* along with *S. ruminantium* were also correlated to the branched chain fatty acid iso-valeric acid and also to valeric acid.

Fibrobacter succinogenes, *S. bovis* and *S. ruminantium* were all significantly correlated to the rumen ammonia concentrations in the samples analysed. All monitored key bacterial populations besides *S. bovis* and *Lactobacillus spp.* were correlated to the concentrations of iso-butyric acid.

5.4.2 **Cluster analysis based data from (Bramley *et al.*, 2008)**

The herd analysis used by Bramley had three clusters (Bramley *et al.*, 2008) with cluster 1 indicative of acidosis , cluster 2 indicative of suboptimal performance in cows that may have been transitioning to or from an acidotic state as stage of nutrition was difficult to verify and cluster 3 classified as normal . Based on previous studies of acidosis, specific bacterial populations such as *S. bovis* would be expected to be in the highest numbers in cluster 1. However, populations of *S. bovis* were highest in cluster 2 although this was not the most "acidotic" group. On the other hand, the two clusters (1 and 2) with the lower rumen pH did have the lowest cellulytic bacterial populations (*F. succinogenes*) as expected. What was unexpected based on the literature was that the *Lactobacillus spp.* populations were not significantly different between the clusters so these populations were not related to the acidosis

category. Cluster 2 thought to be indicative of the transition from or to rumen acidosis had the highest rumen total bacterial populations. This raises the possibility that that bacterial species not monitored here in the rumen samples may be contributing to the D and L- lactate concentrations. To assist with further interpretation of these results it would be useful to fully type and quantify the *Lactobacillus spp.* present which would more effectively classify their role in the rumen.

5.4.3 **Feed additives and the effects on rumen microbial ecology and metabolism in these dairy cows**

As seen in the previous section of this discussion, cows with the highest rumen pH had the highest *F.succinogenes* populations and vice versa for cows in the low pH category which supports by the findings of Tajima *et al.* (2001). However, the populations of *Lactobacillus spp.* quantified were highest in cows with the higher rumen pH which does not support by previous literature. Specifically this finding does not support those of Wells *et al.* (1997) in which cows introduced to an 80% cereal diet showed a modest decline in rumen pH but a dramatic increase in populations of *Lactobacillus spp.*. Importantly, this study of Wells et al. (1997) was undertaken under *in vitro* conditions.

The addition of feed additive such antibiotics or ionophores are thought to reduce the incidence of acidosis through changes in the bacterial ecology. However, this study did not show that the addition of antibiotics or ionophores had any significant effects on the bacterial populations even though the effects of monensin on the rumen metabolites such as increased concentrations and proportions of propionic acid were clearly demonstrated. Most of the previous studies have been undertaken using induced acidosis conditions.

The herd analysis highlighted cows with a feed additive, which is designed to reduce acidosis and maximise production showed the lowest rumen pH as well as the highest volatile fatty acid concentrations and lowest acidosis status (most acidotic). The addition of antibiotics or

ionophores did not show any indication of significant effects on the bacterial populations. The samples taken from these herds had used feeding or dose rates of additives that were not always set at rates recommended for control of rumen pH or rumen function Bramley, Lean et al. (2012). , Of the nine herds where feed additives were used, three herds fed at doses that were below the recommended for monensin i.e. 250-300mg/cow/day and two of the three were in excess of the suggested dose (200mg/cow/day) for virginiamycin (Lean *et al.*, 2007). For instance, Bramley, Lean et al. (2012) found that 60% of farms in some regions were feeding ionophores at rates lower than those recommended to meet nutritional requirements for efficiency. The bacterial populations may have adapted to exposure to these feed additives such as monensin. Henderson *et al.* (1981) showed that growth of *F. succinogenes* was inhibited by monensin but after prolonged exposure i.e. more than 21 days, *F. succinogenes* did in fact grow in its presence. While Hook *et al.* (2009) showed that when methanogens were exposed to monensin long term, monensin did not affect their population concentrations. On the other hand, Guo *et al.* (2010) showed that addition of virginiamycin had a selective influence on the rumen fermentation by changing the bacterial populations including a reduction in populations of *S. bovis* and *Lactobacillus spp.* with a demonstrated increase in the populations of *S. ruminantium.*

It was hypothesised that fibre utilising cellulolytic rumen bacteria (*Fibrobacter succinogenes*) populations will decrease during grain feeding or any associated reduction in rumen pH. The populations of *F. succinogenes* were not correlated to the herd category or to the pH clusters but cows in herd 12 fed only 9% grain and the remainder roughage had the highest populations of *F. succinogenes*. In general, the populations of F. succinogenes were highest when the diets contained the highest forage concentrations but this finding was not always correlated to a low rumen pH. All interpretation should be taken with a note of caution since all

samples were collected at a single time point and it was unclear at what stage of transition cows were with regards to the feeding of the grain based diets.

The hypothesis that lactic acid utilising rumen bacteria (*Selenomonas ruminantium*) populations will increase with an increase in the grain component of the diet was not supported by these results. This findings is supported by Tajima *et al.* (2001) whose work showed that the *S. ruminantium* populations increased initially but by day 28 of grain feeding had returned to their original level. Nevertheless, the populations of *S. ruminantium* were the most predominant bacteria in these cows rather than the traditionally dominant, *P. ruminicola.* The population of S. ruminantium population was significantly correlated to high concentrations of rumen ammonia which supports its role in the deamination of true protein in the diet. The highest populations of *P. ruminicola* (cells/mL) were present in herds 12 and 38 which were the herds consuming the highest forage proportions suggesting a more significant relationship to cellulose digestion.

The hypothesis that *Streptococcus bovis* will increase significantly and possibly pathologically, during the development of subclinical or clinical acidosis in dairy cattle fed grain-based diets was not supported by these results. Previous studies were conducted predominantly through *in vitro* studies often supplying wheat as the grain. For instance, the work by (Min *et al.*, 2006) showed that *S. bovis* exhibited greatest specific growth when grown with wheat as the major substrate source. However, under the dynamics of an in vivo rumen environment as used in this study, this growth in *S. bovis* was not the case. Although Tajima *et al.* (2001) showed a 67 fold increase in *S. bovis* populations by day 3 in cattle fed a concentrate diet, by day 28 the S. bovis populations were actually lower in these animals than in cattle fed a hay diet. Onime *et al.* (2013) also found that there was no difference between *S. bovis* populations in cattle fed a diet containing either forage or concentrate. Kleive *et al.* (2003) also found that unless there was an acidotic animal, increases in grain diets did not lead to an increase

in the populations of *S. bovis.* The confounding factor in this study was the extent and composition of the pasture intake in these herds. Bramley et al. (2008) reported high concentrations of soluble sugars in fresh pasture in these dairy cattle. These sugars may have influenced the growth and pathogenesis of *S. bovis* independent of the grain intake in the milking sheds.

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It was hypothesised that an increase in the populations of *S. bovis* was linked with a decrease in ruminal pH, and an increase in the populations of *Lactobacillus spp.*. There was an indication that *S. bovis* were related to the rumen pH in cluster 2, however there is no indication that this was linked with a significant increase in the *Lactobacillus spp.* populations. In fact, the populations of *Lactobacillus spp.* showed minimal relationships to either ruminal pH or *S. bovis* in most cases.

This study attempted to relate the metabolic changes in the rumen to changes in rumen molecular ecology in cattle. The results presented here did in fact show that metabolic changes in rumen pH such increased ammonia concentrations or changes in the proportions of VFAs and growth factors such branched chain VFA could be related directly to specific species such *F. succinogenes* and *S. ruminantium.* Thus this study is one of the first to successfully link molecular assessment of rumen bacterial populations with rumen metabolism in dairy cows maintained under true production conditions on farm. Possibly the biggest influencing factor in these dairy cows was rumen imbalance. When the rumen was not functioning optimally, the relationships between rumen microbial populations seemed to breakdown leading to an imbalance. Hook *et al.* (2011) in their studies of the impact of sub-acute ruminal acidosis on the microbial population showed that adaptation significantly altered the bacterial density, diversity, and community structure, warranting further investigation into the role bacteria play in the adaption to ruminal acidosis. (Hook *et al.*, 2011) did show that the *Selenomonas ruminantium*

populations became the most dominant species during subacute ruminal acidosis adaption as found in this study. However by limiting the scope of this study to the key species monitored then these species may not be representing a complete or even useful representation of the changes in the overall rumen microbial populations. Further studies should utilise more powerful and representative molecular techniques such as those employed by Golder et al (2014) to more fully characterise the changes occurring during the time of grain introduction and the subsequent feeding of grain to dairy cows.

6 The impact of lupins, soya bean or lucerne fed individually to rumen-fistulated sheep.

6.1 Introduction

Thus far this study has focussed on the role of feeding cereal grains (containing α-linked polysaccharides as starch) in the aetiology of acidosis and the associated molecular ecology of the rumen. This chapter will present information on the molecular ecology of rumen in sheep fed high protein diets containing either fibre (lucerne), β-linked polysaccharides (lupins) or high fat (soya beans). The latter two diets i.e. lupins and soya beans, are fed as diets containing comparable metabolisable energy with cereal grain diets and high protein-N. However, there have been very few published reports on the ecology of the rumen under these feeding regimes.

Feeding lupins is a common practice in Western Australian beef and sheep production systems. Lupins are regarded as a good protein source in feedlot rations, with the added benefit that lupins are promoted as a safe source of energy since they contain no readily fermentable carbohydrate in the form of starch in the grain (Van Barneveld, 1999). Starch in cereal grains is still viewed as the main cause of ruminal acidosis in ruminants fed grain-based diet (Owens *et al.*, 1998).

The main lupin species in livestock diets are *Lupinus albus, L. agustifolius and L. luteus*. Each of these species of lupin are unique grains as they contain low levels of starch but high concentrations of soluble and insoluble non-starch polysaccharides, low levels of sulphur amino acids and variable lipid concentrations (Petterson *et al.*, 1997). *Lupinus angustifolius* is the variety most widely used as a supplementary feed for ruminants in Australia. In addition to their use in feedlot and finishing rations, lupins are also fed to sheep prior to joining to improve body condition and reproduction rates or during periods of shortage of quality roughage, where lupins have improved feed intake and subsequent animal performance depending on the quality of

roughage supplied (Van Barneveld, 1999). Therefore, it is hypothesised that feeding of non-α-linked polysaccharides as that contained in lupins will not decrease rumen pH or increase the *S. bovis* or *Lactobacillus spp*. populations. It is also hypothesised that with the low fibre content of the diets (lupin and soya bean) the *F. succinogenes* population will decrease over the sampling periods.

Soybeans are also high in protein and low in starch polysaccharides but in contrast to lupins, soybeans have high and consistent concentrations of fat rather than non-starch polysaccharides (Table 8.2). Lupins and soybeans are equivalent to, or often higher in metabolisable energy (ME) when compared with starch based cereal grains. In addition to their benefits as sources of protein, soybeans and lupins are viewed as reducing the risk of ruminal acidosis without reducing the energy content of feedlot diets for ruminants. Since energy and not protein is the first limiting factor for growth in ruminants, this criterion would be judged as a great advantage for lupins and soybeans. However fat inclusion at concentrations greater than 9% in high concentrate diets is considered to have a negative effect on efficient rumen fermentation, especially cellulose fermentation, when fed to lambs (Kucuk *et al.*, 2004).

Polyunsaturated fats occur in soybeans and these can act as alternative electron sinks through hydrogenation of their double bonds. However, electron disposal into double bonds of polyunsaturated fatty acids detracts from the deposition of those electrons in more useful end products of rumen fermentation such as propionate in particular (and other organic acids) that can be used for energy and glucose homeostasis by the host ruminant. It is hypothesised that sheep fed soya bean diets will not show increased S. *bovis* populations in the rumen but the populations of *F. succinogenes* population will decrease with increased dietary fat.

The aims of this study as presented here was to determine the impact that feeding grain legumes that contain high ME and high protein but low α linked polysaccharides have on the rumen microbial environment. It was hypothesised that:

1. Feeding grain legumes with low starch content e.g. lupins will not predispose ruminants (sheep in this instance) to acidosis.
2. Fibre utilising rumen bacteria (*Fibrobacter succinogenes*) populations will decrease during high fat feeding i.e. soybeans or any associated reduction in rumen pH.
3. Lactic acid utilising rumen bacteria (*Selenomonas ruminantium*) populations will increase with an increase in the grain legume component of the diet.
4. *Prevotella ruminicola* will be the most prevalent bacteria in the rumen during the feeding of grain legumes and lucerne.
5. *Streptococcus bovis* will not increase significantly during feeding of grain legume-based diets.
6. If increases in the populations of *Streptococcus bovis* are observed with decreasing ruminal pH, then the populations of *Lactobacillus spp.* will also increase significantly.
7. Changes in the rumen D- lactate concentrations will be related to changes in the molecular ecology during the feeding of grain legumes in sheep.

6.2 **Materials and Methods**

The materials and methods in this chapter are based on the experimental design, sampling procedures, and rumen and metabolite analyses conducted as part of an honours project and the complete experiment is outlined in Ms Kelly Guest's Honours thesis (Guest, 2005). However sections relating to the samples specifically analysed for this study are outlined below. It must be acknowledged that all results apart from rumen bacterial populations were supplied by Ms Kelly Guest.

6.2.1 **Feeding allocation**

Rumen fistulated merino wethers were fed on a diet of lucerne chaff (718g/hd/day) for 10 days then they were randomly allocated on ranked live weight to (Table6.1) three treatments with four sheep in each treatment and fed either,

- Lucerne hay (*Medicago sativa)* at maintenance
- Soy beans (*Glycine hispida)* at maintenance or
- White lupins (*Lupinus angustifolius)* at 3 x maintenance.

Table 6.1 Feed sources offered for daily treatment (adapted from (Guest, 2005)).

Feed type	**Quantity fed (g/hd/day)**	**Crude protein** g/hd/day	**Fat** g/hd/day
Lucerne	718	139	N/A
White Lupins at 3 x maintenance	1550	530	126
Soyabean	440	187	99

Table 6.2 Feed analysis of diets consumed by fistulated merino wethers (adapted from Guest (2005).

Attribute	**Lucerne Chaff**	**White Lupins**	**Soyabean grain**
Dry matter (DM, %)	87.7	91.9	90.3
Crude Protein (CP, % DM)	19.3	34.2	42.5
Acid Detergent Fibre (ADF, % DM)	31.7	21.4	n/a
Digestible Dry Matter (DDM, %)	66.9	91.7	n/a
Metabolisable Energy (ME,MJ/kg)	9.9	13.6	n/a
Crude Fat (Fat, % DM)	n/a	8.1	22.6

6.2.2 **Rumen sampling**

Rumen samples were collected from sheep via their rumen fistula. The rumen fistula stopper was removed and rigid perspex tubing was placed into the rumen. Samples were collected by moving the tube in and out of the fistula hole forcing rumen fluid into the sampling tube and then clamping a thumb over the end of the tube to remove the contents which were then strained through muslin cloth into a 250ml plastic beaker.

Rumen fluid was placed into a plastic beaker and pH measured using an Orion portable pH meter with TPS; pH and ORP reference electrode immediately after the samples were collected, the pH meter was calibrated daily.

Rumen samples were collected with samples analysis in this study for days 0, 0.2, 1, 2, 2.2, 3, 6, 6.2, 7, 8, 8.5., 9.5, 13.5, 13.7 and 14 for all diets. However, after this period, sheep fed the soya bean and lupin diets were removed from the treatments and placed onto lucerne due to rumen dysfunction. Consequently, samples were collected at 14.7, 20.7, 20.9, 21.7, 28.9 and 29.7 days only from those sheep on the lucerne diet.

6.2.3 **Feeding regimes (Guest, 2005)**

Once the treatment regimes were in place, then the amount of food in each feed bin was weighed daily, an hour prior to collection of the rumen sample to estimate daily voluntary feed intake. Sheep were removed from the experiment if rumen pH decreased below 5.5 or if they did not eat the entire allocated treatment amount for more than three consecutive days. As a consequence, two sheep were removed from the experiment: one sheep from the lupins fed 3 x maintenance was removed prior to the sampling at 13.5 days and one sheep fed soy beans at 1 x maintenance prior to 14 days of feeding. The sheep were then placed onto the lucerne maintenance diet and monitored. No sheep died during this feeding trial.

6.2.4 **Buffering capacity (Guest 2005)**

Frozen rumen samples were defrosted overnight at 2^{o}C, centrifuged for 15 minutes at 3000rpm, with the supernatant removed and stored at 2^{o}C. A sample of supernatant (10mL) was then poured into a 20mL beaker, placed on a Corning hotplate stirrer and the initial pH recorded. Following this 0.1mL of 1M HCl was added and pH recorded allowing 1 minute to stabilise. This procedure of adding 0.1 mL of 0.1M HCl was repeated serially until the pH decreased below 6 and then pH 5.

6.2.5 **Analysis of bacterial populations**

Rumen samples were extracted for DNA as outlined in chapter 4 and were analysed for bacterial populations using qRT PCR as outlined in chapter five (Table 5.1) for collections up to 13.5 days after commencing feeding soy beans, 14 days for white lupins at 3 times maintenance and 29.7 days for lucerne.

6.2.6 **Statistics**

Residual plots were examined to ensure that statistical tests complied with assumptions of normality and homogeneity of variance, where necessary data was transformed to ensure that this was the case. Therefore, all of the data from this trial, except pH values and liveweight, displayed lognormal distributions and were log transformed (log_{10}) prior to statistical analysis, while total bacterial were transformed to log_{100}. A linear mixed model which included a fixed effect comparing diet and progressive sampling days and an interaction between diet consumed and progressive sampling days was fitted to each variate using the REML procedure in GenStat (edition 14). The model also included an autoregressive covariance structure between sample dates. All fixed effects were tested using F-statistics or Wald statistics.

Correlations between variates were compared to zero using a two sided test. The matrix of correlations between logarithms of the counts of individual bacteria was used to construct a

biplot which showed the relationships between the different bacterial counts and how sample counts varied across sample dates

6.3 **Results**

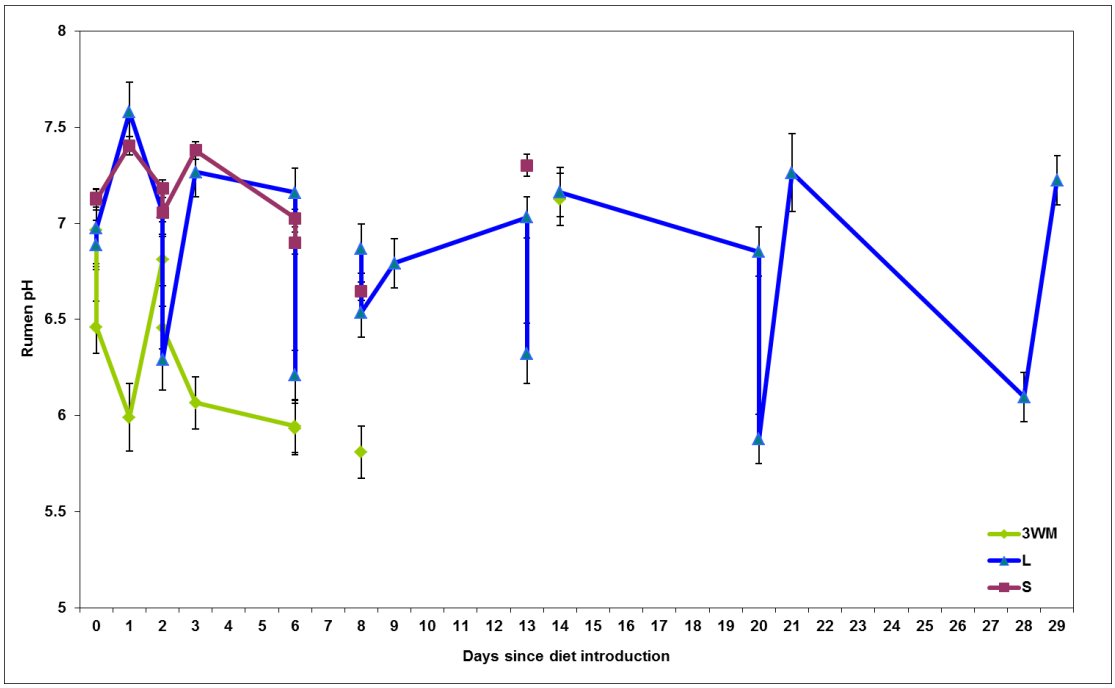


Figure 6.1 Changes in rumen pH (mean ± SEM) for fistulated sheep being fed white lupins at 3x maintenance (3WM), lucerne (L) or soya beans (S) in individual pens at Murdoch University animal house.

Rumen pH in sheep fed the lupin (3 x maintenance lupin) diet decreased to pH of 5.99 at end of day 1, followed by a slow decline to a pH of 5.81 at day 8 after which some of the sheep were removed from the lupin diet and placed on a maintenance diet of lucerne with an associated increase in rumen pH of 7.12 at day 14 (Figure 6.1).

Rumen pH in sheep fed the lucerne (L) diet fluctuated over the sampling period with the lowest pH recorded at 5.88 at 20.9 days, followed by an increase to pH 7.3 for the sample taken at 21.7 days.

The rumen pH in sheep fed soya beans (S) increased to 7.4 with the lowest pH of 6.65 observed at day 8. When removed from the diet after 13.5 days, the rumen pH was 7.3 in one sheep.

The changes in rumen pH in sheep fed the three diets over the sampling period had a significant diet effect as well as a progressive day sampling effect ($P<0.05$). All pH were similar at day 0 ($P>0.05$) after the introductory period on lucerne. The rumen pH in sheep fed the lupin (3 x maintenance lupin) diet was significantly lower than either lucerne (L) or soybean (S) diets at days 0.2, 1, 3, 6 and 8 (LSD 5%). The rumen pH in sheep fed the lucerne (L) diet was significantly lower than in sheep on the soybean (S) diet at 2.2 and 6.2 days (LSD 5%).

The rumen pH in sheep fed the lupin (3 x maintenance lupin) diet during the sampling period was significantly ($P<0.05$) correlated to the bacterial populations of *F. succinogenes* ($R=0.39$), *S. bovis* ($R=-0.46$) and the total bacterial populations ($R=-0.54$). On the other hand, the rumen pH in sheep fed the lucerne (L) diet was not significantly related to any other rumen parameters (rumen pH, buffering capacity or bacterial population) during the feeding period. However rumen pH in sheep fed the soya beans (S) was significantly correlated to the populations of *F. succinogenes* ($R=0.49$) ($P<0.05$).

The rumen pH for the 3 x maintenance lupin diet had the lowest rumen pH at day 8 (5.81±SE), when the sheep were then put back onto a lucerne diet to alleviate the acidosis.

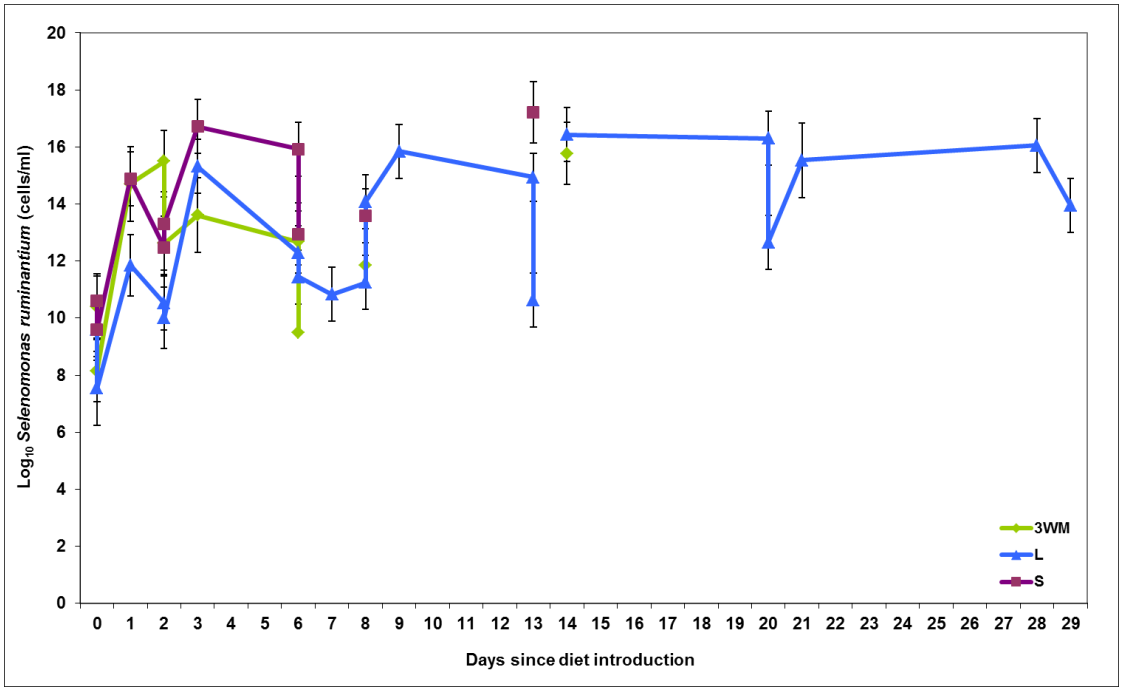


Figure 6.2 Changes in the populations of *S. ruminantium* (cells/mL; mean±SEM) in the rumen of sheep being fed either white lupins at 3x maintenance (3WM), lucerne (L) or soya beans (S) in individual pens at the Murdoch University animal house.

The populations of *S. ruminantium* all initially increased rapidly and significantly to day 1 (Figure 6.2). The *S. ruminantium* populations were dynamic and variable during the period of intense sampling on all three diets. Nevertheless, sheep fed soya bean diets had the highest *S. ruminantium* populations, followed by sheep on the lupin diets and finally sheep on the lucerne diets. The type of diet and duration of feeding each diet had a significant effect on the *S. ruminantium* populations ($P<0.05$).

The *S. ruminantium* populations in the sheep in the 3 x maintenance lupin group were significantly correlated to the populations of the rumen bacteria: *F. succinogenes* (R=0.39), *P. ruminicola* (R=0.61), *S. bovis* (R=0.41) and *Lactobacillus spp.* (R=0.40) ($P<0.05$).

The initial *S. ruminantium* populations (day 0) in the rumen of sheep fed the lucerne diet were significantly lower than the populations at later sampling days. The *S. ruminantium* populations in the sheep fed the lucerne diet showed a significant relationship to the populations of *P. ruminicola* (R=0.48), *Lactobacillus spp.* (R=0.31) and the total bacterial populations (R=0.40) ($P<0.05$).

In the sheep consuming the soya bean diet, the populations of *S. ruminantium* were significantly correlated to the populations of *P. ruminicola* (R=0.40), *S. bovis* (R=0.38) and the total bacterial populations (R=0.49) ($P<0.05$).

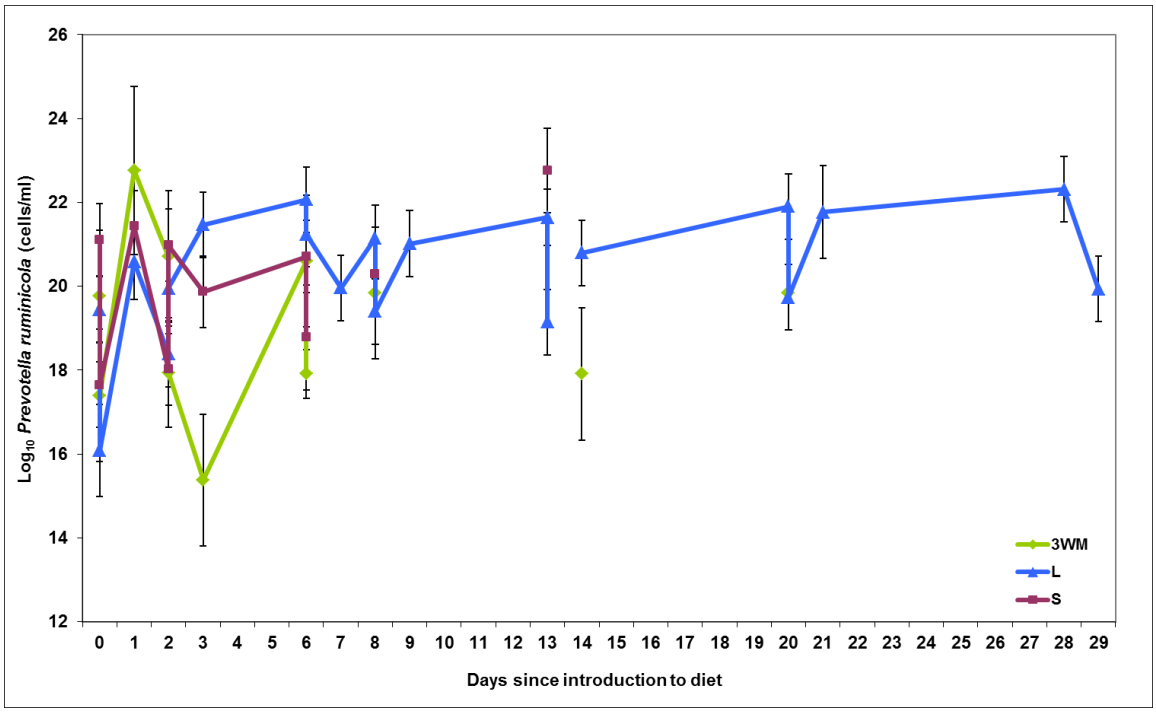


Figure 6.3 Changes in the populations of *P. ruminicola* (cells/mL; mean±SEM) in the rumen of sheep being fed either white lupins at 3x maintenance (3WM), lucerne (L) or soya beans (S) in individual pens at the Murdoch University animal house.

In sheep fed the 3 x maintenance lupin diet, the *P. ruminicola* populations were extremely variable for the first 8 days of feeding (Figure 6.3). The *P. ruminicola* populations decreased at hour 5 (1.56×10^8 cells/mL) from hour 0 (9.23×10^8cells/mL) with the population

then peaking at hour 24 or day 1 (8.5×10^9 cells/mL), and decreasing again to its lowest density at hour 72 or day 3 (8.1×10^7 cells/mL). By 179 hours of feeding (or day 6.2) the *P. ruminicola* populations had established at a density of 6.09×10^8 cells/mL after which the populations remained reasonably constant.

The populations of *P. ruminicola* in sheep on the lucerne diet decreased from (4.26×10^8 cells/mL) at hour 0 to (1.35×10^7 cells/mL) at 0.2 days. The population then increased again at hour 24 to 1.24×10^9cells/mL remaining reasonably constant after that.

The populations of *P. ruminicola* in sheep fed the soybean diet at day 0 (1.96×10^9 cells/mL) continued to fluctuate during the monitoring period.

The *P. ruminicola* population mean showed significant differences between the diets ($P<0.05$). There was though on average no significant effect of progressive days on *P. ruminicola* populations in sheep between the diets ($P>0.05$). The *P. ruminicola* populations for sheep on a 3 x maintenance lupin diet showed a significant relationship to the rumen populations of *Lactobacillus spp*. (R=0.44), *S. bovis* (R=0.53), *S. ruminantium* (R=0.61) and total bacterial populations (R=0.53) ($P<0.05$) during the feeding period.

P. ruminicola populations in sheep consuming the lucerne diet showed significant fluctuation until day 2 (LSD 5%), after which the population then remained reasonably constant. *P. ruminicola* populations sheep on the lucerne diet showed a significant relationship to populations of *F. succinogenes* (R=0.32), *S. ruminantium* (R=0.48), *Lactobacillus spp.* (R=043.) and the total bacterial (R=0.71) populations ($P<0.05$).

Populations of *P. ruminicola* in sheep consuming the soya bean diet significantly increased during the initially sampling (LSD 5%). The populations then remained reasonably constant. Populations of *P. ruminicola* in sheep consuming the soya bean diet showed a significant relationship of *Lactobacillus spp.*, total bacterial population and *S. ruminantium* (R=0.27) to the *P. ruminicola* (R=0.39) population ($P<0.05$).

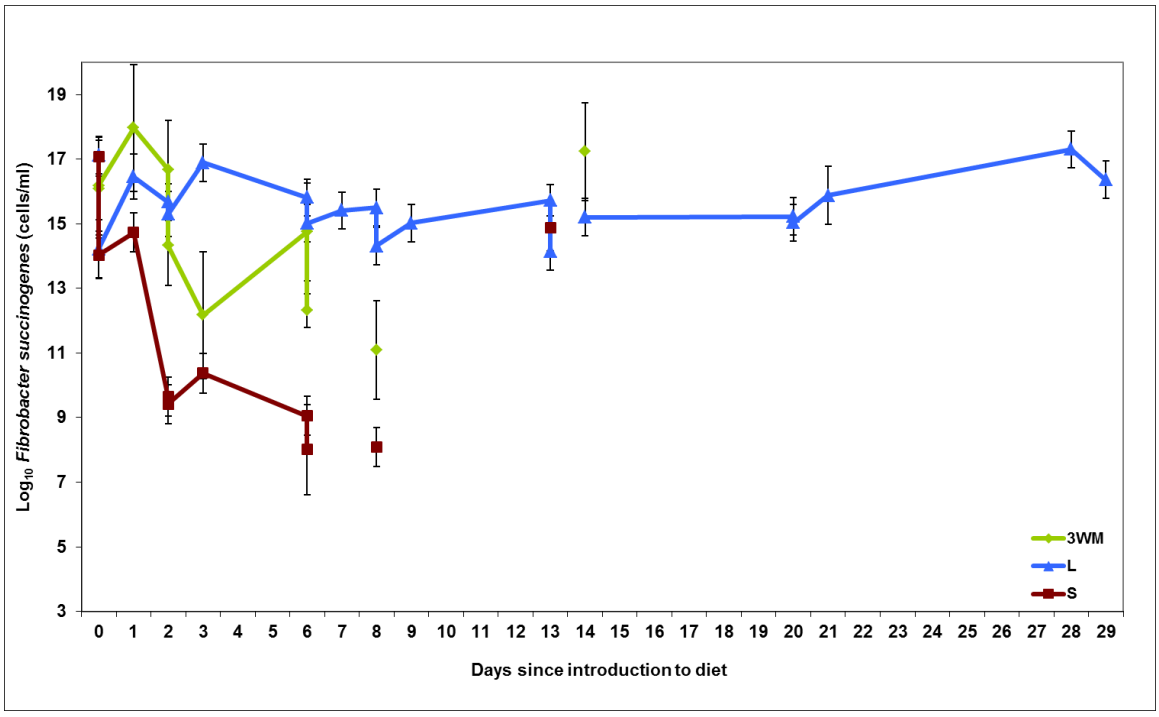


Figure 6.4 Changes in populations of *F. succinogenes* (cells/mL; mean ±SEM) in the rumen of sheep being fed either white lupins at 3x maintenance (3 x maintenance lupin), lucerne (L) or soya beans (S) in individual pens at the Murdoch University animal house.

The *F. succinogenes* populations in sheep consuming the lupin diet increased from 10.5 x 10^7 cells/mL at day 0 to 7.3 x 10^7 cells/mL at day 1. The populations then decreased consistently to the lowest *F. succinogenes* populations at 8 days (4.1 x 10^5 cells/mL). The final population means for F. succinogenes in sheep on the lupin diet was 6.23 x 10^7 cells/mL at day 14 after which the sheep had been removed from the diet due to low ruminal pH (Figure 6.4).

The sheep fed the lucerne diet showed a slight decrease from 2.97 x 10^7 cells/mL at day 0 to 3.39 x 10^6 cells/mL at day 0.2 with slight fluctuations throughout the sampling period.

The *F. succinogenes* populations for sheep on the soya bean diet showed a continual decline over the sampling period starting at 3.32 x 10^7 cells/mL at day 0 and 1.39 x 10^6 cells/mL

at day 2, decreasing to the lowest population values of 4.33 x 10^3 cells/mL at day 8, i.e. less than half of the starting population.

The *F. succinogenes* populations were significantly different between the three diets ($P<0.05$) over the sampling period. The *F. succinogenes* populations were lowest in sheep fed the soybean diets.

The *F. succinogenes* populations for sheep on a 3WM diet were significantly associated with rumen pH (R=0.39) and populations of *S. ruminantium* (R=39) ($P<0.05$). *F. succinogenes* populations in sheep fed the lucerne diet had a significant relationship over the sampling period with populations of *Lactobacillus spp.* (R=0.44) and the *P. ruminicola* population (R=0.32) ($P<0.05$). *F. succinogenes* populations in sheep that were consuming the soya bean diet over the sampling period showed a significant relationship between the rumen pH (R=0.49) and a negative relationship with the *S. bovis* populations (R=-0.29) ($P<0.05$).

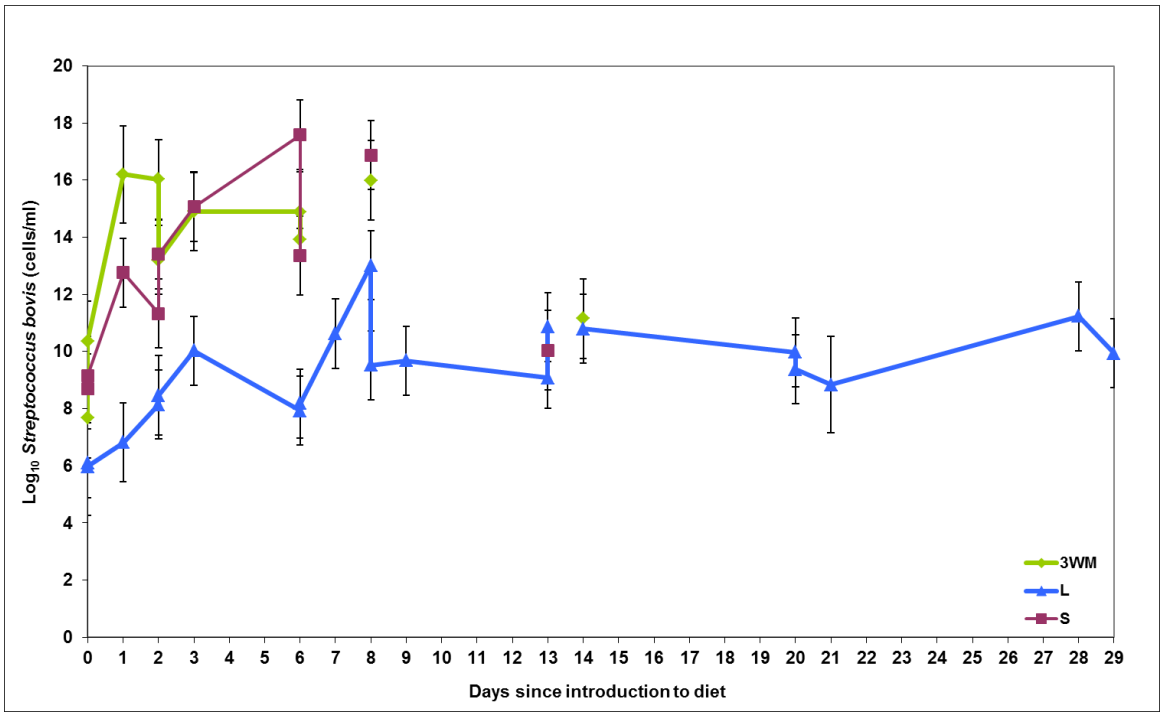


Figure 6.5 Changes in the populations of *Streptococcus bovis* (cells/mL;mean ±SEM) in the rumen of sheep being fed either white lupins at 3x maintenance (3WM), lucerne (L) or soya beans (S) in individual pens at the Murdoch University animal house.

S. bovis populations in sheep fed the lupin diets showed a rapid and significant increase from 1.8 x 10^3 $\log_{10}$ cells/mL at day 0 to a peak of 1.57 x 10^7 cells/mL at day 1, deceasing to 7.21 x 10^6 cells/mL at day 2.2 and remaining reasonably constant after this period (Figure 6.5).

S. bovis populations in sheep fed the lucerne diet increased slightly from hour 0 (2.47 x 10^3 cells/mL) to 3.03 x 10^4 cells/mL at day 3 then remained fairly constant for the remainder of the sampling period.

S. bovis populations in sheep fed soybean diet significantly increased from 4.68 x 10^4 cells/mL at day 0.

The *S. bovis* populations were significantly affected by diet ($P<0.05$) and the duration of feeding, with a significant interaction between the diet and days of feeding the diets ($P<0.05$).

The *S. bovis* populations in sheep fed the lupin diet were significantly higher than in sheep fed the lucerne diet from days 0.2 until day 6 inclusive (LSD 5%). The *S. bovis* populations in sheep fed the soybean diet were significantly higher than in sheep fed the lucerne diet at most sampling times (LSD 5%).

The *S. bovis* populations in sheep fed the lupin diet showed significant correlation to the populations of *P. ruminicola* (R=0.53), *S. ruminantium*(R=0.41), and total bacterial populations (R=0.40) and negative correlation to rumen pH (R=-0.46) ($P<0.05$).

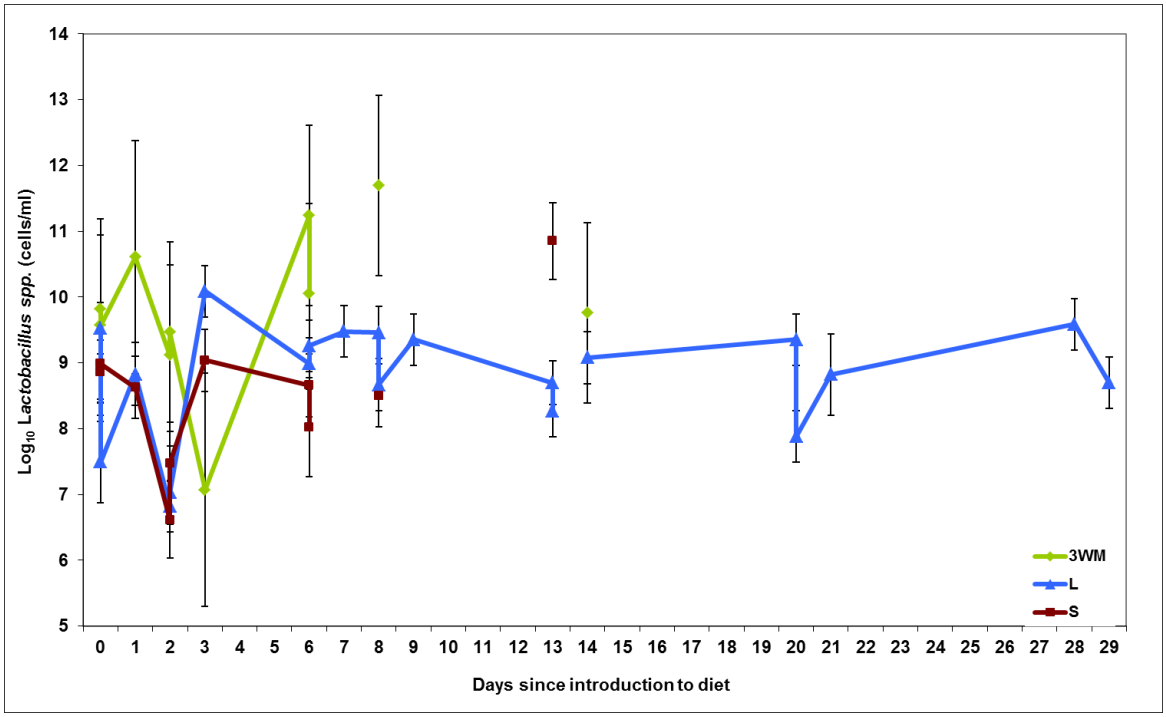


Figure 6.6 Changes in the populations of *Lactobacillus spp*. (cells/mL; mean ±SEM) in the rumen of sheep being fed white lupins at 3x maintenance (3WM), lucerne (L) or soya beans (S) in individual pens at the Murdoch University animal house.

The *Lactobacillus spp.* populations in sheep fed the lupin diet were 1.86 x 10^4 cells/mL at hour 0, increasing to 3.02 x 10^6 cells/mL at day 8 (Figure 6.6). The *Lactobacillus spp.* populations for sheep fed the lupin diet showed significant correlation to the populations of *P. ruminicola* and *S. ruminantium* ($P<0.05$).

The *Lactobacillus spp.* populations in sheep fed the lucerne diet were at day 0 (1.49 x 10^4 cells/mL) decreasing to the lowest population at day 2.2 (2.21 x 10^3 cells/mL) before returning to 1.51 x 10^4 cells/mL at day 3, and then remained fairly constant for the remainder of the feeding period. The sheep fed the lucerne diet showed a significant correlation between the populations of Lactobacillus *spp.* and the populations *F. succinogenes*, *S. ruminantium* and *P. ruminicola* ($P<0.05$).

The *Lactobacillus spp.* populations in sheep fed the soybean diet were 1.49 x 10^4 cells/mL at day 0, and decreased to their lowest population level sampled at day 2, 1.2 x 10^3 cells/mL, before increasing to 3.02 x 10^4 cells/mL at day 3 then remaining fairly constant. By the final sampling at day 14, seven of the sheep fed the soybean diet had been removed from that diet and placed onto lucerne. The sheep fed the soya bean diet showed a significant correlation between the populations of *Lactobacillus spp.* and both the total bacterial (R=46) and *P. ruminicola* populations (R=39) during the sampling period (P<0.05).

The mean of the *Lactobacillus spp.* populations over the sampling period were not significantly different (P>0.05).

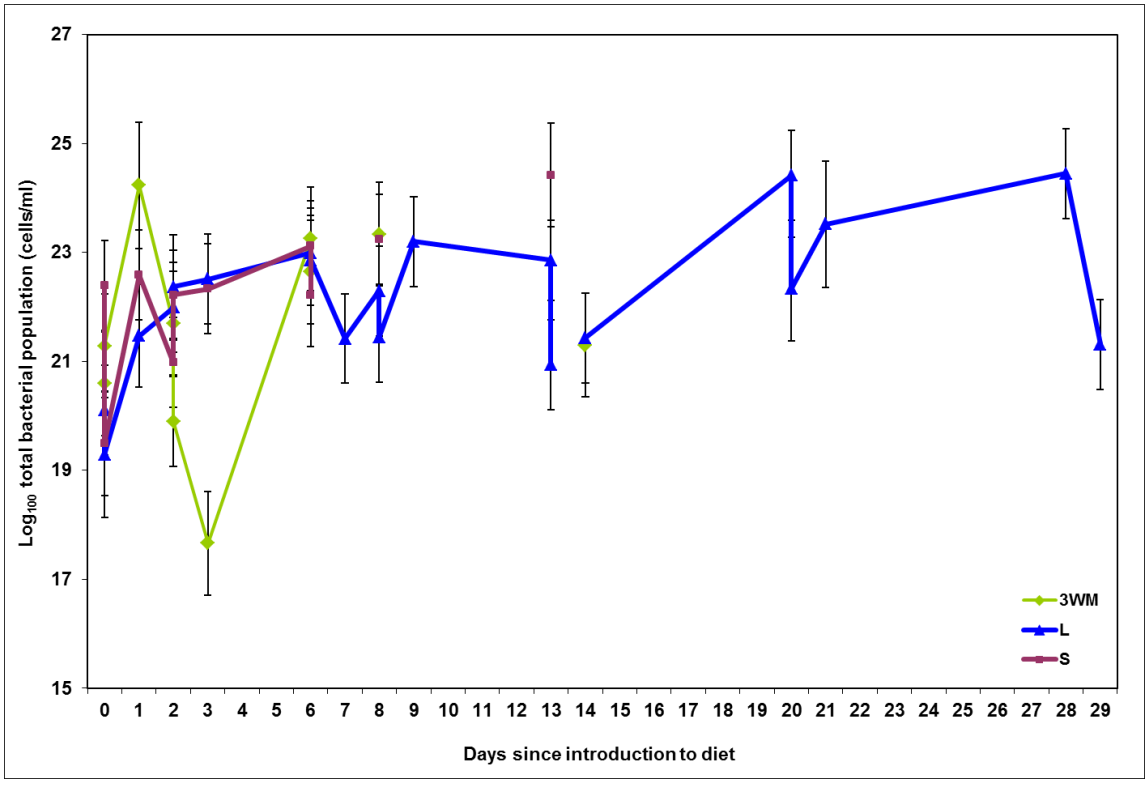


Figure 6.7 Changes in total bacterial populations (cells/mL mean±SEM) in the rumen of sheep being fed white lupins at 3x maintenance (3WM), lucerne (L) or soya beans (S) in individual pens at the Murdoch University animal house.

The total bacterial populations in sheep fed the lupin diet increased from 1.50 x 10^9 cells/mL at day 0 to 6.27 x 10^{10} cells/mL at day 1 before decreasing to their lowest at day 3 (3.02 x 10^8 cells/mL) then the total bacterial population returned to 4.3 x 10^{10} cells/mL at day 6 and remained constant after this period (Figure 6.7). The total bacterial populations in sheep fed the lupin diet showed a significant correlation to the populations of *P. ruminicola* and *S. bovis* ($P<0.05$).

The total bacterial population in sheep fed the lucerne diet decreased slightly from day 0 (6.25 x 10^8 cells/mL) to day 0.2 (4.07 x 10^8 cells/mL) and gradually increased to 1.46 x 10^{10} cells/mL at day 6 with variations and peaks and troughs with the highest population level at 20.7 days (1.46 x 10^{11} cells/mL).

The total bacterial population in sheep fed the soybean diet decreased from day 0 (9.09 x 10^9 cells/mL) to day 0.2 (6.21 x 10^8 cells/mL) before increasing to the original total bacterial population level at day 1 and remaining fairly constant with a gradual increase until day 6 (1.43 x 10^{11} cells/mL) which was the final sample before the sheep were returned to the lucerne diet due to low rumen pH. The total bacterial populations in sheep fed the soya bean diet were significant correlated to the duration of feeding, and the populations of *Lactobacillus spp*., *P. ruminicola* and *S. ruminantium* ($P<0.05$).

There was no significant relationship between the diets and the total bacterial population ($P>0.05$). However there was a significant relationship between the duration of feeding of the diets and the total bacterial populations ($P<0.05$). The sheep fed the lupin diet showed significantly lower total bacterial populations at day 3 than the sheep fed either the lucerne and soybean diets (LSD 5%).

The total bacterial changes for the lucerne diet showed that day 0 had a significant increase to day 3 (LSD 5%), there was also significant declining fluctuations during the

sampling period at days 13.5, 20.7 and 28.9 (LSD 5%).The lucerne diet total bacterial population had a significant correlation to the progressive days of feeding, *P. ruminicola*, *S. bovis* and *S. ruminantium* ($P<0.05$).

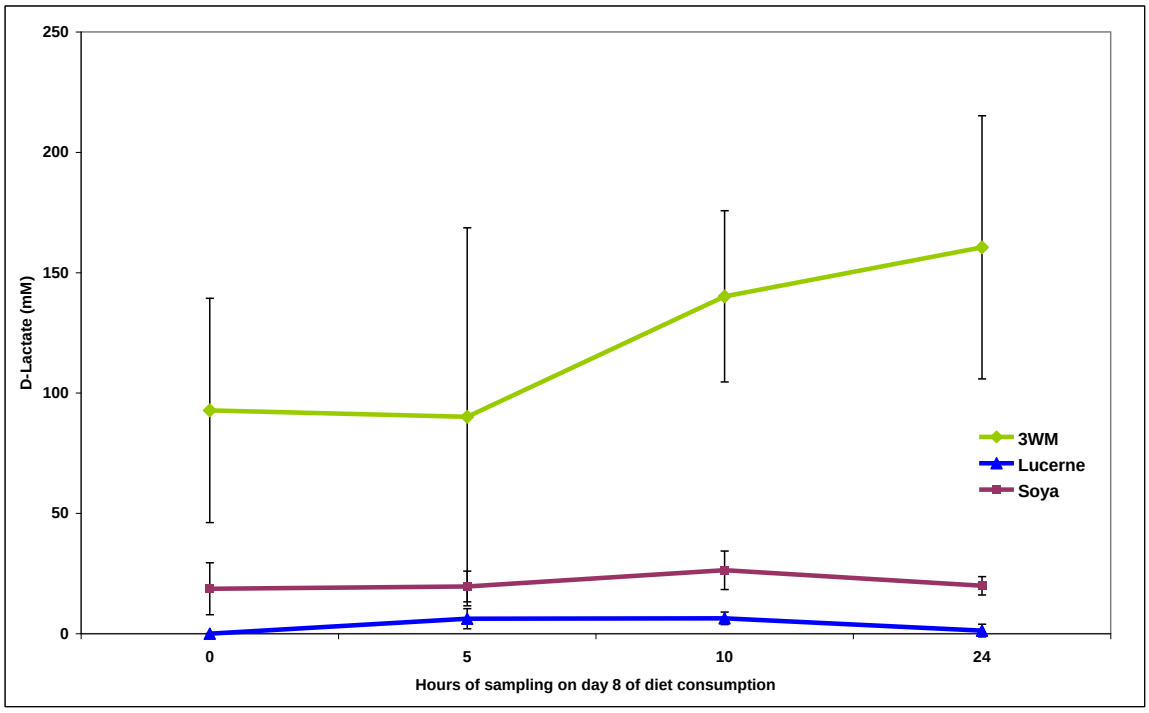


Figure 6.8 Changes in rumen D – lactate concentrations (mean±SEM) at day 8 at hours 0, 5, 10 and 24 post feeding for sheep being fed white lupins at 3x maintenance (3WM), lucerne (L) or soya beans (S) in individual pens at the Murdoch University animal house (Guest, 2005).

The D-lactate concentrations in rumen from sheep fed the lupin (3WM) diet at day 8 taken an hour after feeding was already 92.7 mM and gradually increased over the next 24 hours to peak at 160.5 mM (Figure 8.8). However, D-lactate concentrations, although increasing over the sampling period, were very variable in sheep on the lupin diet. D-lactate concentrations were significantly lower in sheep fed either the soybean and lucerne diets. D-lactate concentrations nevertheless were 18.6 mM at one hour after feeding in sheep fed soybean diet and increased to 26.3 mM 10 hours post feeding. D-lactate concentrations were low in sheep fed the lucerne diet rising to a peak of 6.37 mM, 10 hours post feeding.

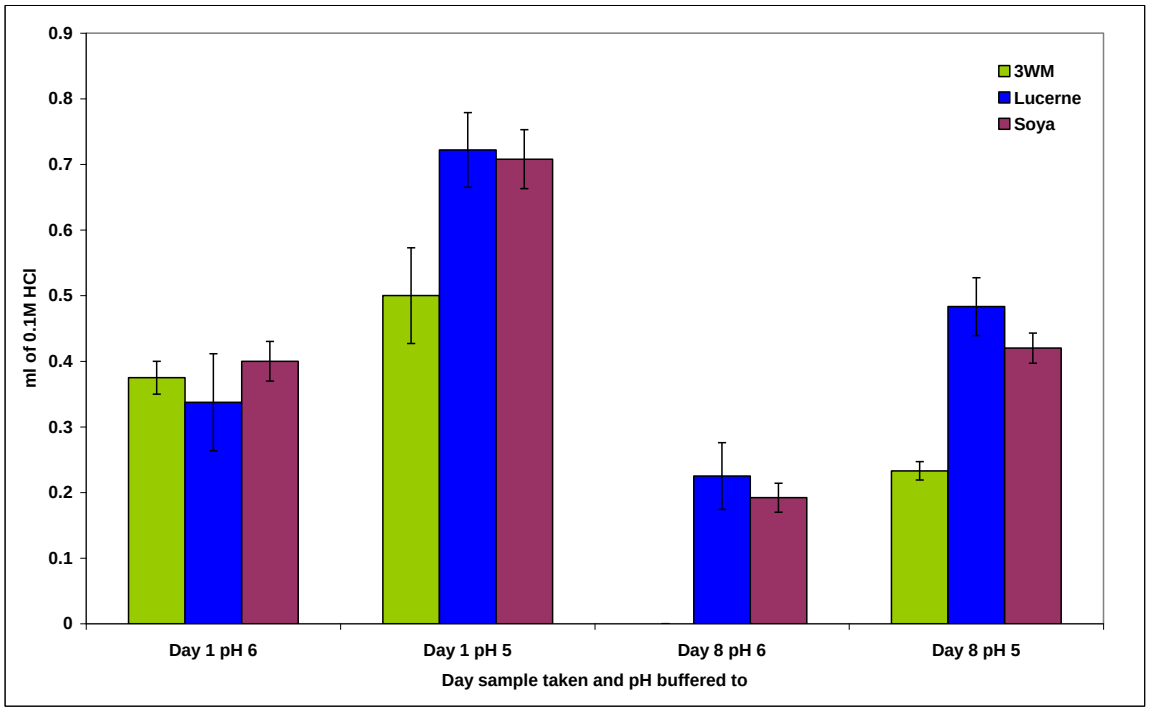


Figure 6.9 Changes in average rumen buffering capacity (mean±SEM) at day 1 and 8 of sampling to pH values 5 and 6 for sheep being fed white lupins at 3x maintenance (3WM), lucerne (L) or soya beans (S) in individual pens at Murdoch University animal house (Guest, 2005).

The buffering capacity calculated by Guest (2005) indicated that the rumen pH adjusted to pH 6 at day one as an indicator of buffering capacity showed no difference between diets (Figure 8.9). The buffering capacity of all diets dropped from day 1 to day 8 after adjustments to both pH 5 and pH 6. When the sheep had been consuming the diet for 8 days the rumen pH in sheep fed the lupin diet was already less than 6, therefore indicating a low buffering capacity compared to sheep on the lucerne diet (0.225 (mL 0.1M HCl) and soybean diets 0.192 (mL 0.1M HCl). When adjusted to pH 5 at day 1, the buffering capacity of sheep fed the lupin diet was 0.5 (mL 0.1M HCl) and at day 8 it was significantly lower than either the lucerne and soybean diets at 0.233 (mL 0.1M HCl). Day 1 buffering capacity at pH 5 was highest in the sheep fed the lucerne diet at 0.77 (mL 0.1M HCl) and soybean diet 0.71 (mL 0.1M HCl). Moreover, at day 8

the buffering capacity at pH 5 was still lower in sheep fed the lupin diet with sheep fed the lucerne diet having the highest buffering capacity 0.48 (mL 0.1M HCl).

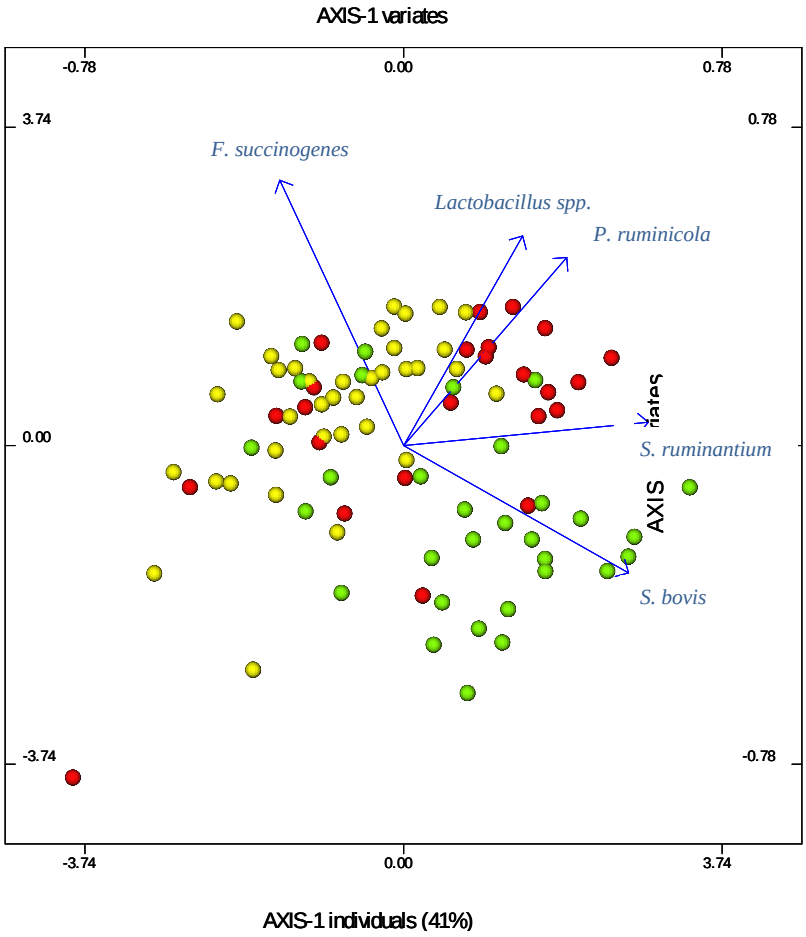


Figure 6.10 Biplot of bacterial populations (cells/mL) for fistulated sheep being fed white lupins at 3x maintenance (Red), lucerne (Yellow) or soya beans (Green) in individual pens at Murdoch University animal house.

The data present in the biplot representing 70% of the total data (Figure 6.10), showed that irrespective of the diets being fed there was no relationship between the populations of *S. bovis* and *F. succinogenes*. The lucerne diet had a higher proportion of cellulytic bacteria than sheep consuming the soya bean diet which had a higher populations of *S. bovis.*

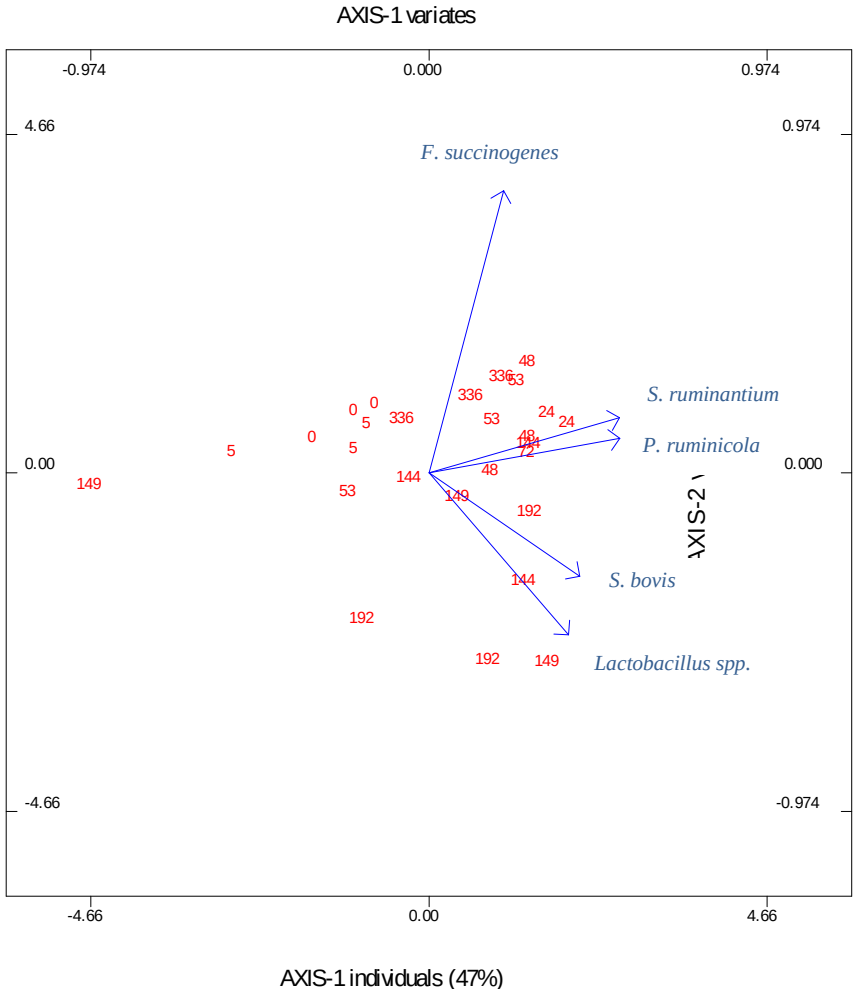


Figure 6.11 Biplot of bacterial populations (cells/mL) in progressive days for fistulated sheep being fed white lupins at 3x maintenance in individual pens at Murdoch University animal house. Numbers indicate days of sampling.

The biplot has 72% of the data from sheep fed the lupin diet showed that the populations of *F. succinogenes* were independent of the populations of *S. bovis* and *Lactobacillus spp.* populations (Figure 6.11). On the other hand, the populations of *Lactobacillus spp*, *S. ruminantium* and *P. ruminicola* were related over the period of sampling (Figure 6.11).

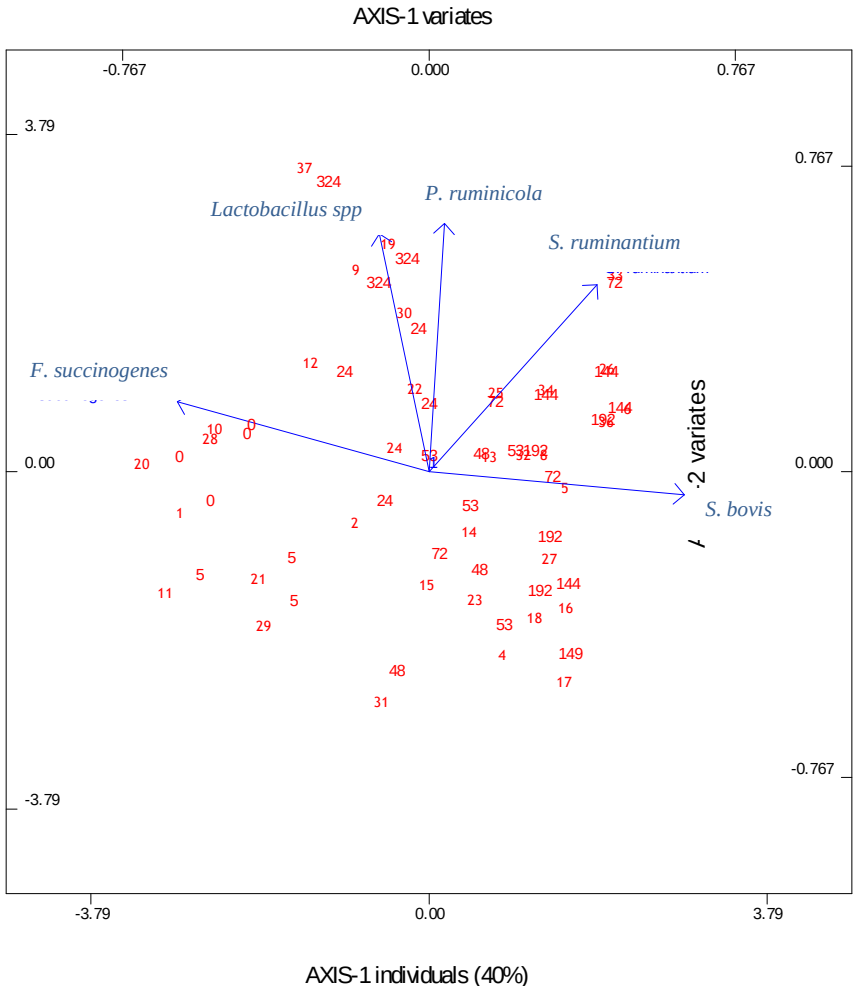


Figure 6.12 Biplot of bacterial populations (cells/mL) in progressive days for fistulated sheep being fed soya beans (S) in individual pens at Murdoch University animal house. Numbers indicate days of sampling.

The biplot data in Figure 6.12 accounted for 74% of the total data in sheep fed the soybean diet and indicated that again the *F. succinogenes* population and the *S. bovis* population were independent of each other over the sampling period (Figure 6.12).

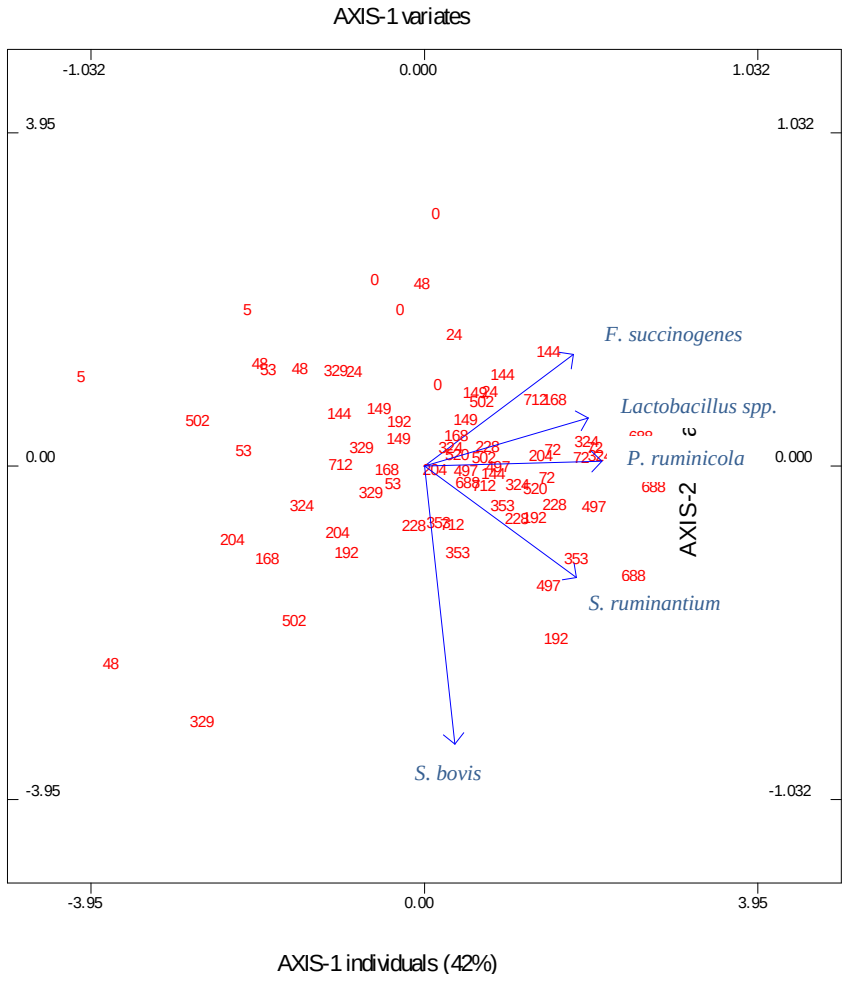


Figure 6.13 Biplot of bacterial populations (cells/mL) in progressive days for fistulated sheep being fed lucerne (L) in individual pens at Murdoch University animal house. Numbers indicate days of sampling.

The biplot data in Figure 6.13 accounted for 64% of the total data from sheep fed the lucerne diet and again showed that the *F. succinogenes* populations and the *S. bovis* populations were independent of each other over the sampling period (Figure 6.13). The populations of *S. ruminantium* were not related to the populations of other bacteria in these sheep (Figure 6.13). On the other hand, the changes in the *Lactobacillus spp.* and *P. ruminicola* populations trended similarly over the sampling period compared to other bacterial changes for sheep consuming a lucerne diet (figure 6.13).

6.4 **Discussion**

The proposition that lupins are a safe feed for ruminants since they contain no fermentable α-linked polysaccharides such as amylose and amylopectin must be questioned given the deceases in rumen pH and increases in D-lactate concentrations observed in this study.

Lupins must contain carbohydrates that are fermented at a rapid rate associated with a lowering of pH, a D-lactic-acidosis and a loss of buffering capacity. Analysis of feedstuff by Knudsen (1997) indicates that lupins were one of the lowest starch-containing grains at 12 g/kg but contained the highest non starch polysaccharide content at 451g/kg. The breakdown of non-starch polysaccharides by a bacterial population was not directly monitored given the species used during this study. The β-glucans in lupins can be fermented rapidly in the rumen to produce organic acids at a rate in excess of the buffering capacity of the saliva. Moreover, the rate of anaerobic glycolysis in the rumen can give rise to carbon being diverted from the succinate pathway into the acrylate pathway and hence a rapid and excessive production of both L- and D-lactic acids. Such an alignment of these three indicators should indicate caution when including *ad libitum* feeding of lupins as a transition energy feed in feedlot rations certainly for sheep and possibly cattle. For sheep at least, diets high in lupins may not be suitable without a roughage component in the diet. Moreover, lupin feeding can lead to the extent of rumen dysfunction as observed here.

These results are similar to those reported by (Allen *et al.*, 1998) in which they fed milled lupins to sheep and recorded a decrease in rumen pH and increase in ruminal and plasma D-lactate levels. This study had levels of ruminal D-lactate approximately double compared to that of the work done by Allen *et al.* (1998) but decreases in ruminal pH over the time period were similar to this study. The ruminal ammonia and urea increased significantly in the work by Allen *et al.* (1998) without the corresponding increases in plasma urea and ammonia indicating that ammonia toxicity may not have been an issue with the excess feeding of lupins. The sheep in the

study by (Allen *et al.*, 1998) were fed on a falling plane of nutrition prior to introduction of milled lupins and these sheep showed indications of acidosis, rumenitis and reticulitus compared to other sheep on a rising plane of nutrition who did not exhibit these symptoms. Milling the lupins can increase the rate of consumption and the surface area for fermentation in the rumen, both of which are factors that can lead to an increase in severity of acidosis, allied to the hunger associated with the lower plane of nutrition in the affected sheep. Although the low plane of nutrition and milling were not part of this trial, it is interesting to note that the sheep in this experiment were fed whole lupins, in which you would expect a lower rate of intake and fermentation. Therefore sheep were in fact able to readily masticate these whole lupins to expose a greater surface area for microbial fermentation leading to a significant decrease in rumen pH and increase in ruminal lactate levels.

There was a significant increase in the populations of *Streptococcus bovis* in the rumen of the sheep fed the lupins *ad libitum.* Increases in the populations of *S. bovis* have been usually associated with rapid fermentation of α-linked carbohydrates normally under conditions of high starch availability (Owens *et al.*, 1997; Owens *et al.*, 1998; Russell and Rychlik, 2001), and significantly correlated with low rumen pH,. This study could have been strengthened by monitoring the total amount and molar ratios of volatile fatty acids in the rumen during lupin feeding. The fermentation of protein of the type and quantities in lupins can give rise to the branched-chain VFAs: iso-butyric, iso-valeric and iso-caproic acids all of which are the β-keto acid breakdown products of their corresponding branch-chain amino acids. Valeric and iso-valeric acids have been implicated as indicators of acidosis and possible rumen dysfunction in dairy cattle (Bramley, 2004). Thus the fermentation of protein in the lupins could also be contributing to the lower pH and loss of buffering in the rumen of these sheep.

Although there are at least two distinct differences i.e. milling of the lupins and the two planes of nutrition, between the study by Allen et al. (1998) and this study, the fact remains that

in both studies, feeding the putatively safe feed, lupins led to rumen dysfunction and lactic-acidosis. Thus the advice to farmers and feedlotters should carry the caveat that care must be taken when either introducing lupins or feeding large quantities (e.g. 3 x maintenance) of lupins to ruminants.

The high oil content in the soy bean diet also resulted in rumen dysfunction although not in the traditional sense of reduced rumen pH and buffering but inhibition of cellulytic rumen bacteria. The increase in rumen D-lactate concentrations in sheep fed soy beans is novel and of concern. The important thing to note in these sheep is that the rumen pH did not decrease yet the populations of *S. bovis* did increase as did the D-lactate concentrations. The study by Yang *et al.* (2009) found supplementation with soy bean oils (4% of ration) increased the amyolytic and proteolytic bacteria (which includes *S. bovis* and *P. ruminicola*) in the rumen while decreasing cellulolytics including *B. fibriosolvens, F. succinogenes* and *R. flavifacienes* similar to the findings in this study. It was also interesting that Broudiscou *et al.* (1990) found soya oil added to the diet did not lower the total VFA concentration but shifted fermentation to increased proportions of propionate and decreased butyrate and acetate proportions. These changes in molar proportions may have resulted in the higher levels of lactate in the rumen of sheep fed soy bean. The source of the carbon for D-lactate in the rumen of these sheep fed soy bean diets has not been established from this study, but it may have been produced mainly from the proteins.Soy beans contain about 30% carbohydrate which is divided between soluble carbohydrate including sucrose (5%), stachyose (4%) and raffinose (1%), while the insoluble fibre fraction makes up 20%. Moreover, microbial lipase is high in activity and oils are digested to release fatty acids. Unsaturated fatty acids get hydrogenated (saturated) in the rumen acting as a sink for H_2, competing with CO_2. This action qualifies vegetable oils that are rich in unsaturated fatty acids for use as a potential strategy to reduce methane emission in ruminants.

The fact that soybean contains 22.6% oil; it makes it unsuitable sole dietary ingredients for ruminants. Ruminant animals evolved as herbivores with a digestive system most suited for the digestion of fibre. Diets rich in starch and fat are not suitable for the ruminant animals. The soy bean diet (S) had very high oil content of 22.6% which as expected lowered the function of celluloytic bacteria in the rumen (Moss *et al.*, 1997; Yang *et al.*, 2009). The most unusual aspect of this diet in comparison to others is the high rumen pH which did not begin to decrease below 7 until hour 144 (6 days). Moreover, the buffering capacity was reduced in sheep fed the soybean diet compared with those fed the lucerne diet, even when the rumen pH was 6.65 (its lowest sampled rumen pH) in the sheep fed soybeans. The lactate concentrations in sheep fed the soy bean diet were higher than those observed in sheep fed lucerne but significantly lower than in sheep fed the lupin diet. In conjunction with the increased lactate, the population of the *S. bovis* increasing rapidly in sheep fed soybean over the sampling period and in fact the population of S. bovis doubled after 6days (Figure 8.3.5). The decrease in *F. succinogenes* populations concentrations over the sampling period was similar to that observed in sheep fed the lupin diet. The high fat content may have inhibited fibre digestion, which in combination with poor substrate availability of fermentable fibre in the soy bean did not support growth of Fibrobacter *succinogenes*.

. It is tempting to speculate that the unsaturated fats in soy beans acted as an alternative electron sink in the reducing conditions in the rumen such that the more usual link between *F. succinogenes* and the methanogenic archaeal species was not operating to support the growth of the cellulolytic *F. succinogenes*. Soy beans are commonly included in feedlot rations for cattle but they have not been included in the aetiology of acidosis under these feeding regimes. Given these findings in sheep, it may be timely to revisit the possible role of soy beans in the acidosis during dietary transitions.

Each of these diets contained protein concentrations higher than the requirement for sheep at this life stage. Thus the consistent presence of high populations of *P. ruminicola* is not

surprising since this species is considered one of the key contributors to the breakdown of dietary proteins to peptides and amino acids in the rumen (Stewart *et al.*, 1997; Yang *et al.*, 2009). The other major proteolytic and peptidolytic species is *Selenomonas ruminantium*. These two species were closely aligned in each of the diets as can be seen in Figure 6.3.10. Thus the functional role of these two species in N metabolism in the rumen may override the effect of pH and to a lesser extent concentrations of D-lactate in the rumen.

The lowered rumen pH was associated with a significant increase in the *S. bovis* populations, with it doubling in the first one to two days in sheep fed the diets consisting of lupins or soy beans (Figure 6.3.5). These increases in S. *bovis* populations have been demonstrated in studies where carbohydrate substrates were available for *S. bovis* which resulted in dramatic increases in the population size over short periods of time (Al Jassim and Rowe, 1999; Rowe, 1999; Krause and Russell, 1996; Russell and Baldwin, 1979). The rumen pH was significantly negatively correlated to the *F. succinogenes* population in sheep fed lupins ($P<0.05$) but not in the sheep fed soy beans. In this study as rumen pH decreased so did the populations of *F. succinogenes.* On the other hand, increases in the *F. succinogenes* population in sheep fed the higher fibre lucerne diet showed a lag period before increasing their population numbers (Bryant and Doetsch, 1955; Stewart *et al.*, 1981). The *F. succinogenes* populations were significantly correlated to the *S. ruminantium* populations during this study. (Caldwell and Bryant, 1966) showed that *S. ruminantium* was highest in the rumen of animal fed cracked corn and urea where they constituted 22-51% of the viable count. The decrease in the populations of *F. succinogenes* may be related to the high lactate content resulting from the growth of the *S. bovis* populations in sheep fed the lupin diet. The rumen pH in these sheep was correlated with the total bacterial populations. This may be related to the ability of bacteria such as *S. bovis* to replicate rapidly as shown by (Russell *et al.*, 1981; Rowe, 1999)where *S. bovis* populations doubled at a rate comparable to *Escherichia coli*. work by (Russell and Baldwin, 1979) showed

the ability to grow in very short generation time of less than 15 minutes. Moreover, sheep fed the soybean diets showed the potential for populations of S. *bovis* to double without a decrease in the rumen pH but with an associated decrease in fibre degrading bacteria represented in the form of *F. succinogenes* populations. It would have been ideal to have additional species of ceulluytic bacteria quantified or employ the use of additional genetic technology to more extensively identify other interactions in the rumen biome.

This work also indicated that there is potential for acidosis with β-linked polysaccharides in the form of the lupins and that a decreasing ruminal pH is not always an absolute indicator of acidosis. In fact, sheep fed the soybean diet had a neutral to high pH but also had a very large increase in the *S. bovis* population. Even with large increases in the *S. bovis* populations, this did not necessarily signify acidosis as shown in the soybean diets. Work done by (Golder *et al.*, 2014) showed that the role of *Lactobacillus and S. bovis* populations in ruminal acidosis was unclear. It is also interesting to note that although the lupin diet had a decrease in rumen pH there was no significant changes observed here in the rumen *Lactobacillus spp.*

Notwithstanding these observations of the links between rumen parameters such as pH, buffering and D-lactate and some rumen bacterial species, the understanding of how these rumen bacteria change and adapt to the different substrates contained in these diets is still relatively unexplored. Most of the previous studies have relied on culture-based techniques Goad *et al.* (1998). Over the last decade or so, qRT PCR has been developed by Tajima and co-workers on a few indicator bacteria in the rumen (Tajima *et al.*, 2001; Tajima *et al.*, 1999; Tajima *et al.*, 2000) to monitor the changes in bacterial populations under dietary transitions. These latter studies have not linked the molecular studies with the physiology and metabolism of the rumen as has been the case here. Furthermore, metagenomic analysis allied to clonal library collections has shown there to be many more potential open transcription units (OTUs) and possibly a much

greater number of bacterial and archaeal species in the rumen than previously reported through culture-based techniques. Metagenomics is a rapidly growing field of research that aims at studying uncultured organisms to understand the true diversity of microbes, their functions, cooperation and evolution, in environments such as the rumen (Huson *et al*., 2009). Thus a metagenomic approach allied to the qRT-PCR methods applied here, and having both of the molecular approaches aligned with the rumen digestive physiology should yield rich and novel insights into the population changes occurring during the feeding of these diets in sheep.

7 Conclusions and Future Directions

Livestock production, specifically the production of red meat and dairy products, is projected to increase to meet the demand of both an increasing world population and a higher proportion of middle income earners. Nevertheless this increase in red meat production may be constrained by concerns about the environmental impact and sustainability of ruminant production systems ((Alexandratos and Bruinsma, 2012; Revell, 2015)). To this end, there is increasing pressure for more efficient production systems for meat, fibre and dairy products. Consequently, there is likely to be an increased dependence on grain feeding to achieve these higher animal production demands with reduced ecological impacts. Grain feeding will continue

to supply the energy and protein required for growth for finishing cattle and sheep and increased milk production in dairy cows. In addition, grain feeding is widely used for supplementation of livestock during periods of low pasture availability. One of the major problems associated with supplementary feeding of concentrate diets based on cereal grain is the associated potential incidence of clinical and subclinical acidosis. Moreover, the economic impact of acidosis, especially subclinical acidosis, is difficult to quantify as the losses can range from unidentifiable production losses to subsequent death of a ruminant.

Acidosis has been extensively studied under conditions where acidosis has been experimentally- induced (Goad *et al.*, 1998; Godfrey *et al.*, 1994; Hook *et al.*, 2011; Horn *et al.*, 1979; Nagaraja *et al.*, 1978; Sauvant *et al.*, 1999) usually by feeding large loads of cereal grain and then monitoring the effects on rumen metabolism and the rumen microbial populations. The decrease in ruminal pH from the normal range of pH 6.4 – 7.2 to below 6.0 and even to pH 5.0 upon introduction to grain based diets has been the consistent observation in these experimental studies. This study was unique in that it monitored cattle in commercial feedlots rather than following experimentally- induced acidosis. The key to this study was monitoring the dietary transition of cattle onto grain based diets rather than understanding the incidence of induced short term acidosis. Therefore, basing the study on commercial feedlots highlighted how differing management techniques impacted not only phenotypic indicators of rumen pH and metabolism but also quantified the genetic changes of key species of carbohydrate and protein fermentation in the rumen.

Two commercial feedlots were studied where cattle were introduced onto either a total mixed ration or hay and grain supplied separately. These commercial cattle managed under commercial conditions showed no signs of acidosis either through changes in rumen bacterial ecology or rumen metabolism. This finding demonstrated that feeding good quality roughage to support cellulolytic fermenters such as *Fibrobacter succinogenes* irrespective of the introductory

method if managed effectively may be a prime determinant in sustaining the rumen in a normal, non-acidotic state. Moreover, the hypothesis that cattle introduced to grain based diets under commercial feedlot conditions will have higher incidence of acidosis when grain and hay was fed separately was not supported. The hay fed separately in this feedlot was of high quality, so it would be valuable to assess whether low quality hay would lead to a higher incidence of acidosis.

Decreases in rumen pH under experimental conditions have been associated with isolation and phenotypic characterisation and quantification of increased lactic acid producing bacteria such as *S. bovis* and *Lactobacillus spp.* as well as lactic acid utilisers such as *S. ruminantium* and a reduction in cellulolytic bacteria such as *F. succinogenes*. Moreover, previous quantification of bacterial changes during acidosis has been carried using phenotypic sub-culture techniques performed on rumen samples collected under experimental conditions rather than commercial feedlot conditions. In contrast this project has focussed on developing genotypic molecular techniques such as qRT- PCR of 16SRNA genes to quantify changes in rumen microbial ecology under commercial conditions, and has aimed to link these genotypic changes to changes in rumen physiology and metabolism.

Several fundamental procedures such as standardisation of enumeration, extraction of DNA and primer design for bacterial quantification using 16S RNA genes needed to be validated and shown to be reliable and repeatable before analysis of field samples could begin. The main task was to establish confidence in the validity of bacterial numbers in cells/mL values that were produced during the qRT- PCR process to reliably enumerate the bacterial species. These relied on quantification of the standards from bacterial culture on a cells/mL basis, complete and consistent extraction of DNA from the rumen samples, both pure cultures and rumen samples and finally the development of effective primers for the 16S RNA genes and quantification of the RT PCR process itself.

To correlate a cells/mL value for a targeted bacterial species that was being monitored, the standard need to be translated into a cells/mL value in the mixed ruminal population samples while utilising the qRT- PCR technology. A Coulter counter was used to quantify the pure cultures on a cells/mL basis and this was compared to a turbidity reading using a spectrophotometer. The turbidity reading showed high R values for the cells/mL but the repeatability was tested further by using the turbidity to determine cells/mL where clumping of bacteria cultured for long periods can result in the blocking of the aperture and of the Coulter counter itself. Once these procedures were performed and validated, any surplus from the pure cultures from the quantified samples was frozen for later DNA extraction rather than relying solely on a turbidity reading to estimate the cells/mL in the sample quantified.

In addition to the quantification of bacterial culture in cells/mL, the DNA extraction process had to be consistent and repeatable for both pure cultures and rumen samples. Consistent extraction of DNA from rumen bacteria proved problematic for some time during this study, with some of gram-positive and gram-negative bacterial species being extracted in a non-consistent manner using various published techniques and commercial kits. Finally, a methodology was obtained from Dr S. Denman of CSIRO (*pers comm*.) that proved effective and consistent for all extractions which highlighted that consistency and repeatability of DNA extraction was crucial to the success of any molecular study of rumen bacteria or the rumen biome.

The instrumental final step was the development of the primers to reliably detect and quantify the targeted key bacterial species of *F. succinogenes, P. ruminicola, S. ruminantium, Lactobacillus spp., S. bovis* and the total bacterial population. Given the technology and software support that was available during 2003-2006, there was no guarantee that the verification tests available at that time were only picking up the targeted bacteria. Verification tests to determine if only the desired regions were being amplified included using melting curve analysis during the

qRT-PCR reaction, which tested the amplicon length and hence the combination of nucleotides giving a unique melt curve. Moreover, keeping the amplicon length as reasonably short as possible through primer design, testing those primers against the pure cultures available or against online sequences still did not guarantee that there was no cross reactivity. However, these results from these techniques were consistent across all samples tested during this study. Molecular techniques that underpin metagenomics have progressed dramatically since the experimental work for this project was completed in 2006 but this study still provides a very good base to the understanding of the rumen microbial populations particularly as these key species have been the focus of microbial studies until mid-2009 when new techniques were being employed. Therefore, the hypothesis that the molecular technique of quantitative real-time polymerase chain reaction (qRT-PCR) of 16S RNA genes can be developed using pure cultures of rumen bacteria as references to then monitor the changes in population ecology of rumen bacteria in mixed rumen samples collected under practical commercial feeding regimes was supported.

Differences in the rumen microbial populations were thought to exist when cattle were raised on varied pastures (dry low quality autumn pasture vs fast growing high quality spring pasture) and then fed subsequently a high grain diet in feedlot. However, this research showed that time of calving did not have a long-term influence on the rumen microbial ecology established post-weaning. In fact, there was a greater influence of the management practices that were put in place when cattle were transitioned onto grain-based diets. Overall cattle from the two calving times showed very successful adaptation to grain introduction without any obvious signs of rumen dysfunction. The other important finding from this data showed that even when there was a decrease in cellulytic bacteria such as the indicator population of *F. succinogenes* or an increase in lactate producing bacteria such as *S. bovis*, this ecological change was not always indicative of acidosis. The interesting outcome is that the rumen protozoal populations remained

significantly different in cattle from the two times of calving over the dietary introduction period in feedlot highlighting that the protozoal ecology was independent of the monitored key bacterial populations. The rumen pH remained at what could be classified as safe hydrogen ion concentrations throughout the introduction and transition to grain diets for both calving groups as indicated by the D- and L-lactate concentrations remaining low throughout the grain feeding period. It would be interesting to be able to monitor the full development of the bacterial ecology from an earlier stage rather than just at weaning time and continue with the cattle that were born onto high quality pasture on an irrigated pivot or associated high quality pasture diet for longer periods prior to grain feeding. In this study, there was only 3 months of feed quality difference between the cattle in the two time-of-calving groups. Moreover, calves in the early calving group were not eating a large amount of roughage as part of their early diet and as such, time of calving may not have been as much of a factor as it could potentially be.

The use of feed additives has become common practice within ruminant feeding systems. However, with legislative restrictions in their incorporation into animal feeding systems, the study also determined if the addition of any feed additive such as antibiotics or ionophores would reduce the incidence of acidosis through changes in the bacterial ecology established in the rumen during any grain introduction. This was not supported in this thesis. However, it should also be noted that introductions were very successful through well implemented management practices. In rumen samples from dairy cattle that received the addition of feed additives were associated not only with increased production indicators such as propionate concentrations and proportions but also with increased acidotic indicators such as reduction in rumen pH, increased populations of *S. bovis* and concentrations of D-lactate. In dairy cattle, addition of good quality hay rather than lower quality straw as a forage source was associated with rumen parameters more indicative of an overall rumen environment representative of a successful transition. This outlines the potential importance of education not just about the use of

feed additives but the importance of using the correct dose, since Bramley *et al.* (2012) reported that 60% of these dairy cattle were fed lower than the recommended dosage of feed additives.

The central dogma in ruminant feeding systems is that cereal grains impact the rumen bacterial populations due to their readily available carbohydrates being fermented rapidly by the rumen microbial population leading to acidosis. Feeding grains with low starch content e.g. lupins or soybeans should not predispose ruminants (sheep in this instance) to acidosis. In fact, these studies feeding lupins *ad libitum* to sheep showed that acidosis occurred in sheep fed with β-linked polysaccharides in the form of a 3x maintenance lupin diet. Moreover, sheep fed high fat diets based on soybeans did develop acidosis as indicated by very large increases in the *S. bovis* population without any associated decrease in ruminal pH and development of clinical signs of diarrhoea and depression. Overall this soybean diet did indicate that there was potential to have populations of *S. bovis* doubling in sheep without rumen pH decreasing below pH 5.5. The monitored cellulytic bacteria *F.* succinogenes decreased dramatically when consuming the soya bean diet. It would have been ideal to have a broader range of cellulytic bacteria quantified, or utilisation of newer technology to more easily quantify the total rumen biome without the need for a large throughput of samples. This study showed that bacterial population dynamics were strongly influenced by feed source and moreover the changes in *S. bovis* and *Lactobacillus spp.* populations did not fit with previous proposals about onset of acidosis mainly from feeds containing rapidly fermented soluble carbohydrates.

This study monitored the key bacterial populations and it was hypothesised that the fibre utilising rumen bacteria (*Fibrobacter succinogenes*) populations will decrease during grain feeding or any associated reduction in rumen pH. This was supported in this thesis under both commercial feedlots, in dairy cattle, and in sheep fed diets that were high in fat, the cellulytic bacteria did decrease. A decrease in the populations of cellulytic bacteria was not always indicative of acidosis as reflected by the rumen pH. This finding should be explored further

using a greater variety of cellulytic bacterial species such as *Ruminoccocus albus* and *flavefaciens*. In addition the latest molecular technologies focusing on genome sequencing, pyrosequencing, proteomics and transcriptomics (Krause *et al.*, 2013) with techniques such as terminal restriction fragment length polymorphism (T-RFLP). T-RFLP is a DNA fingerprinting technique used for comparisons of complex microbial communities and next generation sequencing (NGS) (de la Fuente *et al.*, 2014). T-RFLP will permit monitoring of a much greater variety of bacterial species e.g. the studies by Kim *et al.* (2011) and provide better profiles of the bacteria that are present within the mixed population rumen samples. .

Prevotella ruminicola was the most prevalent bacterial species in the rumen during dietary transition which was supportive of previous work in this area (Griswold and Mackie; Fondevila and Dehority, 1996; Tepsic and Avgustin, 2001; Stevenson and Weimer, 2007a). *Prevotella ruminicola* plays a broad and important role in both carbohydrate fermentation and protein degradation in the rumen. The dominance of *P. ruminicola* in rumen samples may relate to its low sensitivity to rumen pH allowing it to maintain its density during grain introduction. The populations of *Prevotella ruminicola* were often linked closely with other rumen bacterial populations. The bacteria species with which the relationship was strongest was with either *S. ruminantium* or the *S. bovis* populations. The *P. ruminicola* populations also generally increased slightly during the initial period of grain introduction and then remained at a consistent level through introduction while some of the other bacterial populations were more variable during grain introduction. This finding is supportive of the role that *Prevotella ruminicola* plays in primary protein degradation in the rumen during introduction to higher true protein diets.

The hypothesis that lactic acid utilising rumen bacteria (*Selenomonas ruminantium*) populations will increase with an increase in the grain component of the diet was also supported. The *S. ruminantium* populations did increase until approximately day 7 then the populations remained reasonably constant. The relationship with other rumen bacterial populations showed

that the populations of *S. ruminantium* were closely related to the *Prevotella ruminicola* populations but also at times with populations of *S. bovis* but had little relationship to the populations of the cellulytic bacteria, *F. succinogenes*.

The hypothesis that populations of *Streptococcus bovis* should increase significantly and possibly pathologically during introduction to grain-based diets in cattle or due to poor introduction practices was not supported in this study. However, the hypothesis that *Streptococcus bovis* was linked with a decrease in ruminal pH, and an increase in the populations of *Lactobacillus spp*. was supported and demonstrated that *Lactobacillus spp.* populations was in fact independent of the other bacterial populations that were quantified.

The proposal that metabolic changes in the rumen could be related to changes in the molecular ecology during dietary transitions in cattle and sheep was supported in some cases. For instance, the increases in D- and L-lactate concentrations were associated with increased populations of *Streptococcus bovis* in sheep fed ad libitum lupin diets and soybean diets. Moreover, in cattle managed under commercial feedlot conditions, the total VFA concentrations were consistent with high production potential and adapted populations of *P. ruminicola* and *S. ruminantium*. Therefore, in these instances the bacterial populations were follow particular trends consistent with the metabolic indicators. The restriction in the number of bacterial species monitored due to the availability of suitable molecular techniques at the time of the study and the different population dynamics and rates of metabolic pathways in the rumen may have constrained observations of closer and more consistent relationships between the ecology and metabolism of the rumen.

The hypotheses that were posed as part of this Masters study could be further explored with the progression of metagenomics. Since the completion of laboratory work in 2006 more recent studies or rumen microbial populations by (de la Fuente *et al.*, 2014; Petri *et al.*, 2012; Petri *et al.*, 2013b) have profiled a higher proportion of the rumen genome rather than

specifically targeted key bacterial species as undertaken in this study. For instance, profiling of rumen microbial ecology in samples collected under commercial conditions as done by Kittelmann *et al.* (2013) and also in the human stomach as reported by Morgan and Huttenhower (2014b) are readily transferable to ruminant. The techniques of shotgun metagenomics and metatranscriptome sequencing eliminate the possibility of missing whole kingdoms or bacterial clades as a result of PCR bias. Further progression of molecular technologies PhyloChip and GeoChip techniques as outlined by Nikolaki and Tsiamis (2013) will allow investigation of the composition and function of microbial communities and single cell genomics to map genomes from uncultured phyla in environmental samples such as the rumen. The possibilities of molecular techniques have expanded dramatically due to the reduced costs of basic aspects such as sequencing of bacteria that would also have assisted with the development of more appropriate primers. Notwithstanding the limitations of the use of 16SRNA DNA sequences in RT-qPCR as measures of populations of bacterial species in a rumen microbial ecology, RT-qPCR did permit some of the first observations of the dynamics of rumen bacterial ecology in cattle under commercial feedlot conditions and in sheep fed what were previously reported to be 'safe feeds' under experimental conditions. In addition, this thesis was the first molecular study to report on the composition of bacterial populations in rumen samples collected from commercial dairy herds where the feed base, commercial production and rumen metabolism were also being monitored.

Overall this Masters has outlined that acidosis is much more complex in its bacterial changes than previous described. On a practical level, this thesis has demonstrated that management practices and livestock husbandry are crucial in commercial feedlots where livestock can be successfully introduced onto grain based diets without necessarily using feed additives. Moreover, careful management will be required during introduction of supposedly safe feed sources such as lupins and soybeans. Following on from this work, previously utilised

indicators of rumen function such as metabolic indicators and rumen bacteria assumed to result in acidosis were not always straight forward as indicators of rumen dysfunction. For instance, increased populations of *S. bovis* and *Lactobacillus* during a grain challenge were not always apparent even with decreases in rumen pH. This work highlights and supports that the notion that management and husbandry is the key to successful dietary transaction. Moreover, the concentrations of total volatile fatty acids and rumen ammonia concentrations at appropriate levels (i.e. < 3.0mM) were both good metabolic indicators of potential commercial production in cattle and sheep.

8 Appendix

8.1 L (+) or D (-) lactate assay adapted from (*Brandt et al, 1980)*

Standards	S (0)	S (10)	S (25)	S (50)	S (75)	S (100)
Buffer	500	500	500	500	500	500
NAD	50	50	50	50	50	50
L or D lactate std. (1mM)	0	10	25	50	75	100
Water 445	435	420	395	370	345	
L (+) or D (-) lactate Dehydrogenase	5	5	5	5	5	5

Samples	Blank	Samples
Buffer	500	500
NAD	50	50
L or D-lactate std. (1mM)	0	0
Water	445	145
Sample	----	300
L (+) of D (-) lactate dehydrogenase	5	5

NAD solution (made up freshly immediately before use)

20mg/mL water

Hydrazine Glycine buffer (500mL) pH 9.50

13.0mL Hydrazine

Glycine 18.78grams

Make up with water and adjust pH to 9.5

8.2 Ammonia Assay

Use Boehringer Mannheim Ammonia kit, catalogue number 125 857 (19 x 2.0mL).

1. Reagent solution – Dissolve contents of one bottle by adding 2.5mL of buffer from bottle 1a.

2. Enzyme solution- Add 0.5mL buffer from bottle 1a to one bottle 2. Dissolve contents by allowing to stand at room temperature and swirling gently from time to time over a period of 10 minutes.

Always close the bottle after use. Stable for 6 weeks at +2 to 8 ^{o}C or five days at +15 to 25^{o}C.

Sample preparation

Rumen fluid – dilute sample 1: 100 (Take 0.05mL of rumen fluid and add 4.95mL of boiled water).

Wavelength – 340nm

Pipette into cuvettes		
	Blank	Standard/Sample
Rumen fluid	0.00	0.17 mL
Reagents from bottle 1	0.83 mL	0.83 mL
Mix well and leave to stand for 1 minute. Read initial absorbance (340nm) and record as OD_1		
Enzyme solution	0.006mL	0.006mL
Mix well and leave to stand for 8 minutes. Read second absorbance and record as OD_2		

Calculations:

(OD1 – OD2)/0.00622 * dilution factor required = nmoles/mL or µmoles

8.3 Analysing Fatty Acids by Packed Column Gas Chromatography

Bulletin 856B

Analyzing Fatty Acids by Packed Column Gas Chromatography

Described here are packed column gas chromatographic analyses of C1-C7 volatile fatty acids, lactic acid, and longer chain nonvolatile acids, including cis/trans isomers, in various sample matrices. Packings, packed columns, and standards for these analyses are listed after the analyses.

Key Words:
- fatty acids • volatile fatty acids
- *trans* fatty acids • lactic acid

C2-C5 Volatile Fatty Acids

C2-C5 volatile fatty acids (VFAs) in dilute aqueous solution can be separated and quantified as free acids by using a column containing 10% SP™-1200/1% H_3PO_4 on Chromosorb® WAW, a packing developed specifically for this purpose (1,2). The SP-1200 phase separates the free (unmethylated) acids rapidly, with minimal peak tailing, and with good separation of propionic and isobutyric acids (Figure A). Figure B shows a separation of a rumen fluid sample, and Figure C shows the C2-C5 acids in blood plasma. Acid concentrations in the rumen fluid and blood plasma samples were calculated by comparing peak heights to those in the standards. Baumgardt's informative paper on rumen fluid analysis deals with the practical aspects of quantitative GC analyses of VFAs in aqueous solution (3).

Note that 0.6µL samples were used in Figures B and C. Sample volume should be kept small to ensure optimum column performance. If sample volume is excessive, acetic and propionic acids will overload the column and elute with poor peak shape. For rumen fluid, which contains high concentrations of VFAs, we recommend samples in the range of 0.2-0.3µL. Because blood has very low concentrations of VFAs, sample volumes can be larger.

Figure A. C2-C5 Volatile Fatty Acids as Free Acids

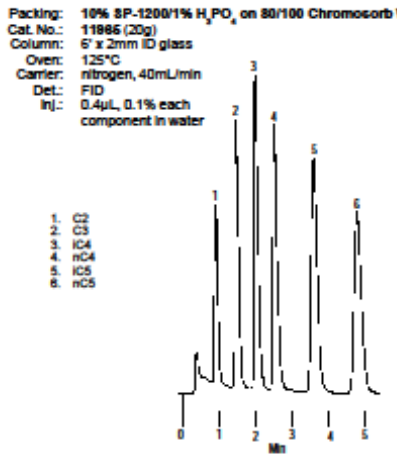
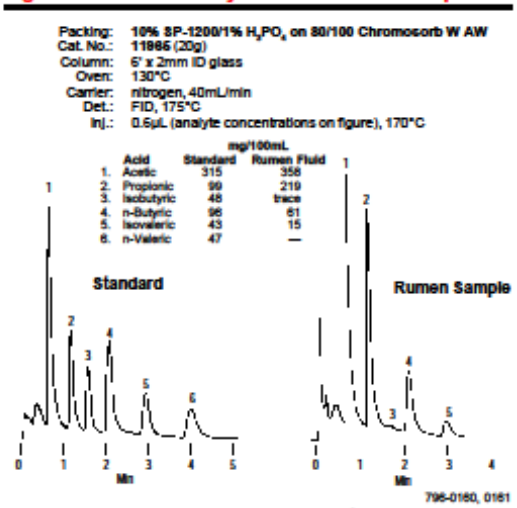


Figure B. Volatile Fatty Acids in Rumen Samples



Chromatograms provided by L.E. Chase, Dept. Animal Science, The Pennsylvania State University, University Park, PA USA.

8.4 **Rumen fluid medium (M10) – Instructions**

Salt Solution A

- 0.3% potassium di-hydrogen phosphate
- 0.6% sodium chloride
- 0.3% ammonium sulfate
- 0.03% calcium chloride
- 0.03% magnesium sulphate

Salt Solution B

- 0.3% di-potassium hydrogenorthophosphate

Rumen fluid medium (based on 100mL)

- 16.50mL of salt solution A
- 16.50mL of salt solution B
- 33.00mL of clarified rumen fluid (centrifuged at 25931 x g for 10 minutes)
- 0.1g peptone
- 0.1g yeast extract
- 0.5g $NaHCO_3$
- 0.2g glucose
- 0.1mL resaurin (0.1%)
- 50mg cycteine-HCl
- 34mL DDI water

Instructions

1. Salt solution A and salt solution B can be made up separately and stored in the fridge.
2. The rumen fluid medium is made up just prior to the medium being made. The rumen fluid is spun down at 4-8^{o}C in a centrifuge at 25000g for 10 minutes, with the supernatant

being removed for use in the medium. If the rumen fluid is still slightly cloudy the procedure is repeated.

3. The medium is then made up in a conical flask based on the instructions above, generally in quantities of 500mL.
4. Boil solution in a conical flask for 30 minutes over bunsen burner with carbon dioxide and condenser in place.
5. Ensure that ice water is flowing through the condenser condenser and remove water from tub as required, ensuring that ice is being replaced.
6. Add resazurin when starts to boil (try and get directing into solution)
7. Cool solution in ice bucket (with carbon dioxide still pumping through and condenser still attached.
8. Add cysteine only when completely cool and swirl until dissolved
9. Keep CO_2 in solution take of condenser and cover with aluminium foil
10. Put calibrated pump into solution, 10mL of the rumen medium was dispensed into 20mL pyrex tubes with CO_2 being pumped and Hungate stoppers (Bellco catalogue number 2047-11600) were used to seal the containers with screw tops placed on the containers
11. Then autoclave the tubes ready for use.

8.5 **Cryoprotectant Instructions**

Before adding water to your rumen medium mixture (appendix one), pour in 100% glycerol so that the final concentration of glycerol 40% v/v. Then top up to desire volume with water. You follow the same process as making rumen fluid medium but you only aliquot 2.5 mL of the solution into the cryoprotectant jar. To use them after autoclaving just add equal volume of culture to the jar so that the final concentration of glycerol is 20 % v/v. e.g. For 100mL of rumen fluid medium broth, you add 40mL of 100% glycerol to the mixture then top it up with water to 100mL. To use them you add 2.5 mL of culture to 2.5 mL of cryoprotectant.

8.6 **Formal Saline solution for Coulter counter (0.9% saline solution containing 0.5% formaldehyde)**

Dissolve in five litres of deionised water:

45grams of sodium chloride

67.57mL of formalin

Filtered through a vacuum pump at 40 pounds' pressure six times with a series of filter papers the upper section had:

8µ filter paper

1.2µ filter paper

0.8µ filter paper

The lower section had:

0.65µ filter paper

0.2µ filter paper

0.2u filter paper

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