Studies of the responses of cattle and sheep to rapidly fermentable carbohydrate challenges

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BSc (Hons)

Submitted in fulfilment of the requirements for the Degree of

Doctor of Philosophy (PhD)

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19th February 2018

Abstract

Subacute ruminal acidosis (SARA) occurs in ruminants following disruption of normal ruminal fermentation and is commonly observed in cattle on diets that are high in rapidly fermentable carbohydrate. SARA has been shown to lower production efficiency of cattle and reported global economic implications of SARA are consistently high. Individual animal responses to diets that are expected to induce acidosis vary but specific mechanisms responsible for this variation are unknown. This project examined the range of responses in variables commonly associated with SARA among animals and among herds - reticuloruminal pH, histamine and LPS, biochemistry and haematology and expression of inflammatory related genes - with the aim of observing how individuals vary in response to a diet expected to induce SARA.

The first trial detailed in this thesis involved the creation of a histological scoring system for the ruminal mucosa of adult cattle, and the subsequent histological examination of rumen wall tissue from animals (n = 195) from diverse management styles and nutritional backgrounds (commercial grass-fed beef, commercial silage-fed culled dairy, commercial mixed fed beef cattle (silage and concentrate), commercial cereal-fed (barley beef) cattle and research cereal-fed beef (high challenge)). Six beef farms were selected to provide broadly similar high and low-risk profiles and were classified by risk (RISK) of developing SARA according to the amount of concentrate in the diet (BARLEY), the proportion of particles <1.2 mm (FINES) and the percentage of straw and silage in the diet. Risk and specific dietary and management practices were related to histological scores and differences in the severity or nature of selected response variables. Effect of farm of origin, RISK, FINES and BARLEY were investigated. Farm of origin affected 32 of 33 dependent variables, many more than the *a priori* risk category, BARLEY or FINES. Animals on high-risk farms showed increased concentrations of reticuloruminal LPS and lactate (p <0.01), thicker stratum corneum (SC) and stratum granulosum (SG) (p <0.05), higher relative expression of TLR4 (p <0.05), reduced reticuloruminal short chain fatty acid (SCFA) concentrations (p <0.05) and reduced caecal LPS concentrations (p <0.05). Initial histological investigations were consistent with previous light microscopic descriptions of the tissue architecture in the literature but the consistent finding of increased SC and SG with increased BARLEY conflicted with current literature. The scoring system was shown to be able to differentiate

between animals according to their specific diet (p <0.001) or based on less specific groupings of FORAGE or CONCENTRATE (p <0.05).

The second study investigated and quantified variation among individual animals in their susceptibility to the effects of high levels of starch supplementation. The efficacy of novel direct fed microbials (DFM) for the control of performance and health effects related to the diet was also assessed. Forty lactating Holstein-Friesian cattle were allocated to 5 groups and fed a basal ration for 3 weeks, a basal ration plus a group-specific DFM for 3 weeks and then 2 weeks on a diet intended to induce ruminal acidosis plus their allocated DFM. The challenge diet resulted in an increase in variables associated with an inflammatory response; including increased plasma concentrations of SAA (p <0.05), increased expression of the *TLR4* and *IFNγ* genes in peripheral blood leukocytes (p <0.01) and increased counts of monocytes in blood (p <0.01). This inflammatory response occurred at the same time as an increase in milk yield and an indication of improved energy balance with decreased βHB and triglycerides (p <0.01). Reticuloruminal SCFA varied significantly with time-point (p < 0.05). Total SCFA decreased when the DFM were introduced (p <0.05) and again when the challenge diet was offered (p <0.01). Introduction of the DFM treatments resulted in a significant increase in reticuloruminal pH values (p <0.001). The challenge ration did not affect mean or median reticuloruminal pH values but did increase the number of observations below pH 6.0 and increased the range of daily mean pH observations. The study clearly showed that several variables associated with an inflammatory response increased rapidly after challenge with a diet high in rapidly fermentable carbohydrates and that this inflammatory response was evident although there was an increase in milk yield, an improvement in the metabolic state of most animals and a complete absence of clinical signs of acidosis.

The final study examined to what extent sheep should be used as a model for dietary manipulation studies in cattle. Six cattle and 4 sheep were maintained on a basal diet for 4 weeks, followed by 1 week on a diet intended to induce acidosis. Changes in reticuloruminal pH, ruminal histology, SCFA proportions, reticuloruminal LPS and histamine concentration and haematology and biochemistry were compared between species. The trial provided baseline data on the comparative rumen microbiome of both species using 16S rRNA sequencing. Both species showed similar response patterns to the challenge diet, despite variations in the severity of responses between species. Both species showed significant reductions in

reticuloruminal pH (p <0.0001) following introduction of the challenge diets. Gene expression levels were not significantly altered following the introduction of the challenge diet for either species. The more substantial change in reticuloruminal pH responses and the greater feed refusal in the sheep, relative to cattle, indicated that the selected treatment rations were disproportionately challenging to the sheep. Other than this, the observation that varied most between species was the 16S rRNA results. Sheep showed a greater loss of diversity following the introduction of the challenge diet. However, a common microbiome was observed and those sheep which were not severely acidotic mirrored the cattle in their 16S results. With care applied to the selection of challenge rations, the work described here suggests that the use of sheep as a model for dietary manipulation studies in cattle is justifiable.

The key outcomes from this thesis were that an inflammatory type response in various parameters may become evident before clinical symptoms in animals suffering ruminal acidosis; that farm of origin has a more substantial effect on all variables than the *a priori* risk classification or other measured dietary inputs; that SCFA concentrations decreased in animals fed high carbohydrate diets; and that a new scoring system for ruminal epithelium is capable of differentiating among diets fed to animals. The reduced SCFA concentrations noted in animals fed on high carbohydrate diets is likely a result of rapid adaptation, including increased SCFA flux across the reticuloruminal epithelium. The inflammatory response noted in cattle prior to any clinical symptoms suggests potential for the identification of inflammatory markers prior to acidosis and for selective breeding as an acidosis resistant phenotype.

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Acknowledgements

There are many people to whom I owe thanks. Firstly, I would like to express my thanks to my primary supervisor Nicholas Jonsson for the patience, motivation, and immense knowledge he provided to me throughout my 4 years. Nick helped push me in both my research and writing skills and put up with endless questions shouted across the office. I may not have ended up in this field were it not for Nick's enthusiasm and encouragement with my initial honours project and I will always be thankful for this.

I would also like to thank my secondary supervisor Peter Hastie. Peter's door was always open to allow a chat, whether about PhD work or otherwise, and his insights helped to shape my thesis and kept me going – particularly during this last hard year – something I won't forget. I wouldn't have gotten to where I am without Peter's help.

Thanks must also go to Caline Koh-Tan for all her guidance and help in the lab. Caline pushed me to understand things that I did not think I ever would. I have learned a huge amount from her and I am grateful for her help in the early stages of my PhD.

The team from Aberdeen, Christine McCartney and John Wallace and everyone else who helped with collection of samples and trials must be thanked too. Abattoir trips, gut rooms and parlours are not everyone's idea of fun, but we kept each other going through the early starts and long days and I appreciate it.

Huge thanks go to my colleagues and friends in the old cell sciences lab, especially the JYT (Aynsley, Callum, Zoë and Amy) and my honorary supervisors Neil, Fiona and Maureen. You all kept me going when I needed it most and made my final few years of my PhD the best. You let me vent, helped me sample, provided a distraction and generally cheered me up.

Finally, I must thank my family for putting up with me when I was crabbit, for forgiving my stressing and late nights and for reading things, though you did not know what I meant half the time. Your support has seen me through this project to its completion – thank you!

“I declare that except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution”

Name: Holly Jane Ferguson

Signature:

Abbreviations

ACTB	Beta actin or βAct
CCL11	Chemokine (C-C motif) ligand 11
DMI	Dry matter intake
DIM	Days in milk
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GWAS	Genome wide association study
IFNγ	Interferon gamma
IL2	Interleukin 2
IL10	Interleukin 10
lL1β	Interleukin 1, beta
LPS	Lipopolysaccharide
NHE3	Sodium hydrogen antiporter 3
PBMC	Peripheral blood mononuclear cell
RFC	Rapidly fermentable carbohydrates
RPLP0	Ribosomal protein lateral stalk subunit P0
SAA	Serum albumin A
SARA	Sub-acute rumen acidosis
SB	Stratum basale
SC	Stratum corneum
SCFA	Short chain fatty acids
SG	Stratum granulosum
SS	Stratum spinosum
TLR4	Toll-like receptor 4
SCFA	Short chain fatty acids, also known as volatile fatty acids

1 Introduction and literature review

1.1 Introduction

The ruminant stomach originates from a single diverticulum at the oesophagus and splits into 3 large and distinct chambers – the rumen, reticulum and omasum. These 3 chambers - known collectively as the forestomach - along with the abomasum, create a structure with 4 chambers in total (Frandson et al., 2003). The rumen contains a range of archaea, bacteria and eukaryota responsible for fermentation of feed and the subsequent creation of short chain fatty acids (SCFA), used by ruminants as their main source of energy. The rumen is present in all species of the suborder Ruminantia, which includes cattle, sheep, goats and deer among others. The key function of the rumen is to allow ruminants to digest complex structural carbohydrates like cellulose via fermentation, thus allowing them to inhabit a niche not available to monogastric animals (Fernando et al., 2010). Despite this key function, the rumen itself has many more important roles than simply just a fermentation chamber.

In modern agriculture, increased yield in areas such as meat, milk and wool production in ruminants is favoured by high levels of dietary supplementation with rapidly fermentable, starch rich carbohydrates. Beef cattle fed a diet high in rapidly fermentable carbohydrates prior to slaughter have shown an increase in carcass weight, conformation, degree of finishing, fat depth and rib eye area (Realini et al., 2004). In dairy cattle, milk parameters are consistently reported to be affected by rapidly fermentable carbohydrates in the diet (Enemark et al., 2002, Danscher et al., 2015, Xu et al., 2016). In both beef and dairy cattle, there is a need to balance an increase in yield with increasing levels of rapidly fermentable carbohydrates and the negative effects noted when these optimum levels of feeding are exceeded.

Above an optimum level, dietary supplementation with rapidly fermentable carbohydrates eventually leads to an increase in protons in the rumen, due to short chain fatty acid (SCFA) production and accumulation (also known as volatile fatty acids or VFA), which eventually leads to decreased reticuloruminal pH. Decreased reticuloruminal pH causes challenges arising from the resulting loss of the diversity of the ruminal microbiome, which in turn affects fermentation, and the overall function of the rumen. Although to some extent increased acid production is a

desired effect of a diet high in rapidly fermentable carbohydrates - SCFA being responsible for a large proportion of the energy requirements in cattle - the accumulation of protons and associated reticuloruminal pH drop and negative consequences is not. Decreased reticuloruminal pH, known as ruminal acidosis or often referred to in literature as sub-acute ruminal acidosis (SARA), can lead to a number of negative consequences for the animal, including poor production, poor body condition, mild anorexia, diarrhoea, depression, decreased ruminal motility, rumenitis, general inflammation, laminitis, epistaxis and in severe cases can even lead to death (Plaizier et al., 2008).

There is substantial variation among individual animals fed the same ration in the extent to which they can maintain the reticuloruminal pH within its “normal” range and prevent a drop in pH due to proton accumulation, or are resistant to ill effects of ruminal acidosis (Garrett, 1996; Morgante *et al.*, 2007; Kleen *et. al*., 2009). Identifying the range of responses among animals, or a specific mechanism present in those animals able to maintain their reticuloruminal pH or cope with negative consequences associated with decreased pH, would allow for selection of animals genetically predisposed to cope better with or not develop ruminal acidosis. This would mean better welfare, better production and better returns for farmers globally.

Adverse effects of ruminal acidosis are commonly reported, particularly in the dairy industry, primarily due to abrupt change in diets in cows post-parturition to a diet higher in rapidly fermentable carbohydrates. Cows in the early post-partum period are particularly vulnerable to ruminal acidosis, due to this immediate diet change post-calving and correspondingly, the number of cases reported is often higher in animals at calving time (Rabelo et al., 2003, Penner et al., 2007). Although a common condition, the prevalence of ruminal acidosis in European and North American dairy production systems has been shown to vary widely. Some herds might show no indication of acidosis, yet similarly managed herds may show a prevalence of up to 40% (Morgante et al., 2007, Kleen et al., 2009, Kleen et al., 2013). Despite variation in reported prevalence, estimates of the financial impact of acidosis are consistently high globally; through production losses, increased culling rate and death losses. It has been estimated that the financial costs associated with ruminal acidosis are between USD $500 million to $1 billion annually (Enemark, 2008).

1.2 The bovine forestomach and abomasum

Microbial fermentation of feedstuffs by the microbiome of the rumen allows ruminants to inhabit a distinct niche that differs from that of the monogastric animal, by providing them the ability to breakdown complex carbohydrates like cellulose and hemicellulose. The bovine forestomach is composed of the 3 distinct and separate chambers (rumen, reticulum and omasum) before the final acidic digestion chamber, the abomasum. The rumen, reticulum and omasum are lined with non-glandular mucous membranes, while the abomasum is lined with a glandular secretory mucosa (Nickel et al., 2013). Food is fermented and broken down in the reticulum, regurgitated as a bolus (or cud) and chewed again to break it down further and retrieve as much nutritional value as possible. The cud is then swallowed again and passed through to the omasum where liquid is reabsorbed before it passes into the abomasum for the final acidic digestion, similar to that of a monogastric animal. Finally, food moves into the small intestine where digestion and further absorption of nutrients occurs (Dehority, 2002).

The reticulum, the first chamber of the bovine stomach, is located in the abdomen close to the heart, adjacent to the diaphragm, lungs, abomasum, rumen and liver (Budras et al., 2003). The reticulum is connected to the rumen and separated from the rumen chamber by a muscular fold (Dehority, 2002). Together with the rumen, it is commonly referred to as one entity - the reticulorumen. The mucosa of the reticulum forms a network of crests, hexagonal in shape, the height and depth of which varies considerably across species. Crests have been shown to be higher in sheep and cattle in comparison to okapi, deer or giraffe (Shorrocks, 2016), though the general size of the reticulum itself is relatively constant across all ruminant species. The reticulum has long been recognised in domestic ruminants as the site of particle sorting (Okine et al., 1998). In bovines, the reticulum plays this role in particle separation though biphasic contractions (Braun and Jacquat, 2011). The first contraction of the reticulum sends large particles from the reticulum back into the rumen and smaller particles on to the omasum. The second contraction contracts the reticulum fully to refill it with contents from the rumen and thus the cycle of contractions and sorting begins again (Braun and Götz, 1994). Heavy or dense feed often settles in the reticulum during these contractions, meaning the reticulum often catches metals or other heavy hardware accidentally or intentionally ingested (boluses). Metal retention can result in traumatic reticulitis or pericarditis,

often known as “hardware disease”, where metal perforates the reticulum during contractions, resulting in inflammation or infection in the surrounding areas and pericardium, which can be fatal (Roth and King, 1991, Samad et al., 1994).

The rumen, the second chamber of the ruminant stomach, is the chamber most commonly referred to in literature when considering bovine acidosis. The rumen is often referred to as a large fermentation chamber, but plays its own very important role in ruminant digestion via absorption of SCFA produced during fermentation and the corresponding sodium bicarbonate secretion. The reticulorumen is discussed in detail in section 1.3.

The omasum is the third chamber of the ruminant stomach, it is almost spherical in shape and the lower end of the omasum is connected to the fundus of the abomasum, the fourth chamber of the ruminant stomach (Budras et al., 2003). The mucosa of the omasum is mostly smooth with small, lenticular papillae. The surface of the omasum resembles “book–like” folds, often referred to as leaves (Prins et al., 1972). The omasum is responsible for absorption of water and SCFA from the rumen (Holtenius and Bjornhag, 1989). Similarly to the reticulum, the omasum has biphasic contractions. On the first contraction, the omasum expels fluid from ingesta that has entered from the reticulum. The second contraction expels solid ingesta left behind, allowing it to move into the abomasum and allowing the omasum to fill again, continuing the cycle of contractions and movement of ingesta (Stevens et al., 1960, Prins et al., 1972).

The fourth and final chamber of the ruminant stomach, the abomasum, is considered separate from the forestomach (rumen, reticulum, and omasum) and lies upon the abdominal floor (Budras et al., 2003). The position of the abomasum varies slightly, dependent on the fullness of other chambers, contraction rate of the rumen and reticulum (due to attachments), age and pregnancy (Constable et al., 2017).The abomasum is split into cranial and caudal sections (cranial further split into the pylorus and body), has a loose folded appearance inside and is coated in mucous (Wolffram, 1996). The abomasum is secretory (secreting rennet), similar to the anatomy and function of a monogastric stomach. The main function of the abomasum is the chemical digestion of food, not the mechanical or fermentation degradation observed in other forestomachs. The abomasum uses chemical digestion, via hydrochloric acid and pepsinogen, of microbial and dietary proteins to

prepare these protein sources for further digestion and absorption in the small intestine (Ash, 1961). Cattle fed diets high in rapidly fermentable carbohydrates are susceptible to a condition of the abomasum due to a build-up of gas from rapid fermentation of the carbohydrates (Sarashina et al., 1990). From this gas build up, the abomasum can become left or right (LDA, RDA) displaced in the abdominal cavity (Sarashina et al., 1990). Displacement can have numerous ill effects for the animal, including loss of appetite, decreased ruminal contraction rate and decreased cud rate and can cause economic losses for farmers due to premature culling (Shaver, 1997). Surgery may be required for serious displacements but some minor LDA can be corrected via manipulation of the cow. This condition is most commonly observed in dairy cattle, as a result of their diet high in rapidly fermentable carbohydrates and sudden diet changes leading up to and postpartum (Shaver, 1997).

1.3 The reticulorumen

1.3.1 Development

At birth, ruminants essentially have a single stomach, the abomasum, with the same function as that of a monogastric animal. In neonates, the rumen is small, underdeveloped and not functional, and forms only a small portion of the total stomach in comparison to later life (Ørskov, 1998). From birth, calves gain nutrition solely from the dam's milk or calf milk replacer (CMR). Under normal circumstances in calves, when drinking, milk bypasses the rumen, reticulum and omasum via the closure of the oesophageal groove, a muscular structure at the lower end of the oesophagus, and goes to the abomasum where it is digested (Ørskov, 1998). Thus, milk bypasses the ruminal fermentation chamber and it is digested fully.

Rumen development in ruminants is dependent on access to a fibrous diet, inoculation by rumen bacteria and essentially, the presence of SCFA to stimulate the rumen wall and papillae development (Van Soest, 1994). If ruminants are maintained on a milk only diet without access to fibrous food, the development of the rumen is greatly limited (Van Soest, 1994). Even if milk were to enter the rumen through improper closure of the oesophageal groove, milk digestion in the rumen leads to lactic acid fermentation with little SCFA production, leading to poor stimulation of the rumen wall (Van Soest, 1994). As calves begin to wean by

consuming small parts of fibrous vegetation or pelleted food, this enters the rumen and becomes a substrate for fermentation by the ruminal microbiome. The microbiome develops after birth from contact with bacteria from the dam and surroundings. Fermentation of these pellets or vegetation, rich in complex carbohydrates, leads to production of SCFA and consequential SCFA induced stimulation of rumen development (Van Soest, 1994). In a high-intensity commercial setting, where calves may be removed and weaned at an earlier age, inoculation of rumen bacteria and development of the rumen, can be ensured via contact with farm staff and the environment alone (Hobson and Stewart, 1997). Although strictly isolated animals will develop a rumen microbiome, species present in the microbiome may differ qualitatively or quantitatively from other animals present in the same herd (Van Soest, 1994).

1.3.2 **Histology**

In addition to the rumen increasing greatly in size with diet and age, the ultrastructure of the rumen also develops. Several studies have allowed for a greater idea of the structure and organisation of the rumen epithelium (Asari et al., 1985, Graham and Simmons, 2004, Scala et al., 2011, Steele et al., 2011, Steele et al., 2012) in juvenile and adult ruminants.

1.3.2.1 **Light microscopy studies of the rumen**

The surface of the rumen is covered with small, leaf-like projections known as papillae, the size and shape of which vary across the rumen, from animal to animal, among species and with diet (Dobson, 1955). Papillae function as absorptive structures for SCFA and papillary proliferation and size has been shown to respond to dietary manipulation that alters SCFA production levels. If an animal were to remain on a milk only diet, rumen papillae would remain small, as a large surface area for SCFA absorption would not be necessary. As vegetation and fibre is included in the diet, papillae grow rapidly to provide a larger surface area for fermentation and absorption of SCFA (Eurell and Frappier, 2013). As the diet becomes more complex and protein and carbohydrate levels increase, papillary size and density increases as a result of the regulation of insulin-like growth factor 1 (IGF-1) production by butyrate and propionate, as shown by Shen *et al.* in the rumens of juvenile goats (Shen et al., 2004). This butyrate and propionate driven

papillary change is key in the rumen's ability to alter papillary structure dependent on diet throughout life, to increase surface area for SCFA absorption and increase the length of papillae, even after the initial juvenile development has taken place (Sakata and Tamate, 1978, Zitnan et al., 2005, Malhi et al., 2013). Variations in the effectiveness of this mechanism may be one of the many factors responsible for variation among cattle in their response to a diet high in rapidly fermentable carbohydrates. Papillae may be roughly 2 mm broad and 6 mm long at longest and leaf-like in shape, but change across the rumen and with diet, from lenticular in shape to leaf-like and conical (Dobson, 1955).

Light microscopy studies of the rumen epithelium have characterised the tissue as keratinised, stratified, squamous epithelium (Graham and Simmons, 2004). Papillae consist of a core of densely packed collagen fibres surrounded by a stratified epithelium and have a rich blood and lymphatic supply in the core that penetrates into the papillary body (Dobson, 1955). The epithelium of the rumen has 3 main functions: absorption, protection and metabolism. Papillae themselves consist of 4 layers, the outer epithelial stratum corneum (SC) closest to the lumen of the rumen, the stratum granulosum (SG), stratum spinosum (SS) and the base layer, the stratum basale (SB) (Graham and Simmons, 2004). The SC forms a barrier against rough ingesta and deeper layers metabolise SCFA produced in fermentation and absorb other products from the rumen (Eurell and Frappier, 2013). Underlying the SB is connective tissue, rich in capillaries (Graham and Simmons, 2004). Individual layers can be seen clearly in figure 1-1 (A-C).

The basal layer of the rumen epithelium is adjacent to the capillaries in the papillary body and consists of columnar cells. The number of cells between these columnar basal cells and the lumen varies greatly between papillae and among animals (Dobson, 1955). Next to the basal layer is the SS where cells often appear to have large interstitial spaces due to the presence of cytoplasmic fibrils. Next to the SS is the SG, shown to have cytoplasmic fibrils and variable interstitial spaces (Dobson, 1955). Keratinisation of the SG is sometimes evident and cells in this layer often appear to have undergone flattening and vesiculation/vacuolation (Dobson, 1955). Next to the lumen of the rumen is the SC that, as with the SG, shows flattened cells and has a high affinity for most stains, which is very apparent in light microscopy (Dobson, 1955). At the edge of the SC, nearest the lumen, there are often swollen

misshapen cells with a high refractive index, as can be seen in figure 1-1C (Dobson, 1955). Cells at the edge of this layer are in the process of sloughing into the rumen cavity and therefore, cell layers may appear to be missing here upon examination under light microscope. Sloughing of the SC can be seen in figure 1-1B.

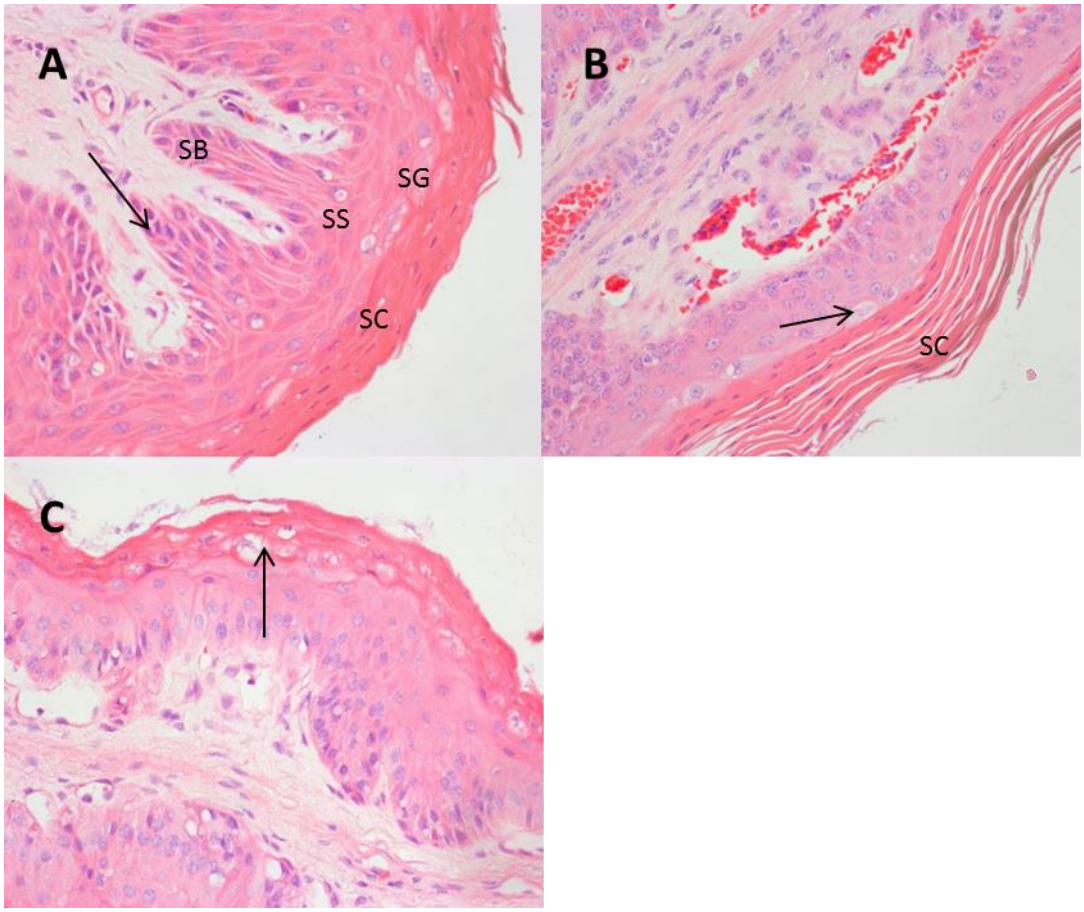

Figure 1-1: H&E section showing (A) Section through the tip of a papillae showing clearly the basal columnar cells round the connective tissue fibres (arrow) and the gradual flattening of the cells as you move towards the outer keratinised layers, particularly in the SC (x40). The intensely stained SC can be seen as well as the limits of the SG, SS, SB and vasculature and connective tissue. (B) Section of a papillae showing sloughing of the outer layers of the SC and clear vacuolation in the SG (arrow) (x40). (C) Section of papillae showing the large swollen cells of the SC which have a high refractive index (arrow) (x40). Images are taken from samples from chapter 3.

Distribution of papillae across the rumen is not uniform. In the majority of ruminants, the dorsal and ventral sacs of the rumen are the most densely populated with papillae (Christie, 2014). Distribution and appearance of papillae across the rumen in cattle was described clearly in a study by Schnorr and Vollmerhaus (1966). This previous study detailed the density, width and height of papillae across the rumen. Figure 1-2 shows images of the varying densities and variations in shape between papillae across the rumen. Figure 1-3 shows the left and right view of the rumen,

detailing the density and height of papillae across the surface. A higher density of papillae was found in the dorsal and ventral sacs of the rumen and a longer length of papillae was found in the dorsal and cranial sac. As a result of this high density of papillae, many histological studies choose the ventral sac as the site of sampling for ruminal epithelium (Steele et al., 2011c).

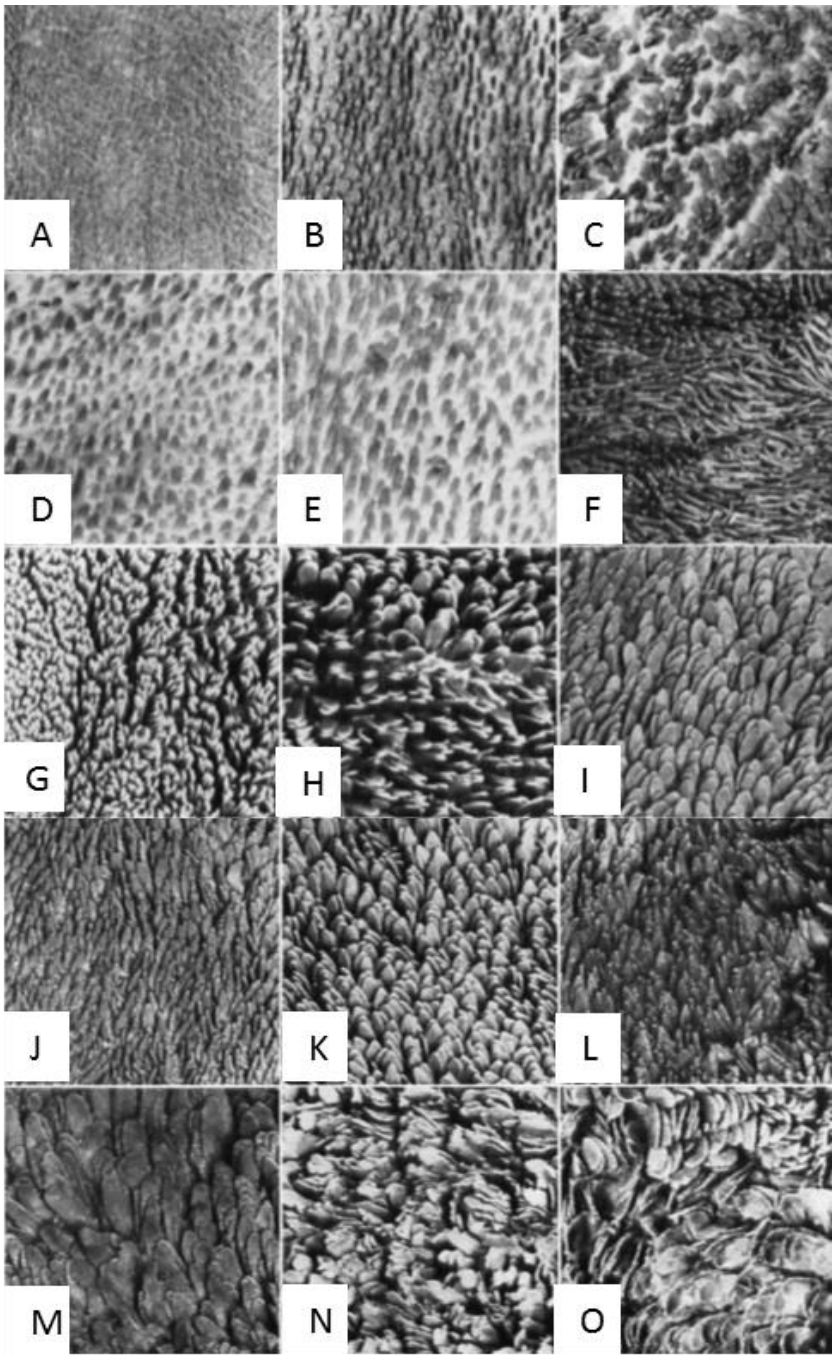

Figure 1-2: Images showing the range in papillae density, width and height across the rumen of cattle. A = papilla-free mucosa; B-I = papillated mucosa with B wart-shaped, C wedge-shaped, D,E, tongue-shaped, F thread-shaped, G,H,I band-shaped, K,L,M, lanceolate, N,O, leaf-shaped papillae. Original size, optical microscope. Figures from "Das Oberflach enrelief der Pansenschleimhaut bei Rind und Ziege" (Schnorr and Vollmerhaus, 1966).

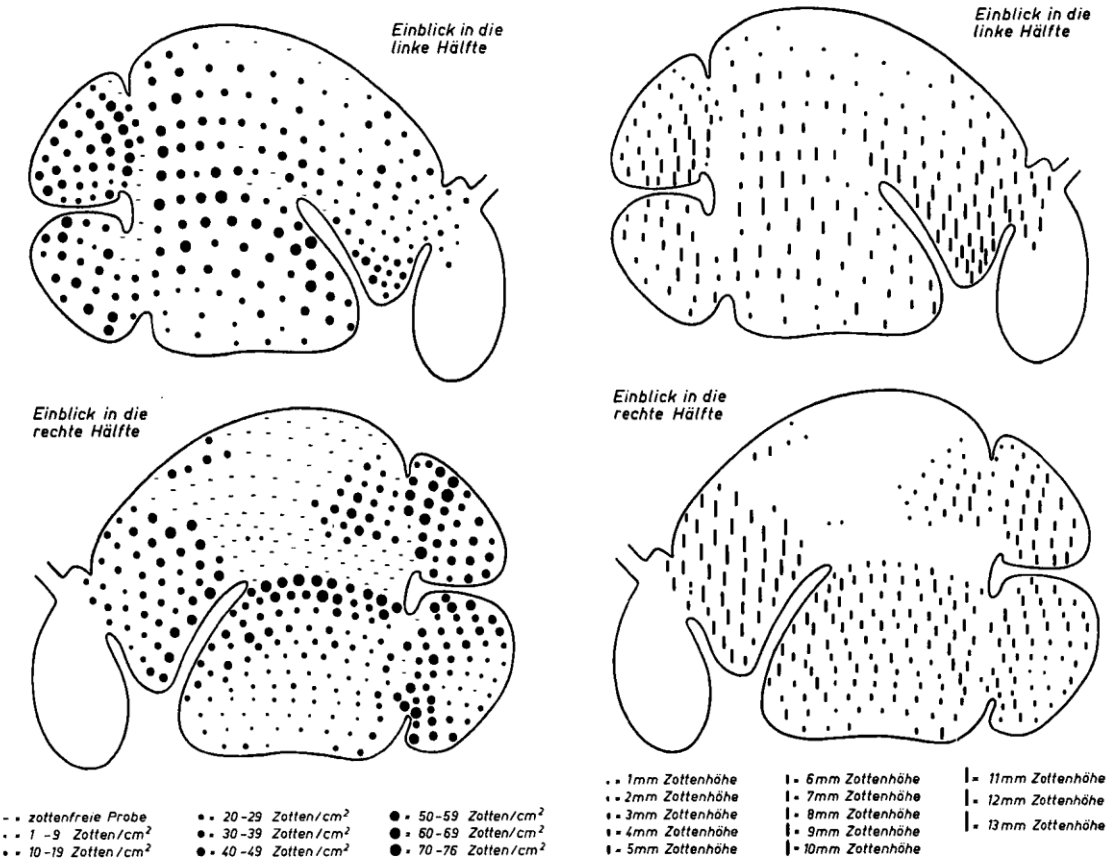

Figure 1-3: Left and right view of the rumen and reticulum, detailing the density and height of the papillae present. Increasing size in black dots reflects increasing density, showing higher density in the central sac. The longer lines reflect higher height of rumen papillae noted across the rumen. Figures from "Das Oberflach enrelief der Pansenschleimhaut bei Rind und Ziege" (Schnorr and Vollmerhaus, 1966).

1.3.2.2 **Electron microscopy studies of the rumen**

Scanning electron microscopy studies (SEM) of the rumen have provided more information about the epithelial structure and organisation of the rumen and confirmed the cell strata organisation noted in light microscopy studies. A study by Steele *et al.* (2011) using SEM to look at the effect of diet on ruminal epithelium found that on the papillae surface, there are deep ridges and indentations where bacteria and protozoa settle. This previous study found that high grain diets affected these ridges, with ridges becoming shallower and consequently microbial colonisation was reduced. The same study found that a high grain diet in comparison to high forage vastly altered the structure of the ruminal epithelium. The high grain diet led to large spaces between the strata with large amounts of bacteria evident in these gaps. Steele et al. (2011) also found an increased sloughing rate of the SC in the animals fed a high grain diet and the adhesion between cells of the SC and SG appeared to be compromised - evident in large gaps between cells. Cell junctions, as shown in the light microscopy, were observed with SEM to become

indistinct on a high grain diet and cells of the SB became pyramidal and displayed large intracellular spaces.

Another study using SEM to investigate the keratinisation of cultured rumen epithelial cells treated with butyrate and lactate found that in butyrate-treated cultures, there were distinct indications of keratinisation, the appearance of refractive granules, the formation of keratohyaline granules and thickening of the cell membrane (Galfi et al., 1983). This previous study also showed the formation and further detachment of keratinised cells, SC-like in structure, in cultures treated with butyrate, thus indicating the progression of keratinisation through sloughing, as shown in the light microscopy studies mentioned previously. This effect of butyrate is important, as it stresses again the key role of SCFA in both the initial development of the rumen and overall function and histological organisation of the rumen epithelium at any age and suggests SCFA may be responsible for epithelial changes such as parakeratosis (Galfi et al., 1983).

1.3.2.3 **Confocal and immunohistochemistry studies of the rumen**

In addition to light microscopy and SEM studies, confocal laser scanning microscopy studies of the rumen has been used in conjunction with FISH (fluorescence *in situ* hybridization) in numerous studies. These studies looked to identify and characterise rumen bacteria and protozoa and define their distributions and ecology within the rumen environment (Lavker et al., 1969, Schelcher et al., 1992, Lloyd et al., 1996, Valle et al., 2015).

Immunohistochemical studies of the rumen epithelium have also been used to look for the presence of inflammatory markers and the effect diet and dietary additives have on these markers; such as cytokines like *CD3*, toll-like receptors (*TLR4*) and markers of antigen presenting cells like MHCII (Chen et al., 2012, Qadis et al., 2014). Immunohistochemistry can also be used in histological examinations to highlight the presence of microabscesses (focal collections of neutrophils) which show strong positive staining for myeloperoxidase.

1.3.3 **Ruminal physiology**

1.3.3.1 **Microbiome**

Considerable work has been undertaken in the isolation, taxonomic identification and enumeration of the bovine rumen microbiome and a large proportion of common ruminal microbes were identified relatively early in the literature (Hungate, 1950, Bryant, 1959) but vast numbers are yet to be identified. The majority of bacteria in the rumen are free floating and detached from plant material but microbiota may be attached to or within feed particles, and attached to the epithelial cells of the reticuloruminal wall (Calabro et al., 2012).

A variety of methods can be used to identify species present in the ruminal microbiome and the structure and composition of a particular microbiome. Older studies typically used culture based methods (Bryant et al., 1958, Fonty et al., 1987, Minato et al., 1992), but more recently, analysis of 16S ribosomal RNA (rRNA) gene sequences and real time PCR is likely to be used (Stevenson and Weimer, 2007, Jami and Mizrahi, 2012, Han et al., 2015). 16S rRNA sequencing is a commonly used sequencing method that can identify and compare bacteria present within a given sample. 16S rRNA gene sequencing is a well-established method for studying the phylogeny and taxonomy of samples from complex microbiomes or environments that are difficult or impossible to study by other methods (Illumina, 2016).

The rumen microbiome develops at birth from nose to nose contact with the dam, contact with surroundings (faeces, other animals, general environment) and contact with farm staff and is vital for fermentation of complex carbohydrates and for altering pH (Ørskov, 1998). Rumen microbial ecology has been extensively researched and numerous studies have been published describing the predominant species of bacteria, protozoa and fungi (Hungate, 1966, Krause and Russell, 1996, Cheng and McAllister, 1997).

Globally, similar bacteria are found in all ruminant microbiomes, but different feeding strategies, diets, climate, farming practices and individual variation all play a role in the microbial community present in an individual animal (Henderson et al., 2015). Ruminant microbial communities are composed of varying groups, mostly

specialised in hydrolysis and fermentation of polysaccharides, so animals fed similar diets are expected to have similar populations present to ferment the particular sugars present in that diet (Firkins and Yu, 2004).

However, variation has been shown in the microbiome present between animals fed on the same diet. In a study to assess similarity between cattle in the microbiota present in ruminal fluid samples, a study by Jami and Mizrahi (2012) carried out 16s sequencing on the V2 and V3 part of the gene and identified and characterised bacteria present, to look for individual operational taxonomic units (OTU). They found that roughly 50% of OTU noted only occurred in up to 30% of the animals sampled, despite similar experimental conditions, diets and sampling procedures, thus showing individual differences between ruminal microbial populations (Jami and Mizrahi, 2012).

In contrast, work by Henderson *et al*. (2015) stated that despite changes in the rumen microbiome composition as a result of diet and individual host, a large core microbiome is commonly found across a wide geographical range. They found a common core microbiome in 742 samples from 32 ruminant species across 35 countries and differences could be attributed to diet, with host being less influential (Henderson et al., 2015). The most commonly found bacteria were *Prevotella*, *Clostridiales*, *Bacteroidales*, *Ruminococcaceae*, *Lachnospiraceae*, *Ruminococcus* and *Butyrivibrio* (most common to least). These “core bacterial species” comprised 67.1% of all sequence data collected. It is expected that variation outside of this core can be explained by climate, location and dietary inputs (Henderson et al., 2015).

1.3.3.2 **Biochemistry**

The ruminant stomach facilitates microbial fermentation and absorption of the end products (SCFA) arising from carbohydrate fermentation (Aschenbach et al., 2011). These SCFA, the largest proportion being acetic, propionic and butyric acid, are absorbed directly from the rumen and are the main source of energy for ruminants (Bhagwat et al., 2012). SCFA provide approximately 70% of the caloric requirements of ruminants, in comparison to approximately 10% for humans and approximately 20-30% for other omnivorous or herbivorous animals (Chen et al., 2014). In ruminants, approximately 90% of glucose is supplied by gluconeogenesis,

with 50-60% derived from propionate (Reynolds et al., 1988). In diets high in rapidly fermentable carbohydrates, there is a consequential and deliberate increase in the amount of propionate produced, which provides more energy for the ruminant, as propionate provides the largest amount of ATP with the least amount of NADH. In addition to the 3 main SCFA, ethanol, propanol, formate, acetate, hexanoic acid, heptanoic acid, lactic acid, valerate, isobutyric acid and isovaleric acid are also produced. Valeric acid, isobutyric and isovaleric acid account for less than 5% of the total SCFA and are thought to be more important for microbial protein synthesis using non-protein nitrogen (NPN) than directly for ruminant energy needs (Husveth, 2011).

Although all 3 forestomach chambers have a squamous epithelial lining, most SCFA produced are absorbed across the rumen wall (66% absorbed ruminally and 34% passed on in the liquid phase) (Peters et al., 1990). Absorption rates of SCFA are higher when reticuloruminal pH is reduced and absorption rates of individual SCFA are higher in SCFA with longer chain lengths (Thorlacius and Lodge, 1973), e.g. absorption rate of butyric acid is higher than that of propionic acid. About 50% of SCFA absorbed are absorbed via passive diffusion in the undissociated state and the remainder are absorbed as anions, by facilitated diffusion in exchange for bicarbonate ions (Husveth, 2011). The granulosum cells of the rumen contain carbonic anhydrase that promotes formation of carbonic acid (Stevens and Stettler, 1967). Carbonic acid associates with SCFA to form undissociated SCFA which diffuses more easily across the rumen epithelium, leaving bicarbonate ions behind in the ruminal fluid (Husveth, 2011). This mechanism therefore not only allows easier SCFA absorption, but also acts as a buffer mechanism in the rumen, by reducing SCFA concentrations in the rumen for carbonic acid, a weaker acid and by the presence of bicarbonate, which acts as a buffer itself. This plus salivary buffering are key ruminant buffer systems for neutralising SCFA.

During absorption of SCFA through the rumen wall, a large proportion of butyric acid - more in sheep than cattle – is metabolised to β-hydroxybutyrate (βHB). Remaining butyric acid is transported to the liver and is metabolised here (Husveth, 2011). Therefore, absorbed butyric acid appears in general circulation almost entirely as βHB. βHB is metabolised by most of the tissues in the body and is used in the production of the fatty acids characteristic of ruminant milk (Husveth, 2011).

A small amount of acetate is metabolised to CO_2 by the rumen epithelium, but the majority is not changed during absorption or by passage through the liver. Acetate is the most abundant SCFA in general circulation and is one of the ruminant's main metabolic substrates, taken up by the tissues. Acetate is also a main precursor in the synthesis of body fat in ruminants (Husveth, 2011).

Lactic acid production is commonly discussed in the literature with regards to lactic acidosis in animals fed a diet high in rapidly fermentable carbohydrates and has been proposed as playing a dominant role in the development of ruminal acidosis in general (Huntingdon and Britton, 1978). Lactate is produced in large amounts by amylolytic bacterial fermentation in the rumen, particularly when rations are high in rapidly fermentable carbohydrates and reticuloruminal pH drops (Møller et al., 1997). Lactate can also account for as much as 15% of the total dry matter of silage (Gill et al., 1984). As lactate proportions increase and the buffering capacity of ruminal fluid is decreased, lactic acidosis may develop (Møller et al., 1997). Under normal circumstances, lactic acid is produced in low quantities and is utilised by bacteria to produce propionate (Gill et al., 1984, Husveth, 2011). However, with diets high in rapidly fermentable carbohydrates, decreased pH leads to inactivation of propionate producing bacteria but not lactic acid producing amylolytic bacteria, leading to accumulation of lactic acid in ruminal fluid (Husveth, 2011). As lactic acid is a stronger acid than other SCFA and is absorbed slower than other SCFA from the rumen, accumulation exacerbates the problem by decreasing reticuloruminal pH further (Møller et al., 1997, Husveth, 2011). Lactic acid has two forms, L(+) and D(-) lactate (Hernandez et al., 2014). L-lactate is metabolised rapidly by the liver to pyruvate, which is converted to glucose, and L-lactate acidosis is relatively common, primarily occurring as a result of tissue hypoxia (Ewaschuk et al., 2005). D-lactate is formed via the methylglyoxal pathway (carbohydrate, fat and protein metabolism) (Ewaschuk et al., 2005). D-lactate is thought to be metabolized to pyruvate at about one-fifth the rate that L-lactate is metabolised (Ewaschuk et al., 2005). D-lactate acidosis arises in ruminants after grain overfeeding and ruminal fermentation of milk and is associated with diarrhoea in neonate calves (Ewaschuk et al., 2005). Production, accumulation, and acidosis from D-lactate is caused by excessive gastrointestinal fermentation of carbohydrate by lactobacilli and the subsequent inability to adequately clear the D-lactate (Ewaschuk et al., 2005). Treatment

includes bicarbonate and fluid infusion, feeding of hay or enhancing lactate-utilising ruminal bacteria.

Decreased pH can have numerous ill effects for ruminants and so the animal must be equipped to try to buffer reticuloruminal pH back to a range most compatible with microbial and host life. Fluctuation in reticuloruminal pH affects the relative abundance and activity levels of ruminal microbiota and a reduction in pH can reduce diversity of species of bacteria and protozoa dramatically, in addition to consequently reducing efficiency of the rumen for digestion of fibrous material (Xu and Ding, 2011). Different species of microbiota thrive and die at different pH levels (Duncan et al., 2009) and a change in reticuloruminal pH would therefore be expected to affect the populations present.

For the rumen to function efficiently, reticuloruminal pH is usually maintained between pH 5.5 and 7.2, with an average of between pH 6.5 - 6.8, varying with time of day, feeding and drinking (Thrall et al., 2012). Under normal feeding conditions, the pH of the reticulorumen is, in simple terms, maintained at an appropriate pH level by the balance between acid production and absorption and numerous types of buffering by the animal. Erdman (1988) states that rumen pH can be explained as a function of production and active absorption/excretion of SCFA and water, saliva flow and its buffers into the rumen, feed acidity and outflow through the omasum to the lower gastrointestinal tract. Failure of regulation by one of these mechanisms leads to deviation of reticuloruminal pH from physiological levels and can lead to development of ruminal acidosis and possible serious effects on animal health and production. Bovidae utilise various types of buffering mechanisms to attempt to combat pH depression.

Saliva in ruminants, in particular secretions from the parotid gland, is an effective buffer of reticuloruminal pH and contains high levels of bicarbonate and phosphate (Van Soest, 1994). Levels of phosphate and bicarbonate in ruminant saliva are present in a much higher concentration than those of non-ruminant species (McDougall, 1948; Turner and Hodgetts, 1955). Saliva contributes roughly half of all bicarbonate entering the rumen, the other being transported across the rumen epithelium in exchange for ionised SCFA (Owens et al., 1998). It should be noted however, that although ruminal saliva is a good buffer, decreased reticuloruminal pH alone cannot trigger increased saliva production to aid buffering. Saliva

production is determined by the length of time an animal spends eating, ruminating and resting (Maekawa et al., 2002).

The ruminal bicarbonate system is considered more sophisticated than the ruminal SCFA and phosphate buffering systems (Aschenbach et al., 2011). Bicarbonate buffering in the rumen is part of a double-open system, where bicarbonate can decay to H_2O and CO_2, and is in equilibrium with dissolved CO_2 in the rumen. Dissolved CO_2 in the rumen may be eructated or absorbed, and as this is steadily removed from the system, the efficiency of bicarbonate buffering is increased again (Kohn and Dunlap, 1998). As a result of this double-open system, the buffering capacity of ruminant saliva is greatly increased. However, despite the buffering capacity of the saliva, conflicting results have been reported concerning the effectiveness of the protection from acidosis provided by saliva. In a study by Penner and Beauchemin (2010), increased rates of salivary secretion were associated with increased severity of ruminal acidosis. However, it became apparent during this previous trial that salivary secretion rate is extremely hard to measure. Sponge gags do not give an accurate representation of salivary rate, as they cannot effectively measure saliva created during eating or ruminating. Parotid gland measurements also proved difficult as secretions dried up and stopped after several days of having a drain in place. Despite saliva being a key buffer, it is hard to measure and it is thought that other mechanisms of buffering may play a more important role in protection against ruminal acidosis.

The rumen epithelium itself plays a very important role in buffering the rumen and has been shown to secrete large amounts of bicarbonate and remove protonated buffer bases (protonated SCFA known as HSCFA) via absorption (Gäbel et al., 1991). Both of these processes contribute to returning and stabilising pH to the correct physiological range (Gäbel and Aschenbach, 2006, Gäbel and Aschenbach, 2007). It is widely accepted that between 50-80% of ruminally produced SCFA are directly absorbed across the ruminal wall and between 15-50% passes to the distal digestive system (Aschenbach et al., 2011). Despite energy intake determining the amount of SCFA available for absorption or passage, there is no linear relationship between energy intake or SCFA production rates and SCFA passage into the omasum (Penner et al., 2009b). This is due to the fact that passage of excess acids into the omasum is counteracted by increased ruminal absorption rates of SCFA at decreased pH (Dijkstra et al., 1992) and decreased liquid movement into the

omasum under high concentrate feeding conditions. However, an extremely high energy intake has been found to increase the amount of SCFA passing into the omasum unabsorbed (Tamminga and Van Vuuren, 1988). A decrease in rumen motility due to decreased pH could account for the decreased movement into the omasum, as ruminal mixing is an important factor for influencing absorption rates (Allen et al., 2009). Passage of SCFA into the omasum however does not equal passage of protons out of the rumen, as not all SCFA passing out will be HSCFA, carrying a proton (~1% of VFA at pH 6.8) (Aschenbach et al., 2011). It is estimated that ruminal buffering via proton removal from SCFA passage and other buffering substances (phosphate/ammonium) is roughly 15% (Allen, 1997). With salivary buffering of up to 40% of protons, the majority of ruminal buffering via proton removal has to come via absorption (Aschenbach et al., 2011).

Despite the importance of ruminal buffering via absorption, ruminal homeostasis is hard to determine by SCFA absorption rates alone as they are influenced by several contributing factors. Absorption rates are affected by SCFA chain length and pH itself can affect absorption. It has also been shown that bicarbonate appears in the rumen when SCFA are absorbed, again altering the pH and therefore absorption rates (Aschenbach et al., 2011).

The current model of SCFA absorption via HSCFA in the rumen describes lipophilic diffusion. It is thought that lipophilic diffusion occurs as permeability of the lipid bilayer of the rumen epithelium to electrically charged SCFA anions is very low, and therefore passive diffusion must occur with the lipophilic pronated form, HSCFA. However, there are both quantitative and qualitative constraints that suggest that lipophilic diffusion is not the only form of SCFA absorption across the rumen wall. A quantitative constraint is that according to the Henderson-Hasselbalch equation, HSCFA constitute only a small portion of the HSCFA: SCFA acid base equilibrium (Aschenbach et al., 2011).

$$pH = pK_a + log_{10} \frac{[A-]}{[HA]}$$

i.e. HSCFA (pK 4.8) constitute 1% of the acid: base equilibrium at pH 6.8 (Aschenbach et al., 2009).

A qualitative constraint is that SCFA production rates are inversely related to their lipophilic permeability (i.e. acid production rates: acetic > propionic > butyric acid

but lipophilic permeability: butyric > propionic > acetic acid) and that rate of intracellular metabolism is inversely related to acid production rates (Aschenbach et al., 2011). These qualitative constraints could eventually lead to selective accumulation of large amounts of acetate in the rumen.

All of these varying factors mean that a clear response parameter for a negative change in the reticuloruminal environment, in response to a diet high in rapidly fermentable carbohydrates, is extremely hard to define. Buffering mechanisms are influenced by one another and differ greatly among individuals in the same herd, receiving the same ration. The variable commonly investigated to determine ruminal acidosis, reticuloruminal pH, is determined by the equilibrium between these mechanisms and the supply of protons from the SCFA produced by feed fermentation and therefore is not a stable or consistent parameter (Calsamiglia et al., 2012).

As well as SCFA, other parameters in the rumen are often investigated in acidosis studies, such as the presence of reticuloruminal lipopolysaccharide (LPS) or histamine. Both LPS and histamine levels in the rumen are affected by acidosis and the effect of SCFA on pH (Aschenbach et al., 2000, Khafipour et al., 2009). As pH decreases, the histamine and LPS levels in the rumen are likely to increase. These parameters are discussed in more detail in section 1.4.2.3.

1.3.3.3 **Motility**

Reticuloruminal contractions are vital for digestion and have various functions; mixing ingesta with digesta, minimising effects of stratification and moving fermentation products to the rumen wall for absorption. They are responsible for particle sorting and passage, for inoculating ingesta with microbes to allow fermentative digestion and are vital for regurgitation for rumination and eructation of free gas arising from fermentation (Waghorn and Reid, 1983).

As with other chambers of the ruminant stomach, the rumen undergoes biphasic contractions. Rumen motility is controlled via the vagal nerve and contractions occur every 90 seconds to 3 minutes. The first contraction, the A-wave or backward moving contraction, originates in the reticulum and passes caudally around the rumen (Waghorn and Reid, 1983). As some parts of the rumen contract, others relax and contractions move in a wave like pattern. The second contraction, the B-wave or forward moving contraction, occurs only in the rumen and is associated with

eructation of gas by forcing the gas pocket at the top of the rumen into the cardiac sphincter to be eructated (Ruckebusch and Tomov, 1973, Waghorn and Reid, 1983). As a result of the A-wave contractions in the reticulum, liquid is rapidly moved into the rumen. Before completion of the second reticular contraction, the anterior pillar of the rumen contracts and forms a barrier to the second reticular outflow (Schalk and Amadon, 1928). The wave of rumen contractions begin at the anterior pillar and proceed posteriorly, involving the dorsal part of the rumen (Schalk and Amadon, 1928). Then, as these structures relax, the ventral sac begins its contraction, forcing ingesta forward and upward into the anterior dorsal region of the rumen (Baumont and Deswysen, 1991). Following on from this the B-wave involves only the rumen. Studies into the rate of rumen contractions have been conflicting; Schalk and Amadon (1928) found that the rate of rumen contractions varies during rumination, resting and feeding, with contraction most rapid during feeding and slowest during rumination. In contrast to this, Magee (1932) stated that rumen movements were most rapid and extensive during rumination. Further work has suggested that contractions are most frequent during eating and slower during rest and rumination (Waghorn and Reid, 1983).

A variety of factors can affect the rate of reticuloruminal contractions, such as reticuloruminal pH, diet and neural conditions. Contraction frequency is highly affected by diet, and both feed type and intake level play a role in contraction rate, with high forage diets resulting in a higher frequency of contractions in comparison to diets high in rapidly fermentable carbohydrates (Waghorn and Reid, 1983). This follows on from the role of mixing in inoculating ingesta for fermentation; diets high in forage require more mixing and more rumination to digest, in comparison to diets high in rapidly fermentable carbohydrates. For example, addition of long forage, such as barley straw, has been shown to increase contraction frequency of the reticulorumen during eating and also at rest (Norgaard, 1989).

1.3.4 **Measurement and assessment of reticuloruminal function**

1.3.4.1 **Clinical observations**

In addition to clinical examination, several methods are commonly used to observe reticuloruminal contraction rates. Fistulated animals obviously offer an easier way to monitor contraction rate, allowing direct access and observations of the rumen

wall. However, it is possible to monitor contraction rates in non-fistulated animals and methods such as electromyography and radiotelemetry do not rely on fistulated animals, but can be used in them too. Other methods require fistulation, such as pressure sensitive recordings of ruminal gas or fluids (Egert et al., 2014). Radiotelemetry measurement of reticuloruminal contractions uses the reticulum's ability to retain weighted objects by administering indwelling transmitters which measure movement or changes in pressure corresponding to reticuloruminal contractions (Cook et al., 1986). Gyroscopic boluses can be used to collect frequency change data corresponding to ruminal contractions, measuring motility (Michie et al., 2015) and can be administered orally, negating the need for fistulated cattle. As the accuracy and battery life of radiotelemetric systems increases, the use of bolus-based accelerometers to measure ruminal contraction rate and function will become easier and more reliable.

1.3.4.2 **Fistulated animals**

Fistulated animals refer to those which have been surgically fitted with a cannula to allow access to a specific area to collect samples, introduce feedstuffs or to allow for transfaunation. In ruminants, cannula are primarily fitted to the rumen but can be fitted to a number of different areas such as the oesophagus (Van Dyne and Torell, 1964) or other parts of the stomach and GI tract (Harmon and Richards, 1997). Cannulas vary in size from very small to allow access for tubing/ a syringe to large enough to allow hand access and bigger.

1.3.4.3 **Fistulated animal uses**

Animals fitted with ruminal cannula are commonly used in dietary studies to allow direct access to the rumen for sample collection, to investigate parameters such as reticuloruminal pH, microbial populations, SCFA concentrations and other parameters often associated with ruminal acidosis or other dietary conditions. Cannulae also allow a portal to insert a dietary challenge directly into the rumen, thus bypassing an animal's possible refusal to eat a ration. Inappetence is often a symptom of ruminal acidosis or rumen dysfunction (Radostits et al., 2007) and may occur in feed trials as a result of the challenge diet fed. There are some limitations to using a fistulated animal to observe the natural function of a rumen. The rumen is an anaerobic environment and the presence of a cannula in fistulated animals can

change this. However, the presence of the rumen mat above the rumen liquor may be enough to keep the lower third of the rumen as an anaerobic environment but no studies address this.

Fistulated animals are also used for collection of ruminal contents to allow for transfaunation (DePeters and George, 2014). Transfaunation refers to transfer of ruminal contents from a healthy to a sick individual. Rumen transfaunation has been used for centuries to alleviate dietary malfunctions. Brag and Hansen (1994) stated that rumen transfaunation was used long before more recent research demonstrating the importance of the rumen microorganisms to metabolism, and the earliest printed reference they found in Swedish literature referring to a similar practice was from 1776. This early reference stated “it is common practice, even in the countryside, to take the fodder out of the mouth of a sheep or a goat to give it to an animal which does not ruminate” (Brag and Hansen, 1994). Subsequent research related to the discovery of the importance of rumen microbial population occurred much later in the 1900’s (DePeters and George, 2014). However, as diet affects the rumen environment and the microbiota, diet has an effect on the success of transfaunation. This is shown in a study into protozoa-free sheep transfaunation by Dehority (1978). In this previous study, transfaunation was carried out from an alfalfa-fed steer into protozoa-free sheep, and found that in sheep fed an alfalfa hay diet, 24 species of protozoa became established. In contrast, in sheep fed a concentrate diet, only 9 species of protozoa were established (Dehority, 1978), showing that diet affects the rumen environment and in turn, the microbiota present.

1.3.4.4 **Reticuloruminal pH measurements**

Before the use of rumen telemetry, taking samples of ruminal fluid was far more invasive, requiring oro/nasogastric tubing, fistulation or rumenocentesis to give access to the rumen to allow repeated measurements to be taken. Reticuloruminal pH measurements taken this way are more likely to vary due to salivary contamination (for orogastric tube) (Enemark et al., 2004) and as a result of the fact that pH varies dependent on the site where the rumenocentesis needle or stomach tube takes the sample from, pH is not constant across the rumen (Abdela, 2016).

With advances in technology, we have the ability to monitor physiological responses in cattle, such as temperature, reticuloruminal pH and heart rate, via a telemetric

system (AlZahal et al., 2009). Telemetric systems have greatly increased the speed of disease detection and therefore increased herd health, productivity and welfare (AlZahal et al., 2009).

The use of rumen telemetry boluses takes advantage of the reticulorumen's ability to retain weighted objects, allowing the device to remain in the same position, transmitting accurate, repeatable recordings for weeks (Mottram et al., 2008). As technology improves to allow longer battery life and ensure no drift in recordings, the technology will become even more useful. It is important to note however that as there are no set standards for ruminal pH yet, interpreting pH observations obtained via telemetry is still subjective.

Indwelling pH boluses have previously shown a drift in measurements over time. Drift refers to offset errors in the pH data, commonly due to degradation or damage to the reference electrode. This degradation or damage can occur due to high temperatures, extreme pH, high ions in the measurement liquid and any material coating the glass of the electrode. As a result of this, the reference electrode cannot function properly and values obtained will begin to gradually increase or decrease - drifting. Various studies have reported different results when looking at pH drift; undirected but not significant drifts were noted after 12 hours (Penner et al., 2006), positive drifts were noted after 10 days (Enemark et al., 2003) and drift was noted after 48 hours (Kaur et al., 2010). However, it is difficult to compare studies to find a consistent effect of drift, as there are significant constructional differences and differences in pH sensors between studies. Glass pH sensors are less likely to drift, as they are less likely to degrade over time. Additionally, these studies often check indwelling pH measurements using manual measurement of liquor from the rumen, and as Enemark et al. (2003) stated there is often a distance between the probe and the site of the sampling which may account for difference in measured pH (often between 0.05 and 0.2 units), which appears as drift. In addition, the reticulum's ability to retain heavy objects means that boluses are often retained there – not in the rumen itself. The use of boluses for measuring temperature is well supported with consistent correlations being shown between indwelling bolus temperature and rectal temperature (Sievers et al., 2004, Bewley et al., 2008).

1.3.4.5 **Continuous monitoring**

In addition to indwelling rumen boluses, another commonly used method of continuous monitoring is activity collars. Collars are commonly used to monitor rumination, eating patterns, activity, oestrus and overall health in cattle (Turner et al., 2000, Ungar et al., 2005, Kamphuis et al., 2012, González et al., 2015). There are two main types of collars; accelerometer based and microphone based. Accelerometer based collars detect changes in movement frequencies, allowing for identification and notification of changes in activity, eating and rumination. Changes correlate to overall animal health and are often observed before any clinical symptoms are observed. This type of collar is able to detect different movement frequencies, identify, and separate them out as moving, eating and ruminating. The collars then detect fluctuations from the animal's norm for each of these types of movements, to identify when the specific pattern is abnormal for each type of signal. This allows swift notification for stockowners of changes in rumination or eating that they may not have detected in a large herd. Microphone style collars work by recording sounds of mastication. The collars consist of a microphone on the side of the cow's neck and the characteristic sounds of regurgitation and rumination are recorded and processed. Data are then presented as a rumination time for each individual (Ambris-Vilchis et al., 2015). Collars used in conjunction with indwelling pH boluses can be a helpful measure for monitoring an animal's rumination, eating and oestrous patterns and can help to detect changes earlier than traditional stockman observations.

1.3.4.6 **Other assays for assessing reticuloruminal function**

In addition to those methods mentioned, there are other methods used to assess reticuloruminal function including protozoal counts, gram-positive counts, starch digestion assays and gas production tests.

Protozoal counts are commonly used in studies into the rumen microbiome and rumen function, as ciliated protozoal counts are often correlated with digestion in the animal – the lower the protozoal count, the poorer the rumen function and fermentation. As protozoal counts are easier and cheaper to carry out than 16S rRNA sequencing to look at the microbiome as a whole, it allows a small insight into the presence of different microbiota and gives an idea of the function of the rumen.

Rumen activity can also be investigated by studying gas production from the rumen using the *in vitro* gas production technique (IVGPT) to simulate the natural environment of the reticulorumen (Calabro et al., 2012). With IVGPT, feedstuff is incubated at 39˚C under anaerobic conditions and buffered rumen fluid is added; gas produced from fermentation of this feedstuff by microbiota present in the rumen fluid is measured and is indicative of rumen activity (Calabro et al., 2012). IVGPT has also been used to highlight differences between the rumen activity in different species of ruminant (cattle, buffalo, ovine etc.) and to allow SCFA determination at the end of the incubation process, to compare the fermentation process of different feeds under incubation with SCFA proportions produced (Calabro et al., 2012).

Starch digestibility is often looked at via near infrared analysis (NIR) with faecal samples. Starch digestibility can be affected by particle size, kernel characteristics, moisture content, and fermentation (Johnson et al., 2003). Grain is often processed to increase starch digestibility, to allow for faster fermentation of soluble starches in the rumen (Johnson et al., 2003). Solubility is therefore important when considering optimum rumen efficiency and energy utilisation and when considering the risk of ruminal acidosis from rapid fermentation. By investigating the starch levels in faeces, the passage and breakdown of starch from the rumen can be tested (Fredin et al., 2014).

1.3.5 **Potential genetic contributions**

Although it has not been shown in the literature, as both ruminal acidosis and frothy bloat are ruminal fermentation disorders associated with dietary input, it is possible that variation in resistance to ruminal acidosis among individual animals may have a moderate heritability, given results of studies on the genetic basis of frothy bloat in cattle. Evidence that susceptibility to bloating is a heritable trait is provided by variations in expression levels of certain salivary proteins (Rajan et al., 1996) and greater bloat incidence in the progeny of certain sires (Knapp et al., 1943). There are several approaches to genetic selection, of which the single nucleotide polymorphism based (SNP-based) genome-wide association study (GWAS) is most likely to yield a functional tool for dairy cattle breeders by incorporation into genomic selection panels. Future studies into the heritability of rumen acidosis may prove that there is moderate heritability, as with frothy bloat, but this is currently unknown. However, it should be noted that all GWAS are potentially limited by the ability to

define a repeatable and valid phenotype, and rumen acidosis is a challenging phenotype to define. Given the commonly used name of the condition (sub-acute ruminal acidosis or SARA), it is surprising that reticulorumen pH is not an excellent measure. However, pH is limited by the difficulty of measurement, variation in relation to feeding, diurnal variation and the fact it reflects a chemical end-point of interactions among SCFA and buffers in the rumen. Possible alternative indicators might include acute phase proteins such as haptoglobin and serum amyloid A (SAA, shown to be elevated in SARA), ruminal fluid and plasma histamine concentrations, ruminal fluid LPS concentrations, expression levels of specific genes (inflammatory cytokines and immune response markers), variations in specific SCFA concentrations or some other functional test.

1.4 Reticuloruminal dysfunction

1.4.1 Acute acidosis

1.4.1.1 Definition, diagnosis and pathogenesis

Ruminal acidosis can be broken down into 4 categories; peracute, acute, subacute and mild, as described by Radostits *et al*. (2007). Acute acidosis is commonly referred to in the literature by a number of different names: grain poisoning, grain overload, lactic acidosis, toxic ingestion, acute ruminal impaction, metabolic acidosis and many more (RAGFAR, 2007). Acute acidosis is referred to as the most “dramatic” form of ruminal microbial fermentative disorders and can be fatal in less than 24 hours (Garry and McConnel, 2009). It occurs when animals have access to a large amount of rapidly fermentable carbohydrates (often grain) which are consumed in large amounts very quickly. Acute acidosis may occur as a result of a number of different circumstances; accidental access to grain stores/silos where the animal may gorge themselves, feeding large amounts of grain without gradual introduction, sudden change in the type of grain/concentrate being fed, stock grazing on newly harvested pasture where there may be spilled grain or unharvested grain present or an animal who is off-feed returning to feed and being given unrestricted access to rapidly fermentable carbohydrates (Garry and McConnel, 2009).

Consuming large amounts of rapidly fermentable carbohydrates quickly leads to a rapid decrease in reticuloruminal pH, development of metabolic acidosis from the

absorption of lactic acid, dehydration as a result of diarrhoea and inappetence and possible endotoxemia from endotoxin release of ruminal bacterial origin as a result of the lowered pH (Gozho et al., 2005, Cockcroft, 2015). In addition to being rapidly fermentable, these types of carbohydrates – such as cereal grains – offer less buffering capacity than high forage/ fibre feeds, due to their decreased particle size, meaning less salivation and less rumination, lowering the pH and effectiveness of the ruminal buffering system further (Navarre et al., 2012). Severity of acidosis therefore depends on the amount and type of carbohydrate ingested and can range from mild indigestion to fatal toxaemia (Navarre et al., 2012). Affected animals may die if acidosis is severe and swift action is not taken to remedy it. Severe cases may require a rumenotomy to remove rumen contents, followed by intravenous (IV) therapy with 5% sodium bicarbonate, followed by isotonic fluids (Radostits et al., 2007, Cockcroft, 2015). Less severe cases may be treated with IV alkalinising therapy, together with a ruminal antacid such as magnesium oxide. Both severe and less severe cases should be fed hay to encourage salivary buffering and rumination. Clinical symptoms of acute acidosis include kicking at the abdomen, grinding of teeth, diarrhoea, inappetence, depression, bloating, elevated heart rate, staggering and even death (Radostits et al., 2007).

1.4.2 **SARA**

1.4.2.1 **Definition and diagnosis**

Subacute ruminal acidosis or SARA is one of the most commonly reported digestive disorders associated with cattle fed a diet high in rapidly fermentable carbohydrates, and has been reported globally (Abdela, 2016). In the literature, a wide range of terms have been used to refer to this condition, leading to confusion over definitions and which term is technically correct; subacute ruminal acidosis (Nordlund, 2003), SARA (Garrett et al., 1999), chronic rumen acidosis (Garry, 2002), subclinical rumen acidosis (Nocek, 1997), as well as chronic latent acidosis and latent acidotic stress (Kleen et al., 2003). SARA is often reported as being a disorder of high producing dairy herds, as farmers push for increased milk production with an increase in rapidly fermentable carbohydrates and a decrease of fibre in the diet to meet energy requirements. However, despite the focus of SARA research often being on dairy cattle, it does not solely affect high producing dairy animals, and has been shown in

both beef and dairy cattle and under different management styles (Nagaraja and Titgemeyer, 2007).

The presence of SARA in a herd or individual is usually diagnosed according to reticuloruminal pH and is characterised by intermittent drops of reticuloruminal pH below a certain threshold. However, there is much disagreement as to the standard definition of SARA and there is no definitive standard of ruminal pH values which are detrimental to cattle health and production (Plaizier et al., 2008).

SARA is often defined as “periods of moderately depressed ruminal pH (about 5.5-5) that are between acute and chronic in duration”,despite SARA often being referred to as chronic acidosis in literature (Garrett et al., 1999, Nordlund, 2003). There are a wide range of current definitions of SARA, based on a varying range of ruminal fluid pH values and they are dependent on both the method by which samples are collected and the time of day of sampling, as reticuloruminal pH varies across 24 hours. Varying thresholds and definitions are noted below in table 1-1.

Table 1-1: Differing pH thresholds as detailed in literature, dependent on collection method and criteria.

pH threshold	Criteria	Collection Method	Reference
5.5	None specified	Rumenocentesis	(Garrett et al., 1999)
6	4 hr post feeding	Oral probe/ stomach tube	(Plaizier, 2004)
5.6	Time spent below pH 5.6	Indwelling probe	(Gozho et al., 2005)
5.6	Average pH vs. area below pH 5.6	Indwelling probe	(Cooper et al., 1999)

Additionally, Duffield et al. (2004) found that ruminal fluid collected via cannula from the ventral sac and via stomach tube were 0.33 and 0.35 pH units higher than fluid collected by rumenocentesis. Therefore, creating a standard for the diagnosis of SARA requires standardising the method and timing of ruminal fluid collection and the threshold pH value needs to consider these factors. Based on these findings, it has been proposed that thresholds for pH indicating SARA should be 5.5, 5.8 and 5.9 when rumen fluid samples are collected by rumenocentesis, through rumen cannula from the ventral sac, and using an oral probe respectively (Abdela, 2016). Multiple definitions and variability in results of sampling methods have led to many differing interpretations of “SARA” in the literature.

1.4.2.2 **Dietary thresholds and risk factors**

Feeding a diet high in rapidly fermentable carbohydrates and low in fibre is extremely common to increase milk production in early lactation dairy cows and is seen in beef cattle, particularly when finishing cattle for slaughter to boost growth (Steen, 2001, Hernandez et al., 2014). As the amount of high energy, easily palatable carbohydrate in the diet increases, reticuloruminal pH decreases due to the production of SCFA (Dijkstra et al., 2012). There is variation among individual animals in the extent to which they are susceptible to the low pH associated with this diet for a number of reasons; feed intake variation, eating rate variation, diet selection, salivation rate, inherent ruminal microbiome population, previous exposure to low pH and outflow from the rumen (Dijkstra et al., 2012). With more rapidly fermentable foods, such as barley and other grains, rate of starch degradation is increased and leads to faster production of SCFA (Dijkstra et al., 2012).

Dietary management is key for preventing ruminal acidosis, especially in dairy herds where dietary changes following calving can increase incidences of acidosis (Beauchemin, 2007). Cattle require a diet that is adequate in fibre for the proper functioning of their rumen; if the quantity and quality of dietary fibre is poor, the normal physiology of the rumen is impaired and there is increased risk of acidosis (Zebeli et al., 2011). Breaking thresholds suggested for the maximum starch and sugar in a diet, in conjunction with disregard for the minimum NDF in modern dairy diets may predispose animals to developing SARA.

The calculation of dietary energy balance for lactating cows is calculated by first predicting feed intake and considering concentrate feeding level, live weight, week of lactation and milk yield (NRC, 2001). Early transition cows (where the transition period refers to the 3 weeks before and after calving) and cows at peak dry matter intake (DMI) are often reported as being at the most risk of developing SARA due to rapid introduction to high energy dense diets (Mulligan et al., 2006). However, if care were taken to ensure an appropriate transition phase in these cattle, instead of allowing a rapid increase in starch and sugar consumption, coupled with low DMI and forage intake, then cows would be at less risk of SARA (Mulligan et al., 2016). As reported by Mulligan *et al.* (2006), in a study into the prevalence of SARA in dairy cattle, Oetzel (2011) found higher prevalence in cows ranging from 80-150 days in

milk (DIM) compared to cows less than 80 DIM, for herds fed on a total mixed ration. Acidosis has also been reported in pasture fed dairy cattle, so management type does not negate the risk of SARA (Bramley et al., 2008). Mulligan *et al.* (2006) also summarised the key dietary targets which should be met to minimise occurrence of SARA in herds; see table 1-2.

Mulligan *et al.* (2006) suggest that monitoring dietary input and meeting these recommended targets for levels of dietary fibre, concentrate levels and feed space, in conjunction with monitoring symptoms such as incidence of SARA, locomotion score, ruminating levels, faecal score and milk fat percentage can reduce incidence of SARA in a herd and increase speed of detection, should it occur.

Table 1-2: Dietary thresholds for monitoring and preventing acidosis, as detailed by Mulligan et al. (2006)

Criteria	Target Value	Reference
Concentrate in diet (%)	< 65 %	
Cereals in concentrate (%)	≤ 40 %	
Diet starch and sugars (%)	< 20-25 %	
Crude fibre	15-17 %	
ADF	19-21 %	
NDF from forage	21-22 %	
Forage length (%)		
>13mm	30 %	
>40mm	5-10 %	
Long fibre in ration	1-2 kg	
Concentrate fed at milking (kg)	≤ 6 kg	
Increase in concentrate post calving	≤ 0.75 kg/day	(Shaver, 1993)
Feed space per animal	0.6 m	(Grant and Albright, 1995)

DMI is fundamental in cattle nutrition; it establishes the amount of nutrients available for health and production and accurate DMI estimate is therefore important when formulating diets to prevent over or underfeeding (NRC, 2001). Feeds low in digestibility are thought to affect DMI because of their slow rate of passage through the reticulorumen - the stretch and fill receptors in the rumen negatively affect DMI as the weight and volume of digesta increases (Allen, 1996). The neutral detergent fibre (NDF) fraction of the diet is considered the primary constituent associated with the fill effect in the rumen due to its slow digestion rate (NRC, 2001). DMI calculations for lactating cows must take into account body weight, milk production level and DIM to provide an accurate estimation of the DMI requirement for an individual animal at a specific time, as their requirements vary throughout stage of lactation. Forage to concentrate ratio (F:C) in lactating dairy cows has been reported

to affect DMI, however NRC suggests that this affect is probably more to do with the amount and digestibility of the forage itself and the limiting effect of propionate on DMI, rather than a specific ratio of F:C having an effect (Allen, 2000, NRC, 2001). Neutral detergent fibre (NDF) is also associated with DMI and Mertens (1994) suggests that NDF be used to define the upper and lower limits of DMI. The NRC (2001) states that with diets with a high NDF concentration, rumen fill limits DMI and in diets with low NDF concentrations, energy feedback inhibitors limit DMI (NRC, 2001). Competition for food and space available for feeding also affects DMI. Behaviour at the feed bunk is affected by dominance and cows with a higher social rank spend more time feeding and eat more than those with a lower rank, regardless of their production level and the intake they should be at (Aafjes, 1967, Garry and McConnel, 2009). Feed bunk surface position can also affect both DMI and buffering which in turn affect SARA risk. It has been shown than cattle eating in a natural grazing position with their heads down produce more saliva than cattle with their heads held horizontally (Garry and McConnel, 2009).

Carbohydrate percentages in diets can be broken down into structural and non-structural carbohydrates (NSC). Non-structural carbohydrates are those found inside the cells of plants and structural carbohydrates are those found in plant cell walls. Structural carbohydrates must be broken down via bacterial fermentation and cannot be broken down by mammalian enzymes (NRC, 2001). NSC such as sugars, starches and SCFA are the major source of energy for ruminants. These NSC are highly digestible, although rate of fermentation varies dependent on source, and they are usually increased in diets while NDF is reduced, to meet energy requirements of high producing cattle. The ideal level of this rapidly fermentable carbohydrate in diets is not well defined and to avoid acidosis an optimal level must be found. Generally, it is suggested that the maximum concentration of NSC should be roughly 30-40% of DM (Shaver, 1993, Nocek, 1997). However, in modern agriculture, animals are often fed at a level which pushes this maximum concentration far higher to provide a larger amount of energy to increase production, which can result in ruminal acidosis (Abdela, 2016). Therefore, accurate calculation of the various percentages of starches, sugars, concentrates and fibre is important to reduce the risk of SARA in a herd.

1.4.2.3 **Pathogenesis**

The pathogenesis of “subacute” ruminal acidosis or SARA is similar to that of acute acidosis. However, there are important variations between the two, such as the presence of clinical symptoms, extent of the pH depression noted and concentrations of SCFA (Nagaraja and Titgemeyer, 2007). Table 1-3 summarises some of the parameters associated with acute acidosis and SARA and details differences between the disorders.

Table 1-3: Parameters associated with acute and subacute acidosis, and how they differ. Table adapted from “Ruminal acidosis in beef cattle: the current microbiological and nutritional outlook” (Nagaraja and Titgemeyer, 2007)

Parameter	Acute Acidosis	Subacute (SARA)
Clinical Symptoms	Present	Absent
Mortality	Yes	No
Reticuloruminal pH	< 5.0	5.0-5.6
Reticuloruminal lactic acid	High (50-120 mmol)	Normal (0-5 mmol)
SCFA	Initial increase then below normal	High
Lactobacillus sp.	Increase	Increase
Ciliated protozoa	Decreased or absent	Decreased or absent
Reticuloruminal endotoxin	Increased	Increased
Blood bicarbonate	Reduction	Normal
Blood lactate (D-)	Increased	Normal
Packed cell volume	Increased	Normal
Inflammatory markers	Increased	Increased

The pathogenesis of SARA is poorly understood but involves failure of one or more of the mechanisms that regulate reticuloruminal pH due to dietary induced changes in the reticulorumen. These mechanisms include salivary buffering, SCFA absorption through the ruminal epithelium and the associated buffering, SCFA metabolism by ruminal microbes and passage from the rumen into the distal gastrointestinal tract.

1.4.2.4 **Pathology**

Numerous ill effects are reported in the literature as a consequence of subacute ruminal acidosis in cattle and sheep; from histological changes, to clinical pathology changes and various associated diseases and conditions.

Histology

Various ultrastructural and histological changes associated with SARA have been reported in the literature. In a study by Steele *et al.* (2009) into the effect of induced ruminal acidosis with a high grain diet on the ruminal epithelium, animals were split into high grain and high forage diets. In high forage animals, papillae obtained showed an intact keratinised SC, as should be expected, with longer and deeper crevices throughout the surface of the papillae (Steele et al., 2009). In comparison, papillae from animals from the high grain group showed evidence of extensive sloughing of the SC and non-differentiated keratinocytes on the surface of the epithelium – something often associated with parakeratosis (Steele et al., 2009). Also evident was a difference in the microbial colonisation of the rumen, with microbiota being far more evident in high forage papillae and a greater number of microbial phenotypes were present (Steele et al., 2009). The extent of the SC sloughing in high grain fed animals was further investigated and showed not only an increase in sloughing but also an increased migration of SB cells towards the lumen, leading to loss of demarcation of different layers. There was also a loss of cell adhesion with large gaps between cells of the SC and SG (Steele et al., 2009). In a further study by Steele *et al.* (2011a) investigating the ultrastructural changes in an animal fed an acidosis inducing high grain diet in comparison to a high forage diet, the study showed significant changes in the thickness of the SC, SB and SS – all of which were reduced on the high grain diet. The study found that the reintroduction of high forage diets into these animals resulted in increased thickness in all layers (Steele et al., 2011a). The high grain diet animals also showed reduced indentations on the surface of the papillae and reduced microflora present in comparison to the high forage diet, similar to the results observed in the 2009 study (Steele et al., 2011a).

Changes in tight junctions in the rumen epithelium have been associated with "barrier failures" – often reported as being responsible for release of endotoxins and ruminal bacteria into circulation from the rumen. Loss of tight occluding junctions was observed in Steele *et al*.'s (2009) study, where high grain animals displayed loss of cell adhesion and large gaps were evident between cells and has been reported in other studies (Steele et al., 2012, Liu et al., 2013). Tight junctions in the middle layers of the rumen epithelium, SG and SS, play a key role in regulating permeability of the ruminal barrier and preventing translocation of endotoxins and bacteria (Liu et al., 2013). Impairment of tight junctions is commonly observed in

intestinal inflammatory diseases, such as Crohn's disease in humans, and similarly, changes in tight junctions in cattle appear to be as a result of local inflammation from high grain diet induced inflammation altering tight junction protein expression and localisation (Liu et al., 2013).

LPS

Soluble lipopolysaccharide (LPS) has long been associated with symptoms thought to be caused by diets high in rapidly fermentable carbohydrates in ruminants, such as liver abscesses and laminitis, and is known to be causative in some forms of liver failure. LPS, also known as endotoxin, is a component of the outer membrane of gram-negative ruminal bacteria, released during bacterial lysis. It can migrate from the rumen into the circulation (blood and lymph) as a result of failure of the rumen epithelial tight junctions, where it can spread to other organs and is capable of eliciting a host immune response (Plaizier et al., 2012). During bouts of high acid in the rumen, the barrier function of the rumen wall is reduced, increasing risk of LPS leaking through and entering the circulation. Despite this, Plaizier (2012) claims there is limited evidence for increases of LPS in peripheral blood during acidosis but suggests this may due to inadequate sensitivities of LPS tests or fast clearance of LPS from blood (Plaizier et al., 2012). Depending on the severity of the leakage of LPS, immune response may be local or systemic. Different types of rapidly fermentable carbohydrates can affect the type of inflammatory response observed in cattle, particularly inflammation/ immune response because of LPS and other endotoxin translocation. In two studies, it was found that diets high in ground alfalfa hay pellets induced rumen pH drops representative of those associated with SARA but without the innate immune response observed in grain induced SARA and associated with shedding of LPS (Khafipour et al., 2009, Li et al., 2012b). This difference between the two types of dietary induced pH drop shows that the current SARA diagnosis, reliant only on pH depression, is perhaps outdated and does not consider where pH drops but there is no immune or negative response.

Histamine

In addition to an LPS inflammatory response, during reticuloruminal pH depression, histamine translocation through the rumen wall can further exacerbate inflammation. Histamine, like LPS, is of ruminal bacterial origin. Studies have shown that under

normal pH conditions, the permeability of the rumen wall to histamine is low but as the pH decreases, absorption increases significantly (Aschenbach et al., 2000). Histamine is associated with the pathogenesis of bronchial constriction and cardiovascular shock and is an important regulator of food and drink intake in cattle (Plaizier et al., 2008). Therefore, changes in histamine levels and absorption across the rumen wall can be expected to have a systemic effect as opposed to a purely localised reaction in the rumen (Plaizier et al., 2008).

Blood parameters

In addition to those responses seen in the rumen wall, there are also changes observed in blood metabolites and haematology and biochemistry profiles. In a study comparing cattle fed high forage and high grain diets, plasma glucose and plasma beta-hydroxybutyrate (βHB) were significantly higher in cattle on the high grain diet than on the high forage diet (Steele et al., 2009). Non-esterified fatty acids (NEFA) levels in the blood did not change. High glucose levels in the high grain diet is thought to be as a result of the increased energy intake but increased βHB is hypothesised to occur from increased ketogenesis in the rumen epithelium as a result of the grain diet (Steele et al., 2009). This hypothesis was confirmed by qPCR, looking at the gene responsible for ketogenesis in the rumen. Expression of this gene increased 1.6 times during the high grain diet in comparison to the high forage diet (Steele et al., 2009). Increased βHB concentrations in blood indicate stimulation of lipolysis or excess absorption of butyrate in ruminants, which can arise by feeding of spoiled silage. Lipolysis is stimulated by any condition leading to negative energy balance such as anorexia, late pregnancy or lactation and exercise. In cows in late pregnancy, negative energy balance may be exacerbated by a state of insulin resistance due to high levels of progesterone. This promotes ketogenesis by causing a shift in energy metabolism from glucose to fat and by decreasing conversion of dietary or endogenous triglycerides to stored fat (Cornell University, 2013). However, despite the increase in βHB and non-response seen in NEFA in the study by Steele et al. (Steele et al., 2009), in another study into the long term effects of SARA in lactating goats, goats fed a high concentrate diet showed a significant increase in NEFA levels in comparison to low concentrate fed animals (Dong et al., 2013). Thus, showing again the variation among animals in their response to a diet expected to induce acidosis.

Extra-ruminal pathology

As well as the pathology associated with the rumen wall and inflammatory responses, there are various forms of extra-ruminal pathology associated with a diet high in rapidly fermentable carbohydrates and acidosis or SARA. Milk fat depression is often referred to as a consequence of SARA in literature. However, there is some disagreement on whether it should be considered as such. Allen (1997) noted that milk fat only partly correlates to ruminal pH and is a difficult measurement to interpret in early lactation cows due to interactions with body fat mobilisation. It is for these reasons that Kleen *et al*. (2003) stated that milk fat depression cannot be considered a direct consequence of decreased ruminal pH and SARA and in fact, low milk fat disorders are likely to occur independently in situations where SARA may also occur – not as a direct result of one another. In comparison, Plaizier *et al*. (2008) stated that milk fat and milk yield depression do occur as a result of SARA and that it may cost farmers up to a loss of $400 (USD) per cow, per lactation. However, Plaizier *et al.* (2008) did acknowledge that several studies (Keunen et al., 2002, Gozho et al., 2007) did not see a decline in milk fat as a result of grain induced acidosis in cattle. It has been proposed that this inconsistency in the literature and results regarding milk fat could reflect the fact that there is a milk yield reduction in some cases, positively correlated with milk fat percentage, or could be due to the variation in durations of the bout of acidosis. Short bouts of acidosis are thought to be unlikely to have a large effect on milk fat in comparison to repeated or longer bouts (Krause and Oetzel, 2005). Due to this inconsistency in the results, milk parameters alone do not make a good candidate as a marker for identifying ruminal acidosis/SARA susceptibility.

Laminitis

Another condition commonly associated with acidosis and SARA in the literature is laminitis. Laminitis is defined as an inflammation of the claw laminae. The corium (layer containing blood vessels, connective tissue and fat) is most susceptible to injury and inflammation, and other regions such as the sole (papillar region) are also often affected in bovine laminitis (Greenough and Vermunt, 1991). Release of vasoactive histamines and endotoxins, like LPS, from the rumen due to decreasing pH and resulting degradation of the rumen wall are thought to be responsible for bovine laminitis in animals on a diet high in rapidly fermentable carbohydrates

(Nocek, 1997). The vasoactive actions of the substances lead to ischemia and inflammation in the hoof, eventually leading to physical damage of the structures between tissues, affecting locomotion. In time, this can result in permanent anatomical damage to the animal (Nocek, 1997). Symptoms of subclinical laminitis include haemorrhages on the soles, yellow discolouration, double soles, heel erosion and ridging of the dorsal wall (Nocek, 1997). Despite the possible correlations, Brent (1976) stated that laminitis was most likely not caused as a result of the leakage of ruminal histamine because histamine is poorly absorbed and any that is absorbed is rapidly metabolised. Brent (1976) also stated that ruminal histamine was unlikely to be the sole cause of laminitis in cattle, as high levels of orally administered histamine have failed to create a laminitis type response. The relationship between laminitis and acidosis is poorly understood and even the mechanisms of laminitis itself are often reported with contradictory definitions. In addition to this, laminitis does not always occur, even in animals with acute acidosis. These various reasons lead to it being a poor parameter to consider alone as a marker for identifying ruminal acidosis or SARA susceptibility.

Liver abscesses

Also commonly associated in the literature with cattle with decreased reticuloruminal pH are liver abscesses. These are thought to be caused by translocation of rumen bacteria, such as *Fuscobacterium necrophorum*, to the circulation as a result of decreased ruminal barrier function, due to decreased pH causing leakage (Plaizier et al., 2008). Nagaraja and Chengappa (1998) found an incidence of liver abscesses ranging between 12-32% on average in feedlot cattle, on a diet high in rapidly fermentable carbohydrates, and found *F.necrophorum* to be the main cause. Abscesses in cattle can cause major financial losses due to reduced performance and reduced carcase yield. As with laminitis, liver abscesses are not consistently reported in cattle diagnosed with acidosis or SARA and this, coupled with the fact that they are visible only at slaughter, makes them a poor parameter to investigate as a diagnostic marker.

1.4.3 **Motility disorders**

Reticuloruminal contractions, as well as being affected by local pH changes and dietary differences, have been shown to be controlled by the central nervous system

(Rousseau and Falempin, 1985). The vagus nerve is involved with both the stimulation and inhibition of contractions and the splanchnic nerve is involved in inhibition (Rousseau and Falempin, 1985). Stretch receptors in the rumen stimulate contractions when the rumen is full via the vagus nerve, and stimulation of epithelial receptors near the cardiac sphincter stimulates contractions. Duncan (1953) demonstrated the role of the vagus nerve in a study where sheep underwent a vagotomy to observe the effect on contractions. These sheep had complete disappearance of the normal contractions and movements of the rumen and reticulum, a complete stop in rumination and decreased contraction in the abomasum, eventually leading to death. This previous study found that after some weeks, in sheep that survived the vagotomy by directly feeding into the abomasum, some activity returned to the reticulorumen. However, this movement never matched the normal, rhythmic contractions of prior to the vagotomy and it was not propulsive enough to move digesta through the animal (Duncan, 1953). In the same study, sheep also underwent a sphlanchnotomy to observe the role the splanchnic nerve plays in contractions. The sphlanchnotomy did not have a significant effect on rumen or reticular motility or movement through the animal and did not have an increased effect in animals who had already underwent a vagotomy (Duncan, 1953). Thus, it appears that stimulation via the vagus nerve is vital for proper reticulorumen function.

There are a variety of different disorders and mechanisms which cause reticulorumen dystonia and affect the normal rhythmical contractions of the reticulorumen. These include acidotic conditions, microflora inactivity, acidosis and alkalosis (Sederevicius and Kantautaite, 1993), general anaesthesia and disease which causes pain or fever and can inhibit the hindbrain reflex centre responsible for contractions in the rumen (Leek, 1983). Indigestion, ruminal impaction and hypocalcaemia/ milk fever can also all cause ruminal contraction stasis and upset (Leek, 1983). Grain engorgement and bloat also excite the epithelial receptors of the ruminal smooth muscle, which inhibits motility (Leek, 1983). In addition, vagal indigestion (VI) following surgical correction of right abomasal displacement or abomasal volvulus can affect reticuloruminal motility (Sattler et al., 2000). VI has been shown to occur in 14-21% of cases following surgery (Smith, 1978, Constable et al., 1991) and has a high fatality rate in cattle, with only 11.5-20% of affected cattle returning to production (Constable et al., 1991). As these various conditions

affect ruminal motility, monitoring ruminal contractions alone, although giving an overall idea of ruminal health, is not suitable as a sole marker for acidosis or SARA.

1.4.4 **Reticuloruminal alkalosis**

Reticuloruminal acidosis is characterised by an increase in hydrogen ion concentration in the rumen and an associated pH decrease. Conversely, reticuloruminal alkalosis is characterised by a decrease in hydrogen ions in the rumen and an associated pH increase. Metabolic alkalosis occurs commonly in domestic animals and is associated with digestive disturbances in ruminants (Dehkordi and Dehkordi, 2011). It commonly occurs when microbial fermentation is reduced while the animal continues to ingest saliva and its buffers (Garry and McConnel, 2009).

A ruminal fluid pH of between 7 and 7.5 in cattle with alkalosis is often caused by prolonged anorexia and microbiome inactivity, caused by poorly digestible forage and simple indigestion. The low rate of fermentation does not generate enough SCFA to neutralise the alkaline pH of the bovine saliva and absorption of SCFA continues, causing additional bicarbonate production in the rumen (Aafjes, 1967). Acetate absorption is associated with greater generation of bicarbonate in the rumen than other SCFA, and acetate is the SCFA produced in the largest amounts during the fermentation of forage (Garry and McConnel, 2009). Excessive bicarbonate administration as an additional dietary buffer is also a potential cause of metabolic alkalosis (Radostits et al., 2007). The clinical symptoms of reticuloruminal alkalosis include tetany, elevated serum bicarbonate, elevated blood pH and an increased abomasal pH and reduction in respiratory ventilation (Busch et al., 2011).

Ruminal alkalosis can also occur in conjunction with the generation of excessive ammonia. Ammonia concentrations in the rumen increase when high protein diets are fermented but the pH usually does not rise above neutral, as these diets also contain enough rapidly fermentable carbohydrates to produce enough SCFA to counteract the pH increase (Garry and McConnel, 2009). However, with diets high in protein or diets high in non-protein nitrogen sources such as urea and ammonium phosphate, much more dramatic increases in ammonia concentration and pH can occur. Due to this interaction, some of the signs of urea poisoning are similar to

those seen in non-urea caused alkalosis, such as bloat, abdominal pain, inappetence, tympany and diarrhoea (Schelcher et al., 1992).

1.5 Strategies for controlling SARA

1.5.1 Mineral buffers

Commonly used as exogenous buffers, sodium bicarbonate ($NaHCO_3$) and magnesium oxide (MgO) are utilised to stabilise the reticuloruminal pH of cattle suffering or at risk of ruminal acidosis. $NaHCO_3$ can enter the rumen via the diet or in saliva, it is a weak base which buffers hydrogen ions and is characterised by an acid dissociation constant (pk_a = 6.25), similar to that of the normal rumen pH (Mao et al., 2017). $NaHCO_3$ is recognised as an efficient buffer because of its high acid consuming capacity in the rumen, and its mode of action is well documented in the literature (Marden et al., 2008). Feeding $NaHCO_3$ has been shown to raise ruminal pH (Le Ruyet and Tucker, 1992) by neutralising excess protons in the rumen, increasing water intake and increasing the rate of passage through the rumen (Mao et al., 2017). Some published work has argued that addition of $NaHCO_3$ to ruminal rations has little direct impact on reticuloruminal pH, as the rumen is already saturated with CO_2 (Russell and Chow, 1993). However, further studies have argued that $NaHCO_3$ directly neutralises ruminal fluid pH even under high CO_2, as CO_2 is eructated (Kohn and Dunlap, 1998, Mandebvu and Galbraith, 1999).

MgO supplementation of the diet is common as both a magnesium source for ruminants and as a rumen alkalising agent to help raise reticuloruminal pH (Tsiplakou et al., 2017). As cattle have limited availability to store magnesium, MgO supplementation of the diet allows them a daily intake to maintain normal body function as well as acting as a slow realise neutralising agent (Tsiplakou et al., 2017). MgO supplementation has been shown to increase feed inversion ratio and average daily gain in sheep and increase feed intake in cattle (Stokes et al., 1986, Hashemi et al., 2012).

1.5.2 Caustic treatment

In addition to dietary additives, methods of grain processing can also be used to influence reticuloruminal pH, diet effectiveness and voluntary intake of grain. There are 2 main types of grain processing; physical and chemical. Physical processing

such as rolling, crimping or heating of grain, break down the outer layers of the grain (de-lignifying) and increase starch digestibility by increasing ease of access for ruminal microbiota (Theurer, 1986). Chemical treatments with alkalis e.g. sodium hydroxide (NaOH) (caustic treatment or soda grain), ammonia and urea are also used to de-lignify grain and increase starch digestibility (Campling, 1991).

In addition to raising reticuloruminal pH, NaOH treated grains were shown to significantly increase carcass weight in lambs in comparison to lambs fed untreated grains (Ørskov et al., 1981). However, there were large differences between lambs fed NaOH treated oat and NaOH treated barley. Lambs fed treated barley had larger gut content weight, negating their carcass gains but the treated oat group did not (Ørskov et al., 1981). Therefore, type of grain being treated and fed is important to consider when interpreting output of carcass gains from studies. Overall however, there were no significant differences in food utilisation between NaOH treated barley, wheat and maize, and caustic treatment of the grain did not affect the reticuloruminal appearance at slaughter and appeared to cause no negative effects (Ørskov et al., 1981).

As well as caustic treated grains, grains are urea treated, traditionally as a preservative to stop spoilage. In addition to helping store grain, this added non-protein nitrogen obtained from the urea can increase crude protein content. There is a strong anecdotal belief among farmers that urea treated grains are associated with an increase in reticuloruminal pH. However, there appears to be no literature-based evidence of this effect and previous limited studies have shown no effect of urea treated grains on reticuloruminal pH (Ørskov and Fraser, 1975).

1.5.3 **Clays**

Oral supplementation of diets with clay in cattle is relatively common. Many different types of clays are used as buffers; phyllosilicates, which can be further broken down into digestive aids like kaolinite, smectites, chlorites and micas (Adamis and Williams, 2005) and enterosorbents like smectite bentonites.

Bentonites are clays with a high adsorbent capacity and are not necessarily regarded as a ruminal pH buffer (Trckova et al.). This capability to bind liquids allows them to be utilised widely to adsorb toxins such as heavy metals and bacteria

(Hassen et al., 2003) or toxic agents such as endotoxins and aflatoxins (Ditter et al., 1983). In a study into the adsorbent capacity of many different types of clay and their ability to adsorb and remove toxins, bentonite was shown to be the most effective at prevention of endotoxemia by adsorbing the endotoxin (Ditter et al., 1983). In animal diets, bentonites are added to function as an enterosorbent that rapidly bind toxins from the digestive tract, often aflatoxin, reducing their absorption by the animal and minimising adverse effects on the host (Phillips et al., 2002). Bentonite is often used in conjunction with $NaHCO_3$ to regulate ruminal acidosis. The reported effect of bentonite itself on acidosis varies widely, with some studies stating there is no effect on acidosis from clay inclusion in the diet and that is purely an adsorptive measure and others stating that inclusion of clay reduced time spent at an acidotic pH (Dunn et al., 1979). Sulzburger et al. (2016) found that addition of clay to a high grain diet in dairy cows, in comparison to a high grain diet with no clay, showed that cows receiving clay had higher reticuloruminal pH, higher faecal pH, less time spent below pH 5.6 and had higher milk yield and milk fat.

1.5.4 **Ammonia**

Ammonia treated grains in the UK are commonly used as a source of non-protein nitrogen for ruminants (Loosli and McDonald, 1968). Non-protein nitrogen refers to components in a diet that are not a protein but can be converted to protein by rumen microbiota and has a lower cost compared to animal and plant proteins in diets. As with caustic treated grains, there is an anecdotal belief that ammonia treated grains act as buffering agents, raising ruminal pH and they are marketed as such. However, there is no literature-based evidence of this effect. The pH of ammonia treated grain is typically between pH 8 and 9 (Lowman, 2017). Ammonia treated grains are alkaline, have an increased protein content and increased digestibility, as ammonia has a de-lignifying effect on the grain (Lowman, 2017). In the UK, 2 commercially available products, “Maxammon®” (Harbro Ltd.) and “Home n’ Dry®” (FiveF Alka Ltd.), are most commonly used on farm to increase nitrogen content of grain and are marketed as rumen buffers, capable of raising reticuloruminal pH (Maxammon - unpublished, commercial in confidence, 2018) . Treatments are also often reported as preserving grain, as the urea in these treatments breaks down to ammonia, which destroys microbes, moulds and mycotoxins in the grain – effectively sterilising it.

1.5.5 **Particle size**

The design of a cattle ration and the manner in which it performs in the rumen is determined by a balance between the physically effective fibre from neutral detergent fibre (peNDF) present and its associated salivary flow, and the amount of rapidly fermentable carbohydrates and their resultant SCFA produced (NRC, 2001). Mertens (1997) describes physically effective NDF as being specifically "the fraction of fibre that stimulates chewing and contributes to the floating mat of large particles in the rumen". In diets high in rapidly fermentable concentrates, there is often low fibre content from forage, as the diet focuses on maximising the energy intake of the cattle - easier to do with higher proportions of concentrates.

Addition of NDF from forage, with an adequate particle length, in these diets is necessary to allow proper ruminal function, as peNDF, particularly coarse or long particles, influences the animal's intake of dry matter and stimulates chewing activity and rumination and increases salivary output (Beauchemin et al., 2003, Oh et al., 2016). Finely chopped forages may arise due to the specific type of processing before silage fermentation. It has been shown that long forage chop lengths may result in poor silage fermentation due to difficulties packing and maintaining the anaerobic conditions needed and therefore this may push the industry processing towards the use of a shorter chop length (Johnson et al., 2003). Feeding finely processed forages with a small particle size has been shown to decrease the time spent chewing, lower reticuloruminal pH and can alter the fat percentage of milk (Yang et al., 2001, Krause and Combs, 2003). The relationship between particle size and pH is well documented in the literature and smaller particle sizes have been shown to be associated not only with a decrease in mean ruminal pH but have been shown to affect the postprandial pH pattern (Grant et al., 1990, Beauchemin, 1991, Krause et al., 2002). Conversely, as would be expected, increased particle length has been shown to increase milk fat percentage (Yang et al., 2001). Therefore, chop length and particle size, along with any treatment of grains is important when considering the effects of a diet and trying to combat or prevent the occurrence of acidosis or SARA.

1.5.6 **Direct fed microbials**

In an effort to aid and further natural buffering in cattle, some farms choose to include a manufactured pre/probiotic additive in the diet. Some commonly used commercial additives include various types of yeast and bacteria. These additives have a non-nutrient role and are thought to increase the buffering capacity of the rumen through various different mechanisms, dependent on the type of additive.

Yeast, bacteria, fungal and protozoal additives, particularly yeast, are very commonly used in dairy rations globally and are also known as direct fed microbials (DFM) and sometimes probiotics. The term probiotics has several different definitions and has been used to refer to microbial cultures, culture extracts, enzyme preparations and various combinations of these. Due to this confusion, the term DFM must be used by feed manufacturers in the USA and the term is used throughout the UK and by the government body the Food Standards Agency (FSA). DFM are defined by as a “source of live, naturally occurring organisms” (Yoon and Stern, 1995, Pendleton, 1998). DFM have been hypothesised to increase ruminal buffering capacity, increase DMI, increase milk production, reduce the use of antibiotics in neonates and stressed calves and to improve daily gain in cattle via increased feed efficiency (Krehbiel et al., 2003, Nocek and Kautz, 2006).

1.5.6.1 **Bacterial DFM**

Specific strains of *Enterococcus* bacteria have been shown to increase mean rumen pH and increase mean daily pH (Nocek et al., 2002) and specific *Enterococcus faecium* strains have been shown to increase prepartum intake and postpartum production in high producing dairy cattle (Nocek et al., 2003). These DFM are intended to exploit interactions between microorganisms present in the rumen and drive populations and interactions in a direction most favourable to rumen and host health and production. Cultures of *Lactobacillus acidophilus* have been shown to reduce faecal shedding of *Escherichia coli* O157:H7 (Shiga toxin producing strain, which causes major disease outbreaks in humans and survives in the bovine GI tract (Lim et al., 2010)) in feedlot cattle (Krehbiel et al., 2003, Cull et al., 2015). DFM have also been used to reduce stress-related rumen changes in beef calves entering feedlots. Young cattle entering the feedlot undergo a variety of stressors including recent weaning, transport, fasting, vaccination, castration and dehorning

(Krehbiel et al., 2003). However, some studies have shown that the microbial concentrations did not decrease substantially or immediately on arrival at the feedlot. They suggest that the use of DFM is not necessary or helpful, and that depression in some animals' protozoa counts is not as a result of the stressors but the animals decreased DMI on entering the feedlot (Fluharty and McClure, 1997, Loerch and Fluharty, 1999). Literature regarding the use of DFM in young cattle is contradictory and results from trials very often are not repeated in another. It has been suggested that the wide range in results from DFM trials may reflect the use of non-host specific species or insufficient levels of dosing (Krehbiel et al., 2003, McAllister et al., 2011).

DFM have been reported to function by modifying the balance of the reticuloruminal microbiota populations, by adhering to the intestinal mucosa and preventing pathogen adherence or activation, influencing gut permeability and modulating immune function (Salminen et al., 1996, Holzapfel et al., 1998). Adherence by bacterial DFM to the reticuloruminal and intestinal wall is thought to be beneficial to the host, as enterotoxin producing strains of *E.coli* need to adhere to the gut wall to induce diarrhoea through enterotoxin production. Therefore, if the DFM are directly competing for adherence sites, there is a benefit to the host in preventing toxin-producing bacteria from attaching, producing and shedding (Krehbiel et al., 2003). Modifying the balance of the reticuloruminal microbiota populations is one of the key functions reported as a benefit of using DFM to prevent or control the effects of a diet high in rapidly fermentable carbohydrates and ruminal acidosis. Strains of lactate-producing bacteria such as *Lactobacillus* and *Enterococcus* may help prevent SARA by preventing accumulation of lactate in the rumen by utilising it (Nocek et al., 2002). Studies have shown that inoculation with a lactate utilising bacteria (*Megasphaera elsdenii*) has been shown to prevent accumulation of lactate and corresponding reticuloruminal pH decrease, when used alongside a diet with high amounts of rapidly fermentable carbohydrates (Kung and Hession, 1995). However, in other studies, *M.elsdenii* dosing did not affect ruminal pH or SCFA proportions and did not affect milk fat (Weimer et al., 2015, Hagg et al., 2010). Success of DFM in preventing ruminal acidosis and increasing yields in live weight and milk parameters is variable and needs further investigation to prove their worth.

1.5.6.2 **Yeast DFM**

Yeast strains, often varying strains of *Saccharomyces cerevisiae,* live or hydrolysed dead yeasts are one of the most commonly used commercially available fungal DFM. The use of *S.cerevisiae* as a "growth promoter" for ruminants was first reported in 1925 (Eckles and Williams, 1925). As with bacterial DFM, benefits from yeasts appear to be due to changes in ruminal microbiota activity from changes in populations; leading to increased degradability of forage and utilisation of SCFA. A review by Robinson (2002) summarised the findings in the literature between 1981 and 2002 on studies into the effect of commercial yeast product use in dairy cattle, and found the majority of published studies showed the use of yeasts resulted in consistent improvements in DMI and animal production. Reticuloruminal responses attributed to the use of yeast are usually related to the stimulation of cellulolytic bacteria (Newbold et al., 1996), which increases the potential for fibre digestion in the rumen and the ability to prevent a decline in reticuloruminal pH by decreasing lactic acid production and/or increasing the utilisation of lactic acid by ruminal bacteria (Chaucheyras et al., 1996, Guedes et al., 2008). As with bacterial DFM, there are often inconsistent results between similar studies utilising *S.cerevisiae*. It is suggested that this variation in results could be due to confounding effects of differing ration compositions, amount of yeasts included in the ration and the source of the yeast culture product being used (Williams et al., 1991). This last source of variation is perhaps due to the use of non-specific strains, as mentioned by Krehbiel et al. (2003).

1.6 **Aims**

The goal of the work carried out in this thesis was to examine the range of responses to a diet expected to induce ruminal acidosis. Sources of variation in the response to these diets may include variation in the absorptive capacity of the ruminal epithelium to SCFA, variation in the extent to which individual animals are able to bind or neutralize specific toxins such as lipopolysaccharide (LPS) and variation in the rumen epimural microbiome. The main objectives were to examine the range of responses in commonly studied variables, in response to a challenge diet; to assess the ability of different additives believed to aid in controlling the effects of ruminal acidosis in cattle, increasing productivity and feed efficiency; to identify the extent to which sheep can be used as a model organism for cattle in studies on rumen

function; and to try to define a phenotype that is a strong indicator of resistance to ruminal acidosis for use in future genetic studies.

With variation in the literature concerning the naming of the disorder most commonly referred to as sub-acute ruminal acidosis or SARA (grain poisoning, grain overload, lactic acidosis, toxic ingestion, acute ruminal impaction, metabolic acidosis (RAGFAR, 2007)), for the purposes of this thesis the disorder will be referred to as SARA throughout.

2 General materials and methods

Materials and methods detailed in this chapter were used across various studies. For those techniques used in one study only, the protocol is detailed in the materials and methods of the relevant chapter. The centrifuge used in all protocols was an Eppendorf 5810R with a 30-place fixed-angle rotor for 1.2 - 2 ml tubes (F45-30-11) or a high-capacity swing-bucket rotor with four 250 ml buckets (A-4-62) for blood tubes.

2.1 Post-mortem sampling

Cattle in these studies were slaughtered via standard humane stunning and exsanguination techniques in accordance with U.K. legislation at different times, in 3 commercial abattoirs (Woodhead Brothers - Morrisons PLC, Turrif, Scotland; Highland Meats - Dawn Meats, Saltcoats, Scotland; ScotBeef – Bridge of Allan, Scotland). The same protocols were used at each location and for every sample, as far as was possible. In the lairage, cattle from the farm involved in the study were separated if possible from other cattle due to be slaughtered and put through the abattoir as one group. The sampling team were split between the “clean” part of the abattoir and the “dirty” part (gut room). In the clean part of the abattoir, animals involved in the trial were followed from the kill point to evisceration and the rumens marked clearly with a cable tie to allow identification in the gut room. Cattle containing a ruminal pH bolus were marked using another cable tie.

2.1.1 Rumen sampling

Following evisceration, marked rumens of cattle involved in the studies moved into the gut room and were separated from the hindgut by abattoir staff. Once the rumen was separated from the hindgut and cut open, a sample of rumen contents was taken and strained through muslin into 2 x 50ml Falcon tubes. The rumen contents were immediately placed in a passive cool box with ice blocks (<4˚C). If the cow were bolused, the pH bolus was located and removed.

Abattoir staff emptied the rumen and it was photographed to show the size and shape of papillae and to look for any obvious damage on the surface, such as bald patches or ulcers. A sample of rumen wall was taken. As abattoir staff carried this

out, the area taken each time could not be completely standardised, however an attempt was made to take the sample from the same area of the ventral sac each time. The rumen wall sample was washed quickly in water to remove any excess ingesta and 2 samples were taken, placed in bijou in either RNAlater (Qiagen, Ref. 76104) or formalin (Sigma Aldrich, F1268). The empty rumens were marked again with a cable tie and numbered metal washer to ensure correct identification after the blanching process. They were blanched at 80 – 90 °C for 10 min as per abattoir standard protocol. Photographs were taken post-blanching, to compare with the initial photographs and to assess for any changes that may become apparent post-blanching, such as changes in papillae colour or bald spots that may have been hidden by ingesta prewashing.

2.1.2 **Hindgut sampling**

Initially, it was intended to collect hindgut samples for all animals and the data was to be used primarily by consortium partners. However, due to lack of staff and time at abattoir samplings, hindgut was only sampled and examined for those beef animals detailed in the trial in chapter 3. Samples of caecum wall were taken and caecum contents collected and strained through muslin into 50 ml Falcon tubes. The caecum wall was washed in water to remove any excess digesta and then 2 samples taken and placed in bijou, in either RNAlater (Qiagen, Ref. 76104) or formalin (Sigma Aldrich, F1268), as with the rumen samples.

2.1.3 **Processing**

All post-mortem samples were transferred on ice blocks in a passive cool box (<4˚C) back to the laboratory. In the laboratory, tissue samples stored in RNAlater (Qiagen, Ref. 76104) were stored at -20°C for gene expression studies. Tissue samples in formalin (Sigma Aldrich, F1268) were fixed for between 1-2 days, dependent on size, then removed and stored in 1x PBS (phosphate buffered saline) until they could be blocked in paraffin for histology. Rumen fluid samples were stored plain for SCFA and histamine analysis and diluted 1:1 in 1 x PBS for LPS analysis. All rumen fluid samples were frozen at -20°C until further use.

2.2 Histochemistry

2.2.1 Preparation of paraffin embedded tissues

Following fixation in formalin (Sigma Aldrich, F1268), tissue samples were cut into smaller pieces (1-3 pieces roughly 2 cm long and 0.5 cm thick), removing any rough edges and ensuring papillae and muscle were present in each sample. Excess fat was removed , so it would not interfere with future staining. Tissue was processed using the Shandon Excelsior™ tissue processor (Thermo Scientific, UK) on a pre-set programme for 17 hours. The paraffin-embedded tissues were cut on a microtome (Leica RM 2125RT) at 3µm and mounted onto glass slides (Superfrost™, Thermo Scientific, UK). Slides were dried in an oven at 60˚C for between 1-2 hours, until excess wax had evaporated and tissues were fixed to the slide. Haematoxylin and Eosin staining (H&E) and Elastin Martius Scarlet Blue staining (EMSB) were carried out manually by one operator. EMSB staining is an MSB (Martius Scarlett Blue) protocol modified to include a Miller's Elastin stain (Koh-Tan, 2015).

2.2.2 Haematoxylin and eosin stain

3µm baked sections were deparaffinised by immersion in Histo-Clear (National Diagnostics, New Jersey, USA) and then rehydrated by passing through 100%, 95%, and 70% ethanol (non-molecular grade) before rinsing in distilled water. Sections were immersed in Gill's 2 Haematoxylin (Thermo Scientific, USA, Ref. 6765007) for 1 minute, followed by dipping in 1% acid/alcohol (hydrochloric acid/ethanol, made in-house) for 30 seconds to eliminate excess haematoxylin, then washing in running tap water. After washing, sections were placed in 70% ethanol for 1 minute, followed by 1 minute in Eosin Y solution (Sigma Aldrich, USA, Ref. HT110116) for counterstaining. Sections were dehydrated with 70%, 95% and 100% ethanol before being cleared with Histo-Clear (National Diagnostics, New Jersey, USA) and coverslip mounted using Histomount (National Diagnostics, New Jersey, USA).

2.2.3 EMSB (Elastin Martius Scarlet Blue) stain

3µm baked sections were deparaffinised by immersion in Histo-Clear (National Diagnostics, New Jersey, USA) and then rehydrated by passing through 100%, 95%, and 70% ethanol (non-molecular grade) before rinsing in distilled water. Sections were immersed in Miller's Elastin stain (VWR, UK, Ref. 351154S) for 30 minutes,

followed by washing in running tap water. Sections were placed in Celestin blue stain (Sigma Aldrich, USA, Ref. 206342-5G) for 5 minutes, followed by washing in running tap water. Sections were placed in Mayer's haematoxylin stain (Sigma Aldrich, USA, Ref. SLBC2066) for 5 minutes, dipped briefly into tap water, dipped briefly into 1% acid/alcohol (hydrochloric acid/ethanol, in-house) then washed in running tap water. Sections were immersed in Martius Yellow stain (Sigma Aldrich, USA, Ref. 377767-25G) for 2 minutes and then washed in running tap water. Sections were immersed in Brilliant Scarlet (Crystal Ponceau 6R, Sigma Aldrich, USA, Ref. C0644-5G) stain for 10 minutes, followed by washing in running tap water. Sections were immersed in Phosphotungstic Acid (Sigma Aldrichm USA, Ref. P4006) for 1 minute, followed by washing in running tap water. Sections were then placed in Soluble Blue stain (Aniline Blue, Sigma Aldrich, Ref. CI42755) for 1 minute, followed by washing in running tap water. Sections were dehydrated by placing in 70%, 95% and 100% ethanol before being cleared with Histo-Clear (National Diagnostics, New Jersey, USA) and were coverslip mounted using Histomount (National Diagnostics, New Jersey, USA).

2.3 **Immunohistochemistry**

Immunohistochemistry staining was carried out by Veterinary Diagnostic Services (VDS), at the University Of Glasgow's School Of Veterinary Medicine. Sections were cut at 2.5µm and placed on charged slides. All slides were incubated for 1hr at 56°C prior to staining. Five slides were stained per sample for all animals; major histocompatibility complex class 2 (MHCll), myeloperoxidase, cluster of differentiation 3 (CD3), a negative control for rabbit and a negative control for mouse. For the negative control, the primary antibody was omitted. The MHCll was carried out at a dilution of 1:20, Myeloperoxidase at a dilution of 1:1000 and the CD3 at a dilution of 1:100. Staining was carried out using a Dakoautostainer (Dako, Agilent Technologies LDA, UK Ltd). All antibodies used were manufactured by Dako (Agilent Technologies LDA, UK Ltd).

All immunohistochemistry was carried out at room temperature using Tris buffer (pH 7.5) + Tween for all buffer rinses. Heat induced epitope retrieval was carried out using an Access Retrieval Unit (Menarini, UK) and sodium citrate buffer (pH 6) for 1 min 40 sec at 125˚C on full pressure. Enzymatic antigen retrieval was carried out using Proteinase K (Dako, Agilent Technologies LDA, UK Ltd). Sections were

loaded onto a Dakoautostainer (Dako, Agilent Technologies LDA, UK Ltd) and rinsed with buffer. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in PBS (made in house) for 5 min, followed by a rinse with buffer. The appropriate primary antibody was then diluted (universal diluent, Dako Agilent Technologies LDA, UK Ltd) and applied for a 30 min incubation, followed by buffer rinses. The appropriate secondary antibody was then applied for 30 min, followed by buffer rinses. Detection was carried out by using K5007 DAB (Dako, Agilent Technologies LDA, UK Ltd) for 2 x 5 min periods. Sections were then rinsed in water, counterstained for 27 seconds with Gills haematoxylin (made in house) and washed in water. Sections were dehydrated and mounted in synthetic resin (DPX, Sigma) ready for scoring. Table 2-1 details the primary antibody, enzymatic antigen retrieval agent and secondary antibody for each stain.

Table 2-1: The primary antibody and dilution, secondary antibodies and enzymatic retrieval agent used by VDS. Where RTU = ready to use. (Dako, Agilent Technologies LDA, UK Ltd)

	Myeloperoxidase
Primary Antibody Enzymatic Antigen Retrieval Agent Secondary Antibody	Dako Polyclonal Rabbit Anti-human (Ref: A0398) (1/1000) Dako Ready to Use Proteinase K Dako Secondary anti-rabbit Horseradish Peroxidase (Ref: K4003) RTU
	MHCII
Primary Antibody Enzymatic Antigen Retrieval Agent Secondary Antibody	Dako Monoclonal Mouse Anti-Human (Ref: M0746) (1:20) Sodium Citrate pH 6 (in house) Dako Secondary anti-mouse Horseradish Peroxidase (Ref: 4001) RTU
	CD3
Primary Antibody Enzymatic Antigen Retrieval Agent Secondary Antibody	Dako Polyclonal Rabbit Anti-human CD3 (Ref: A0452) (1/100) Dako Sodium Citrate pH 6 Dako Secondary Antibody anti-rabbit Horseradish Peroxidase (Ref: K4003) RTU

2.4 **Image viewing and capture**

All sections, histology and immunohistochemistry, were initially scanned under low and high power using an Olympus CX41 microscope. Images of typical and atypical examples of all features of interest were captured using GXCam (GtVision, UK) and ImageJ (Rueden *et al*., 2017) software. Five slides of each type (H&E, EMSB, CD3, MHC2, and MYELOPEROXIDASE) were examined for each animal and images taken for each parameter. Slides were scored according to the scoring system discussed in more detail in chapter 3.

2.5 Blood testing

2.5.1 Collection and processing

Biochemistry and haematology testing was carried out by VDS (University of Glasgow). Bloods were collected on farm into specific vacutainers (EDTA, Heparin) (Becton Dickinson, BD Vacutainers, USA) from either the jugular or coccygeal vein by a Home Office licence holder. Blood tubes were kept in a passive cool box with ice blocks (<4˚C) or in the fridge, until they could be returned to the lab. Once in the lab, heparin tubes were centrifuged at 664 x g for 20 min at 4°C and 4 ml of the resulting plasma was removed and stored. The rest of the plasma was passed to VDS for further processing. 2ml of whole EDTA blood was removed and centrifuged at 664 x g for 20min at 4°C. The resulting plasma was removed and stored and the buffy coat removed and stored in Qiazol Lysis Reagent (Qiagen, Ref. 79306) for RNA extraction. The remainder of the EDTA blood was passed to VDS for further processing.

2.5.2 Biochemistry

Biochemistry was carried out by VDS using the Dimension Xpand plus (Siemens) and Dimension Flex reagent cartridges (Siemens) which set the parameters of the machine automatically. For tests such as GLDH (Glutamate dehydrogenase) and βHB (β-hydroxybutyrate), where cartridges are not manufactured by Siemens, the machine allows for 10 open channel tests, which allows the use of Randox lab cartridges and manual parameter setting. All cartridges follow protocols recommended by the International Federation of Clinical Chemistry (IFCC).

2.5.3 Haematology

Haematology was carried out by VDS using the ADVIA 20.120 Haematology System (Siemens). The machine has a “Vet Package” added to it, to allow for discrepancies in cell sizes between different species. Although the vet package can usually account for any differences, a manual 5-part differential was carried out for all haematology samples by VDS staff.

2.5.3.1 **Quality checks and sample integrity**

Internal quality checks (QC) were carried out daily by VDS to ensure accuracy for all reported results. Specific internal calibrators were used, including specific bovine calibrators (Randox). Many of the kits used were manufactured and sold for human use but QC checks ensure that they can accurately be used to test animal samples. To ensure sample integrity, all samples were given an individual lab number, in addition to the corresponding animal ID number. This meant that any sample from an animal at any given time could be differentiated and retested easily.

2.5.4 **Acute phase proteins**

2.5.4.1 **Haptoglobin and SAA**

Heparin serum was assayed by another laboratory for haptoglobin (Hp) using the assay method described by Eckersall et al. (2006), and the concentration of serum albumin A (SAA) was determined (Eckersall et al., 2006) using a commercial ELISA kit (Tridelta Development plc, Dublin, Ireland) according to the manufacturer's instructions.

2.6 **Semi-Quantitative PCR**

RNA was extracted from both rumen tissue and EDTA blood for use in gene expression for different studies using the protocols detailed below.

2.6.1 **RNA Extraction from rumen tissue**

RNA extraction from rumen tissue was carried out using a miRNeasy® mini kit (Qiagen) and DNase® kit (Qiagen) and a polytron homogenizer (VWR). A small piece (approximately 1 cm^2) of rumen tissue with muscle and papillae from the frozen RNAlater (Qiagen, Ref. 76104) samples was added to Qiazol Lysis Reagent (Qiagen, Ref. 79306). The tissue was homogenised immediately using a VWR VDI 12 S2 homogeniser (VWR, UK) in a fume hood and stored on ice. The homogenate was split into 2 aliquots; 1 frozen at - 80°C and 1 used to continue the protocol for RNA extraction, which was left at room temperature for 5 minutes. 140µl molecular grade chloroform was added and the homogenate vortexed fully and transferred to a pre-spun phase lock gel heavy tube (PLG-H) tube and incubated at room

temperature for 3 minutes. The homogenate was then centrifuged at 20,817 x g for 15 minutes and the resulting aqueous phase transferred to an RNase-free tube.

For precipitation, 1.5 volume of 100% ethanol (molecular grade) was added to the aqueous phase, mixed and the resulting solution pipetted into a column in a 2 ml collection tube (Qiagen, kit) which was centrifuged at 10,621 x g for 10 minutes and flow through was discarded. The Qiagen miRNeasy® mini kit with DNase® kit was then followed according to the kit protocol. The concentration of the RNA created and the A260/280 and A260/230 ratios was tested using a NanoDrop Microvolume Spectrophotometer and Fluorometer (Thermo Fisher, UK). If the RNA concentration was high (>1000 µg/µL), RNase-free water was added to the eluent and it was placed on the NanoDrop again. RNA was stored at -80˚C until further use.

2.6.2 **RNA Extraction from EDTA blood**

2.6.2.1 **Processing**

EDTA blood was brought to the lab and centrifuged at 664 x g for 20 minutes. The resulting plasma was removed and frozen for future use and the white blood cells and platelets (WBC/buffy coat) removed and stored in 1.2ml Qiazol Lysis Reagent (Qiagen, Ref. 79306). The WBC/Qiazol mixture was vortexed fully and stored at -80˚C for up to 1month before RNA extraction.

2.6.2.2 **Extraction method**

For RNA extraction from EDTA blood, a phenol-chloroform method was used (In-house method, Koh-Tan, 2015). Once thawed, 300 µl molecular grade chloroform was added to the Qiazol/WBC homogenate. The homogenate was vortexed thoroughly until creamy in colour, immediately transferred to a pre-spun PLG heavy tube and left at room temperature for 5 minutes to separate into layers. The top layer from the PLG tube was transferred to an RNase-free tube. If the PLG homogenate had not split, the tube was centrifuged at 4°C for 5 minutes at 15,294 x g, as stated in the manufacturer's instructions. The resulting top layer was then added to the fresh 2 ml RNase-free tube along with the previously removed top layer. An equal volume of chloroform was added and mixed by inversion before being centrifuged at 15,294 x g at 4°C for 15 min. The resulting top layer was removed and transferred into fresh RNase-free tube. An equal volume of isopropanol was added and mixed

by inversion then left at room temperature for 3 minutes, before being centrifuged at 15,294 x g at 4°C for 15 min. The supernatant was poured off carefully without disturbing the pellet and 1ml 70% ethanol added and vortexed briefly. The tubes were centrifuged again at 15,294 x g at 4°C for 10 min, supernatant discarded and the resulting pellets left to dry in the freeze dryer. Once dried, pellets were re-suspended in RNase-free water and centrifuged briefly, before being analysed using a NanoDrop to check concentrations and the A260/280 and A260/230 ratios. RNA was stored at -80˚C until further use.

2.6.2.3 Reverse transcription

Reverse transcription (RT) was carried out using TaqMan Universal Master Mix kit (ThermoFisher, UK) with the extracted RNA from tissue or blood. The kit protocol was followed. The RT reaction mix was prepared following the manufacturer's instructions and made up to 20 µl by combining the non-enzymatic components and the enzymatic components (MultiScribe Reverse Transcriptase, RNase Inhibitor (kit)) and the extracted RNA. The volume of water added to the RT reaction mix was calculated using the following equation:

$$\mathrm{H2O} = \frac{7.7}{\frac{1000}{RNA\ conc\ ng/\mu l}}$$

Components were mixed by inversion. Contents were transferred to a MicroAmp Optical 96 well Reaction Plate (ThermoFisher, UK) and sealed with an Optical Adhesive Cover (ThermoFisher, UK). The plate was centrifuged briefly to remove any air bubbles and ensure liquid collected at the bottom of the plate. The plate was transferred to the thermal cycling block (SimpliAmp Thermal Cycler, Applied Biosystems). RT was carried out following the kit protocol: 10 minutes at 25˚C for inhibition, 30 minutes at 48˚C for RT followed by 5 minutes at 95˚C for RT inactivation. Following thermal cycling, 30µl of RNase-free water was added to each sample to create 50µl cDNA in total. All cDNA products were stored at -20°C until further use.

2.6.2.4 **TaqMan assay**

TaqMan qPCR was used to identify peripheral blood leukocyte gene expression levels from cDNA - created from RNA from EDTA blood samples and from rumen

tissue samples. qPCR was carried out using the TaqMan Gene Expression Master Mix assay kit (Applied Biosystems). The assay was carried out using a 384-well microplate (Thermo Fisher, UK) with 3 technical replicates for each sample to reduce measurement error. For genes run in duplex, TaqMan master mix was prepared according to manufacturer's instructions and included: 0.25 µl of first gene probe, 0.25 µl of second gene probe, 2.5 µl of the TaqMan® Gene Expression Master Mix (Applied Biosystems). Where genes were run in singleplex, the second gene probe was replaced with 0.25 µl of RNase-free water.

After thawing, cDNA was extracted from each well and pooled together into a sterile microcentrifuge tube as an undiluted sample. 3-fold serial dilution was then carried out with the neat sample to create a dilution curve, which would be run on the plates to create a standard curve. Each sample from the 96-well plates was then transferred into the corresponding well in the 384 microplate. In order to make up a reaction volume of 5 µl, 3 µl of the master mix was added and plates were sealed with Optical Adhesive Cover (Thermo Fisher, UK). The qPCR assay was then carried out using the 7900HT Fast Real-Time PCR System (Applied Biosystems) and thermal cycling conditions set out in the kit protocol (table 2-2). qPCR results were analysed using relative expression.

Table 2-2: Thermal cycling condition for TaqMan Assay

Step	UDG Enzyme Incubation	AmpliTaq Gold, UP Enzyme Activation	PCR	
	HOLD	HOLD	Cycle (40 cycles)	
			Denature	Anneal/Extend
Time	2 minutes	10 minutes	15 seconds	1 minute
Temperature	50°C	95°C	95°C	60°C

Gene expression for chapter 5 differed slightly as it was carried out in 10ul reaction volume, all other aspects of gene expression were the same as detailed above.

Tables 2-3 and 2-4 below list details about the genes studied in this thesis including the type of gene (target or housekeeping), accession number, amplicon length, location on the chromosome, location of the gene probe by the exon and context sequence. Location refers to the nucleotide location that is the midpoint of the context sequence for the associated accession number. The 25bp context sequence contains the primer sequence. Primers used in this thesis were manufactured by Applied Biosystems (Thermo Fisher, UK) and were either inventoried, pre-designed

primers or made to order. Primers were chosen based on previous work by the group and consortium partners. The forward and reverse primer sequences are not reported here as primer sequence information is considered proprietary, commercially sensitive information by Applied Biosystems and as such, are not readily available.

Table 2-3: Genes studied in this thesis, the type of gene, accession number, amplicon length and location of the gene probe by the exon.

Gene	Type of gene	Accession number	Amplicon Length	Location	Location of probe by exon
IL1β	Target	NM_174093	129	Chr.11: 48244257 – 48252646	Between exon 6 and exon 7
IFNγ	Target	NM_174086	73	Chr.5: 49223227 – 49228049	Between exon 3 and exon 4
TLR4	Target	NM_174198	74	Chr.8: 112326488 – 112337501	Between exon 2 and exon 3
IL-2	Target	NM_174088	107	Chr.16: 3568211 – 3572149	Between exon 2 and exon 3
CCL11	Target	NM_205773	121	Chr.19: 15521334 – 15524231	Between exon 2 and exon 3
NHE3	Target		100		Between exon 10 and exon 11
RPLP0	Housekeeping	NM_001012682	114	Chr.17: 65627474 – 65631496	Between exon 2 and exon 3
GAPDH	Housekeeping	NM_001034034	66	Chr.5: 10852909 – 10857192	Between exon 3 and exon 4
Act β	Housekeeping	NM_173979	141	Chr.25: 40639997 – 40643416	Between exon 3 and exon 4

Table 2-4: Gene studied, status, product code and probe sequence, as reported by Applied Bioscience (Thermo Fisher, UK).

Gene	Status	Product Code	Probe Sequence
IL1-β	Inventoried	4331182	CTGCAGCTGGAGGAAGTAGACCCCA
IFN-γ	Inventoried	4331182	GCTGATTCAAATTCCGGTGGATGAT
TLR4	Made to order	4331182	AGTGCTGGATTTATCCAGATGTGAA
IL-2	Inventoried	4331182	CGTGCCCAAGGTTAACGCTACAGAA
CCL11	Made to order	4448892	ATGTCCTCAGAAAGCTGTGATATTC
NHE3	Made to order	4351372	CATCAGCTACGTGGCCGAGGGAGAG
RPLP0	Made to order	4448484	CTTAAGATCATCCAACTTCTGGATG
GAPDH	Made to order	4448484	TCATTGACCTTCACTACATGGTCTA
Act β	Inventoried	4331182	CTTCCTGGGCATGGAATCCTGCGGC

2.7 **Consortium work - Aberdeen**

Initially, LPS, SCFA and microbiome identification work was carried out by consortium partners at the Rowett Institute (University of Aberdeen) using the following protocols.

2.7.1 **LPS analysis - Aberdeen**

Rumen and caecum contents samples were kept on ice after post-mortem collection before processing. The protocol was the same for both rumen and caecum contents. Digesta was strained through muslin cloth into separate Falcon tubes. Strained digesta was diluted 1:1 with 1 x PBS and mixed before being centrifuged at 20,817 x g for 45 minutes. The supernatant was taken up using a syringe, filtered through a 0.2µm filter into a pyrogen-free glass tube (Lonza, Switzerland) before being boiled for 30 minutes, cooled and stored at -20°C.

2.7.1.1 **Limulus Lmebocyte Lysate (LAL) Kinetic-QCL™**

The concentration of LPS was determined by consortium partners using the LAL Kinetic-QCL kit (Lonza, Switzerland). An inhibition assay was carried out to test for any substances in the ruminal/caecal fluid that may interfere with the LAL assay, by testing pooled samples against samples spiked with endotoxin standard (kit). A dilution curve was created using endotoxin standards to give a reference value to calculate the endotoxin concentration of unknown samples. The curve was optimised to be linear from 0.005EU/ml to 50 EU/ml. As LAL reacts with substances such as β-D-glucans or peptidoglycans, unknown samples were diluted to reach a non-inhibitory dilution. Dilutions for samples were calculated by following the scheme suggested by Lonza (LAL Kinetic QCL Kit). The kit protocol was followed and plates were run on a SpectraMax 190 Microplate Reader with SoftMax Pro 6 microplate data acquisition and analysis software installed. The microplate reader was set following the parameters outlined in the kit protocol.

Results of the test LPS assay were calculated using the standard curve created from standards run on the plate and using the straight-line equation to calculate concentrations of the unknowns. To accept results, the correlation coefficient of the calibration curve had to be ≥ 0.995 and the coefficient of variation of the standards had to be less than 4%. The negative controls had to have significantly longer “onset time” than that of the least concentrated standard on the curve.

2.7.2 **SCFA analysis – Aberdeen**

Initial SCFA analysis was carried out by the consortium partner using gas chromatography.

2.7.2.1 **Procedure**

Internal standard of 0.1M 2-ethyl butyric acid was made up using distilled water. A short chain fatty acid (SCFA) standard was made up by pipetting the following into a volumetric flask: acetic acid (final conc. 30mM), propionic acid (20mM), isobutyric acid (5mM), butyric acid (20mM), isovaleric acid (5mM) and valeric acid (5mM). To the same volumetric flask, sodium formate (final concentration 10mM), lithium lactate (10mM) and sodium succinate (10mM) were added. Both standards were stored at 4°C. The same protocol was carried out for the standards, rumen and caecum fluid samples.

1ml of rumen, caecum fluid or SCFA standard was added to a Sorvall tube and 50µl 2-ethyl butyric acid (0.1M) added. In a designated fume hood, 0.5 ml concentrated hydrochloric acid and 2ml ether were added and the solution vortexed and centrifuged at 956 x g for 10 minutes. The resulting ether layer was removed and stored in a new Sorvall tube. 1 ml ether was added to the original Sorvall tube, which was vortexed and centrifuged again at 956 x g for 10 minutes and the resulting ether layer was once again removed and placed in the Sorvall tube containing the first ether aliquot. 800 µl of the pooled ether was placed in a Wheaton vial and 100µl N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) added. The tube was heated 80°C for 20 minutes and left to incubate at room temperature for 48 hours to fully derivatise any lactate. The sample was then transferred to a screw top vial and gas chromatography analysis was carried out by the analytical department of the Rowett Institute.

2.7.3 **Microbiome analysis – Aberdeen**

Initial microbiome analysis by consortium partners at the Rowett Institute was carried out using the following protocol.

2.7.3.1 **Sample preparation**

5ml of the rumen fluid sample stored in PBS/glycerol (detailed in section 2.1.3) was centrifuged at 106 x g for 20 minutes to create a pellet and the majority of the supernatant removed, leaving approximately 1ml, dependent on pellet size. The pellet was re-suspended as fully as possible in the 1ml using a plastic Pasteur pipette.

2.7.3.2 **Cell lysis**

0.25g of the sample was added to a screw cap tube containing 0.4g sterile zirconia beads (a mix of 0.01mm beads and 0.5mm beads). 1ml lysis buffer was added (500mM NaCl, 50mM Tris-HCl pH8, 50mM EDTA, 4% sodium dodecyl sulphate (SDS)) and the sample was homogenised at high speed using a vortex for 3 minutes. The sample was incubated at 70°C for 15 minutes, centrifuged at 17,947 x g for 5 minutes and the resulting supernatant transferred to a microcentrifuge tube. This was repeated and the supernatant pooled.

2.7.3.3 **Precipitation of nucleic acids**

1260µl 10mM ammonium acetate was added to the tube, mixed well and incubated on ice for 5 minutes. The solution was then centrifuged at 17,949 x g for 10 minutes and the resulting supernatant transferred to 2 microcentrifuge tubes. One volume of isopropanol was added to the tubes, mixed and incubated on ice for 30 minutes. The samples were then centrifuged at 17,949 x g for 15 minutes and the supernatant removed using aspiration. The nucleic acid pellet was washed with 70% ethanol and dried under a vacuum. The pellet was then dissolved in 100µl Tris-EDTA buffer and the two aliquots pooled.

2.7.3.4 **Removal of RNA, protein and purification**

2µl DNase-free RNase (10mg/ml) was added to the pooled aliquot and incubated at 37°C for 15 minutes. 15µl Proteinase K and 200µl Buffer AL (QIAamp DNA Stool Mini Kit, Qiagen) was added, mixed and incubated at 70°C for 20 minutes. 200µl ethanol was added and mixed well before the solution was transferred to a QIAamp column (kit, Qiagen) and centrifuged at 17,949 x g for 1 minute. The QIAamp DNA Stool Mini Kit was then followed according to manufacturer's instructions. DNA was eluted by centrifugation at 17,949 x g for 1 minute and the resulting DNA solution was aliquoted and stored at -20°C for further use.

2.8 **Histamine ELISA assay**

2.8.1 **Rumen fluid histamine ELISA**

Analysis of the histamine concentration in rumen fluid was carried out using the Abnova Histamine ELISA Kit (Abnova, Cat. No. KA1888) intended for the analysis

of histamine in stool and a LabTech LT-4500 microplate reader. Duplicate measurements were used for all standards, controls and samples. Before utilisation, optimisation was carried out to find the best dilution factor for the rumen fluid, testing the rumen fluid at various dilutions to see which fell on the kit's standard curve most closely. Optimisation showed that 1:300 dilution worked best and this was then used for all samples. The kit was used according to manufacturer's instructions.

2.8.1.1 **Assay**

Duplicate measurements were used for all standards, controls and samples. 100 µL of standards, 100 µL of controls and 100 µL of controls were pipetted into the respective wells of the reaction plate. 25 µL Acylation reagent (kit, Abnova) was then added to all wells, followed by 200 µL of acylation buffer into all wells. The reaction plate was shaken shortly by hand and incubated at room temperature for 15 minutes. 25 µL of the acylated standards, controls and samples were pipetted into corresponding wells of the microtiter strips provided in the kit. 100 µL of the antiserum (kit) was added to all wells and strips were shaken shortly by hand and incubated for 40 min at room temperature. The contents of the wells were then discarded and each well washed 3 times thoroughly with 300 µL wash buffer and blotted dry by tapping the inverted plate on absorbent material. 100 µL of the enzyme conjugate was pipetted into all wells and incubated, without shaking, for 20 min at room temperature. The contents of the wells was then discarded and again and each well washed 3 times thoroughly with 300 µL Wash buffer and blotted dry by tapping the inverted plate on absorbent material. 100µL of the substrate was then pipetted into all wells and incubated, without shaking, at room temperature for 17 minutes whilst avoiding exposure to sunlight. 100 µL of the Stop Solution (kit) was added to each well and the microtiter plate shaken to ensure a homogeneous distribution of the solution. The absorbance of the solution in the wells was then read within 10 minutes, using a microplate reader set to 450 nm. Standard curves were plotted and unknown concentrations were calculated from the regression.

2.8.2 Plasma histamine ELISA

The analysis of histamine concentration in plasma fluid was carried out using the Abnova Histamine ELISA Kit (Abnova, Cat. No. KA2589) for the analysis of

histamine concentration in EDTA plasma and a LabTech LT-4500 microplate reader. Duplicate measurements were used for all standards, controls and samples.

2.8.2.1 **Assay**

Frozen EDTA plasma aliquots processed as detailed in section 1.5.1 were thawed at room temperature. Kit components; wash buffer, acylation reagent, were reconstituted and brought to room temperature. Standards (kit), controls (kit) and samples were pipetted into the respective wells of the reaction plate (kit). The same protocol was followed as for the ruminal fluid histamine but samples were not diluted. The plate was then read within 10 minutes of the ELISA, according to limits outlined in the kit.

Both the histamine ELISA for rumen fluid and plasma were analysed by creating a standard curve and the function and regression coefficient, calculated by fitting to the straight-line equation, as detailed below:

$$y = mx + c$$

Using the equation, the values for each sample were calculated and the results multiplied by the dilution factor (if required) to give the true result.

2.9 **LPS EndoLISA endotoxin detection**

LPS work was previously carried out by the University of Aberdeen's Rowett Institute using the protocol detailed in section 2.7.1. For LPS measurements taken for chapters 3 and 5, measurements were carried out using the protocol below and the EndoLISA Endotoxin Detection Assay kit (Hyglos), based on ELISA technology and the LUX Multimode Microplate Reader (Varioskan). The method is a fluorescent ELISA.

2.9.1 **Processing**

Samples of strained rumen fluid, collected as detailed in section 2.1, were returned to the lab in a cool box and diluted 1:1 with 1 x PBS. The samples were centrifuged at 20,817 x g for 45 minutes. Supernatant was taken up using a syringe, filtered through a 0.2 µm filter into a pyrogen-free glass tube (Lonza, Switzerland) before being boiled for 30 minutes, cooled and stored at -20°C.

2.9.2 **Assay procedure**

For the assay, samples were thawed at room temperature and diluted 1:100,000 with endotoxin-free water. Six serial dilutions were created using LPS endotoxin (500 Endotoxin units/ml (EU)/ml) (kit). One EU corresponds to 0.1 ng LPS (FDA RSE *E. coli* O113 EC-6). The reaction plate was filled with samples and standards in duplicate, binding buffer was added (kit) and the plate incubated on a shaking incubator (Grant-bio PMS-1000i and Hybrid Shake 'n' Stack, ThermoFisher). Following incubation, the assay was carried out as per manufacturer's instructions and the plate read on the LUX according to the protocol. Serial dilutions and blanks were used to plot a standard curve of log (endotoxin units or EU/ml) against log (relative fluorescence units or RFU/ml), and the unknown concentrations were calculated from the regression.

2.9.3 **Quantification of results**

Data were transferred to Excel and the results for 0 minutes and 90 minutes averaged for each individual sample. The 0-minute values were subtracted from the 90-minute values and the results log transformed. The standard curve of log(EU/ml) vs. log(RFU) was calculated and the function and regression coefficient calculated by fitting to the straight line equation, as detailed below:

$$y = mx + c$$

Using the equation the EU/ml values for each sample were calculated and the results multiplied by the dilution factor to give the true result. The readings from the Varioskan LUX are reported in relative fluorescent units (RFU).

2.10 **SCFA and ethanol analysis**

SCFA analyses in reticuloruminal fluid for all studies were analysed using gas chromatography (GC). Plain rumen fluid samples were shipped to Sciantec Analytical (Sciantec Analytical Services, Dalton, UK) on dry ice and tested via GC for the presence and concentration of 10 SCFA plus ethanol. Concentrations of ethanol, propan-1-ol, acetic acid, propionic acid, isobutyric acid, propane-1,2-diol, butyric acid, isovaleric acid, valeric acid, hexanoic acid, heptanoic acid and lactic acid were determined. Any concentration < 25 mg/kg, fell below the detection level of the GC and could not be quantified. Molar concentrations of the VFA were

obtained by dividing the measured mass of each VFA in mg/kg by its molecular weight as follows: Acetate - 60.05; Butyrate - 88.11; Propionate - 74.08; Valerate - 102.13; Isobutyrate - 88.11; Isovalerate - 102.13 (g/mol).

3 On farm and *post-mortem* observations associated with sub-acute ruminal acidosis in beef and dairy cattle

3.1 Introduction

It has been known for many decades that the rumen epithelium is capable of change in response to dietary input - from initial development of the rumen epithelium at weaning, to changes in later life in adult ruminants (Sander et al., 1959, Tamate et al., 1962, Lane and Jesse, 1997, Steele et al., 2009). In adult ruminants, a change in the rumen epithelium in response to dietary input, specifically a diet high in rapidly fermentable carbohydrates, has been shown in the literature (Shen et al., 2004, Steele et al., 2011a, Eurell and Frappier, 2013). Observed changes in the rumen epithelium in response to a diet high in rapidly fermentable carbohydrates include elongation of papillae (Dirksen et al., 1984), proliferation of papillae (Shen et al., 2004), branching of papillae (Beharka et al., 1998) and thickening of the outer, cornified layers of the epithelium (Metzler-Zebeli et al., 2013).

Many studies in the literature have allowed for a greater understanding of the structure and organisation of the cellular components of the rumen epithelium. Methods used in these studies include scanning electron microscopy (SEM), transmission electron microscopy (TEM) and bright field microscopy (Asari et al., 1985, Graham and Simmons, 2004, Scala et al., 2011, Steele et al., 2011a, Steele et al., 2012), as well as Western blot (Scala et al., 2011) and confocal laser scanning microscopy (Stumpff et al., 2011). An in depth overview of the functional organisation of the bovine ruminal epithelium was carried out by Graham and Simmons (2004) using electron microscopy, SDS-PAGE and Western blotting. The vast majority of these methods are highly useful in research settings, but are time consuming and thus considered unsuitable for a high volume investigation or screening of many samples at once. Therefore, based on the current literature and methods, it is difficult to provide a repeatable and relevant classification of gross or microscopic features of the bovine rumen epithelium as being common, incidental or indicative of pathology. There is therefore a need for a novel scoring system for the adult bovine rumen that considers this time constraint and would allow a fast, repeatable and relevant classification of rumen epithelium as normal, incidental or pathological.

When considering the development of a scoring system for adult bovine ruminal pathology, the in-depth works of Steele et al. (2011a) are a useful basis. This previous study investigated differences in the histological appearance of the bovine rumen epithelium from animals maintained on both forage and grain based diets. The study allowed a better understanding of the baseline, normal appearance of the rumen epithelium that could be associated with each diet group. Steele et al. (2011a), used a combination of bright field microscopy, SEM and TEM to investigate the rumen epithelium of cattle exposed abruptly to high forage and high grain diets. Results showed that the thickness of the stratum corneum (SC) did not vary between diets but the thickness of the stratum granulosum (SG), stratum basale (SB), stratum spinosum (SS) and the entire rumen epithelium was reduced in animals on a high grain diet. The study also found that the presence of lesions was not significant between diets but during the initial introduction of the high grain diet, the highest incidence of ruminal lesions was observed. They noted that papillae from cattle maintained on the forage diet showed more and deeper ridges and indentations on the surface of the papillae – containing bacteria and protozoa - in comparison to grain fed animals. The high grain diet was associated with increased sloughing of the outer keratinised layers from the SC, particularly during the initial grain introduction period. Additionally, they noted that in high forage animals, tight junctions and desmosomes between cells were tighter, the SC was intact and fewer transition cells were noted between the SC and SG. In contrast, in animals fed high grain diets, there was a loss of tissue structure and an increase in spaces between cells and layers. These findings were similar to a previous study by Steele et al. (2009) investigating histological changes in the epithelium of a single cow. Additionally, though not detailed in the text of the 2011 study, illustrations clearly showed dilatation of vasculature in the papillae of the animals in which symptoms of grain-induced acidosis were shown. This work by Steele et al. (2011a) focussed only on the acute response to an abrupt change in diet and used SEM, which as previously mentioned, is not suited to a high volume or screening approach of many samples.

Although the 2011 work by Steele et al. is in depth and clearly describes changes in the epithelial tissue structure in response to dietary inputs, there is no reference to any rumen epithelial immune or inflammatory processes. Both local and systemic inflammatory responses in response to a challenge diet high in rapidly fermentable

carbohydrates have been shown previously in cattle (Gozho et al., 2007, Emmanuel et al., 2008, Bondzio et al., 2011). Despite this, few studies detail the immune components of the bovine forestomaches. One of the few studies in the literature uses gene expression, immunoblotting and flow cytometry of rumen tissue and rumen contents (Trevisi et al., 2014). This previous study suggests that the immune response of the bovine forestomach plays an important role in overall bovine health. Therefore, from evidence in the literature of an immune involvement in the pathology of the rumen response to a diet high in rapidly fermentable carbohydrates, it would be expected that variation in the number or the location of immune cells in ruminal tissue might relate to the overall health status of the animal. However, as far as it was possible to determine, there are no published reports in the English literature that describe the distribution of any immune cells in rumen tissue.

The majority of the work that has been carried out investigating the histological appearance of ruminal tissue through bright field microscopy uses standard stains like haematoxylin and eosin (H&E). H&E is good at detailing cellular structure but does not define collagenous connective fibres well. Trichrome stains such as Masson's are better at highlighting collagenous/connective structures (O'Connor and Valle, 1982). Martius Scarlet Blue (MSB) is a trichrome stain in which methyl blue is used to stain collagen, crystal scarlet stains fibrin, and picric acid stains red blood cells. Variants of the MSB stain are commonly used in clinical cardiology research to identify areas of clotting and fibrosis (Tyrankiewicz et al., 2016) and MSB has also been combined with Verhoeff's iron haematoxylin stain to study connective tissue and vascular pathology (Buk, 1984). Therefore, it would be expected to be a useful stain to investigate pathology in the ruminal epithelium.

In addition to changes in the rumen histology associated with a diet high in rapidly fermentable carbohydrates, fermentation disorders such as sub-acute ruminal acidosis (SARA) also commonly arise from the challenges presented by this type of diet (Kleen et al., 2003). SARA is widely reported globally, in both dairy and beef cattle fed on high concentrate rations (Nagaraja and Titgemeyer, 2007, Kleen et al., 2009, Kleen and Cannizzo, 2012, Kleen et al., 2013). SARA is associated with numerous ill effects, such as decreased feed intake, laminitis, diarrhoea and changes in milk parameters, leading to increased culling rates in dairy herds and consequential financial losses (Plaizier et al., 2008). It has previously been shown

that susceptibility to SARA varies greatly among farms, and within herds, with some individuals more likely to develop SARA than others (Morgante et al., 2007, Kleen et al., 2009, Penner et al., 2009a). SARA has been shown to lower production efficiency (Abdela, 2016) and welfare (Enemark, 2008) of cattle in a variety of production systems and estimates of the financial impact of SARA are consistently high globally through production losses, increased culling rate and death losses. It has been estimated that the financial costs associated with SARA are between USD $500 million to $1 billion annually (Enemark, 2008). The occurrence of SARA in dairy herds varies globally and was suggested in a review by Kleen and Cannizzo (2012) to be 14% in Friesland (Kleen et al., 2009), 19-40% in the USA (Garrett et al., 1999); and over 33% in Italian herds (Morgante et al., 2007).

Despite the majority of studies in the literature focussing on the effects of SARA in dairy cattle, the condition has been shown to occur in beef cattle (Nagaraja and Titgemeyer, 2007). Finishing diets in beef cattle are often high in rapidly fermentable carbohydrates such as grain, to ensure rapid liveweight gain in preparation for slaughter. As acidosis-related illnesses are painful and negatively affect the overall health of the animal (Garrett, 1996), there is a need to increase the work done in this area, investigating the condition in finishing beef cattle. In addition to a primarily dairy focus, the majority of current studies investigating SARA look at the effects of experimentally induced SARA. Therefore, there is a need to determine how these observations of experimentally induced ruminal pathology relate to on-farm, commercial conditions.

3.2 **Aims**

There were 3 main aims for this work:

1. To develop a histological scoring system for ruminal mucosa of beef and dairy animals
2. To test the performance of the newly created scoring system on a large subset of animals and describe the histological and immunohistochemical findings from the rumen of animals with diverse management styles and nutritional backgrounds
3. To quantify dietary inputs from beef farms and classify predicted risk for developing SARA and attempt to relate histological score results and differences in the severity or nature of these responses, to specific dietary or management practices

3.3 Materials and methods

3.3.1 Study design

This study was split into 3 main sections: initial observations and preliminary sample analysis with the novel scoring system; full sample set analysis using the scoring system and analysis of 6 beef farms using the scoring system and other commonly studied response variables.

In the year prior to the study, dietary and farm management information was obtained via questionnaire from 10 beef farms in Aberdeenshire, Scotland. From these, 6 farms were selected based on dietary composition and perceived susceptibility to SARA. Farms were classified as “high” or “low” risk of SARA based on the questionnaire, the proportion of barley, straw and silage in the diet and on-farm observations of diet appearance and animal behaviour (feed guarding, access to feed rail). From the 6 selected farms, a total of 119 steers and heifers with an average age of 700 days and carcase weight of 371 kg were sampled at slaughter. Breeds included Aberdeen Angus, Blonde d’Aquitaine, Charolais, Limousin, Salers, Simmental, British Blue and Shorthorn crosses. All animals were reared indoors in the Grampian region, close to Aberdeen, between January and July 2013.

In addition to the 119 animals from these 6 beef farms, additional *post-mortem* samples were collected from animals from a wide range of management and feeding systems - including dairy, grass fed, barley beef and research cereal fed animals - to give a full sample set size of 213. Dairy animals were Holstein-Friesian, other groups were a mix of aforementioned breeds.

The first stage of the study involved histological and immunohistochemical examination of rumen samples from a small sample set of 25 animals from the total 213. The primary examination of the histological appearance of these rumens was used to further develop a scoring system for ruminal histology. Samples were split into one of 5 categories. Each of the 5 categories reflected different feeding systems, with broadly distinct concentrate to forage ratios, as detailed below.

A. Commercial grass-fed beef cattle (CGFB)

B. Commercial silage-fed culled dairy cattle (CSFD)

C. Commercial mixed diet (silage and concentrate) fed beef cattle (CMFB)

D. Commercial cereal-fed beef (barley beef) cattle (CCFB)

E. Research cereal-fed beef (high challenge) cattle (RCFB)

The exact proportion of rapidly fermentable carbohydrates (barley) in each of the diets was not measured in every case, but based on information provided by farmers and abattoirs was believed to lie within the following ranges: CGFB: 0%; CSFD: 0-30%, CMFB: 40-90%, CCFB: 80-90%, RCFB: 90%. Additionally, animals were grouped into “CONCENTRATE” (groups C-E) and “FORAGE” (groups A-B).

The next stage involved the histological and immunohistochemical examination of the rumens from the 6 beef farms, together with their dietary input information and results from other commonly tested parameters, such as ruminal SCFA proportions, ruminal histamine concentration, ruminal LPS concentration and others. These results were investigated to see whether there was a correlation between proposed “risk” factor, farm of origin, proportion of barley in the diet, proportion of fine particles in the diet and both the histological score and results from other tests.

The final stage of the study involved histological and immunohistochemical examination of the rumens from the 6 beef farms, together with the full set of samples collected from animals from a wider range of management backgrounds. This full sample set was again classified into the 5 categories, based on the individual feeding systems with broadly distinct concentrate to forage ratios. The newly created scoring system was applied to the full sample set (n=213) to determine whether the scoring system could effectively differentiate between animals based on their concentrate to forage ratios.

3.3.2 **Feed analysis**

In addition to the questionnaires from the 6 beef farms, total mixed rations (TMR) were assessed for composition, visual physical characteristics, near infrared (NIR) and particle size (Penn State sieve test, as carried out by consortium partners). All

cattle on the 6 beef farms had *ad libitum* access to feed, and were estimated to consume 11-13.5 kg dry matter (DM) feed/day. Cattle on 1 farm (BH6) also had *ad libitum* access to barley straw. The main ingredient of all diets on all 6 farms was barley, grown and processed on each individual farm. Diets varied greatly between farms; sugar beet pulp, soya hulls and a ruminal buffer intended to prevent acidosis were fed on 1 farm each and yeast was fed on all farms (as a direct fed microbial (DFM)). Rumitech feed additive (Harbro, Scotland) was used on 2 high-risk farms only. Full dietary input information was not provided by 2 farms: BL7 and BH1. It was not possible to verify statements provided by the farmer regarding the ration fed to the cattle on BL7 but it was possible to measure the particle size of the TMR and obtain a measurement of crude protein and fibre. Any unverified data were not used in any statistical analyses but were used to support the *a priori* risk classification of the farm (low).

Penn State sieve test

Penn State sieve testing of the TMR was carried out by consortium partners using the methods set out by the Pensyllvania State University Penn State Particle Separator methods (Penn State, 2018). The separator boxes were stacked on top of each other, with the sieve with the largest holes on top, followed by medium-sized holes, smallest holes and the solid pan on the bottom. Approximately 3 pints of forage or TMR was placed on the upper sieve as fed – no physical or chemical alteration. On a flat surface, sieves were shaken in one direction approximately 5 times, then rotated one-quarter turn. This process was repeated approximately 7 times, as is recommended in the standard protocol. After shaking, the material on each sieve and on the bottom pan was weighed and the percentage under each sieve calculated.

3.3.3 **Post mortem sampling**

All cattle were slaughtered via humane stunning and exsanguination in accordance with U.K legislation at 3 commercial abattoirs (Woodhead Brothers/Morrisons PLC, Turrif, Aberdeenshire; Highland Meats/Dawn Meats, Saltcoats, Ayrshire and Scotbeef, Bridge of Allan, Stirlingshire). Samples were collected at varying times throughout the year dependent on availability of animals on specific diets. The same protocol was used for collecting samples, processing at each location and for every

sample in the lab, as far as was practicably possible. *Post-mortem* sampling was carried out as detailed in section 2.1. Samples were collected of the rumen wall at slaughter for all animals (section 2.1.1) and hindgut was collected from animals from the 6 beef farms (section 2.1.2). Photographs were taken of the ruminal surface pre and post-blanching as described in section 2.1. Rumen tissue samples were processed in the laboratory as detailed in section 2.1.3.

3.3.4 **Histology and immunohistochemistry**

Samples collected at post mortem were processed for histology (H&E and EMSB) and immunohistochemistry as detailed in sections 2.2 and 2.3.

3.3.5 **Grid-Sampling**

Following initial pre and post-blanching photographs taken of the rumen at slaughter, black patches were noted on the rumen post-blanching in some animals. To investigate processes responsible for this black appearance, a grid sampling approach was devised. Tissue samples were obtained from a notional grid with 100 mm centres over the entire ruminal wall, pre-blanching, from 3 convenience-sampled animals. Post-blanching, blackened areas were noted and from the original set of samples, samples corresponding with white and black areas were examined histologically. Samples were also taken from the post-blanching tissue immediately adjacent to the sites from which the original (pre-blanching) sample was obtained.

3.3.6 **Image capture**

Slides were scanned under low and high power using an Olympus CX41 microscope. Images of typical and atypical examples of features of interest were captured using GXCam and ImageJ software. Five slides of each type (H&E, EMSB, CD3, MHC2, and myeloperoxidase) were examined from each animal and images captured of each slide.

3.3.7 **Scoring system**

The previously described study by Steele et al. (2011) and an example of a gastrointestinal inflammation scoring system by Day et al. (2008) was used alongside input from pathologists to create a histological scoring system for adult

bovine rumen mucosa. Typical and atypical image examples were used to select parameters of interest which could identify variation in features between animals and which should be included in the system. The initial outline for the scoring system was tested by University of Glasgow pathologists and by 3 members of the research team, to ensure that a repeatable score for the same sample could be created. The scoring system makes use of a modified MSB stain (addition of Miller's elastin) in addition to standard H&E to investigate a wider range of tissue structures.

The scoring system measured average stratum corneum (SC) thickness (SCT), average stratum granulosum (SG) thickness (SGT), clefting of papillae (CLEFT), integrity of the stratum corneum (SCINT), presence/absence of microabscesses, cytoplasmic swelling (SWELLSCORE), perinuclear vacuolation (VACSCORE), sloughing of the SC (SLOUGH), average vascular diameter (VASCD), CD3+ count and MHC2+ count. The full scoring system and definitions of each variable and how it was measured is detailed in table 3-1.

Table 3-1: Histological scoring system. Table showing a summary of the variables that were measured in rumen wall samples from 25 cattle and considered for inclusion in a standardised scoring system

Name & Abbreviation	Variable Type	Definition and How Measured	Levels	Level Definitions
Stratum corneum thickness (SCT)	Continuous	Mean of 5 measurements in µm across the SC evenly divided over 2 fields, using × 40 magnification and H&E stain.	NA	NA
Stratum granulosum thickness (SGT)	Continuous	Mean of 5 measurements in µm across the SG evenly divided over 2 fields, using × 40 magnification and H&E stain.	NA	NA
Clefting and complexity (CLEFT)	Ordinal categorical	Presence of clefts, buds, branches along the papillae, using × 4 magnification and EMSB stain.	1-3	1 – All or almost all papillae are simple with no clefts, buds, branches 2 – Minority of papillae show some degree of cleft, bud or branch formation 3 – Majority of papillae show some degree of cleft, bud or branch formation
Integrity of the stratum corneum (SCINT)	Ordinal categorical	The extent to which the SC forms a complete, uninterrupted layer over the papillae, using × 10 magnification and EMSB stain.	1-5	1 – All or almost all papillae show intact SC as a single, scarlet band on EMSB stain 2 – Minority of papillae show vacuolation in the SC but the SC remains clearly identifiable as an intact, single, scarlet band on EMSB stain 3 – Majority of papillae show vacuolation in the SC but the SC remains clearly identifiable as an intact, single, scarlet band on EMSB stain 4 - – Minority of papillae show vacuolation in the SC and interruptions to the integrity of the SC, which is not clearly identifiable as an intact, single, scarlet band on EMSB stain 5 – Majority of papillae show vacuolation in the SC and interruptions to the integrity of the SC, which is not clearly identifiable as an intact, single, scarlet band on EMSB stain
Microabscess (MICROABSCESS)	Binary categorical	Presence of microabscesses in any papillae observed using × 10 magnification and myeloperoxidase stain	0,1	0 – No microabscesses seen 1 – Microabscesses of any number seen
Cytoplasmic swelling (SWELL)	Ordinal categorical	Loss of normal appearance of intercellular space (evidenced as observable space between cells and loss of the characteristic appearance of tight junctions among the cells of the SB, SS and SG, using × 40 magnification and H&E stain.	1-3	1 – Intercellular spaces clearly visible among the majority of cells in all layers 2 – Intercellular spaces clearly visible among some cells but not consistently among layers 3 – Intercellular spaces not visible at all

Cytoplasmic swelling score (SWELLSCORE)	Ordinal categorical	Loss of normal appearance of intercellular space (evidenced as observable space between cells and loss of the characteristic appearance of tight junctions among the cells of the SB, SS and SG, using × 40 magnification and H&E stain.	2-6	Sum of SWELL results from two slides
Perinuclear vacuolation (VAC)	Binary	Presence of perinuclear vacuoles in the stratum basale and stratum sinosum using × 40 magnification and H&E stain.	1,0	0 – No vacuolated cells seen 1 – Vacuolated cells noted
Perinuclear vacuolation score (VACSCORE)	Ordinal categorical	Presence of perinuclear vacuoles in the stratum basale and stratum sinosum using × 40 magnification and H&E stain.	0-4	Sum of binary results from two slides, each treated as per VAC
Sloughing (SLOUGH)	Ordinal categorical	Retention or partial retention of sheaths of SC on papillae, observed using × 10 magnification and EMSB stain	1-3	1 – No papillae show evidence of retained sheaths of cornified cells 2 – Few or occasional papillae show evidence of retained sheaths of cornified cells 3 – Many papillae show evidence of retained sheaths of cornified cells
Vessel size (VASCDIAM)	Continuous	The diameter of the single largest vessel in each of two papillae using × 40 magnification and H&E stain.	NA	NA
CD3 cell density (CD3)	Count	Count of the total number of CD3+ cells in a single image taken at × 40 magnification.	NA	NA
MHC II cell density (MHCII)	Count	Count of the total number of MHC II+ cells in a single image taken at × 40 magnification.	NA	NA

3.3.8 **Semi-Quantitative PCR**

Gene expression was carried out as detailed fully in section 2.6. RNA extraction from rumen tissue collected post mortem was carried out using the miRNeasy® mini kit (Qiagen, UK) and DNase® kit (Qiagen, UK). Reverse transcription (RT) was carried out using TaqMan Universal Master Mix kit (ThermoFisher, UK) on extracted RNA from tissue, following the kit protocol. TaqMan qPCR was used to quantify gene expression levels from cDNA created from the extracted RNA using TaqMan Gene Expression Master Mix assay kit (Applied Biosystems). The assay was carried out with 3 technical replicates for each sample to reduce measurement error. For genes run in duplex (*TLR4, IL1β, NHE3*), TaqMan master mix was prepared according to the manufacturer's instructions and included: 0.25 µl of first gene probe, 0.25 µl of second gene probe, 2.5 µl of the TaqMan Gene Expression Master Mix (Applied Biosystems). Where genes were run in singleplex (*CCL11, IL2, IFNγ*), the second gene probe was replaced with 0.25 µl of RNase-free water. The qPCR assay was carried out using the 7900HT Fast Real-Time PCR System (Applied Biosystems) and thermal cycling conditions set out in the kit protocol.

3.3.9 **Histamine in reticuloruminal fluid**

Analysis of histamine concentration in reticuloruminal fluid collected *post-mortem* was carried out using the Abnova Histamine ELISA Kit (Catalogue Number KA1888), designed for analysis of histamine in human faeces. Duplicate measurements were used for all standards, controls and samples. The ELISA and subsequent analysis was carried out as detailed in section 2.8.

3.3.10 **Lipopolysaccharides in reticuloruminal and caecal fluid**

Lipopolysaccharide (LPS) in reticuloruminal fluid collected *post-mortem* was determined using the Hyglos EndoLISA endpoint fluorescence, microplate method, with a Varioskan Fluorescent microplate reader. Duplicate measurements were used for all standards, controls and samples. The method is a fluorescent ELISA and was carried out and analysed as detailed in 2.9.

3.3.11 Short chain fatty acids (SCFA) and ethanol in reticuloruminal fluid

SCFA in reticuloruminal fluid collected *post-mortem* were analysed using gas chromatography (GC) as detailed in section 2.10. Concentrations of ethanol, propan-1-ol, acetic acid, propionic acid, isobutyric acid, propane-1,2-diol, butyric acid, isovaleric acid, valeric acid, hexanoic acid, heptanoic acid and lactic acid were determined. Any concentration < 25 mg/kg fell below the detection level of the GC and could not be quantified.

3.3.12 Statistical analyses

Feed NIR analysis, particle size testing via Penn-State sieve test, assessment of visual characteristics of feed were performed by consortium partners at the Rowett Institute, Harbro and SRUC. Data from these analyses were used in further analysis in this chapter.

Initial histological examination (n=25)

Distribution of continuous data was checked by examination of histograms and Shapiro-Wilk (SW) testing of normality. Data that had a significantly non-normal distribution according to the SW test were natural log-transformed and examined again. Log transformation resulted in a distribution close to normal for the majority of variables, given the small numbers available, to enable parametric statistical analysis. Effect of diet on each log-transformed variable was analysed using one-way ANOVA, as was the effect of high forage or high concentrate diets. Where data was not normally distributed, Kruskal-Wallis testing was used to investigate the effect of the factors. Due to very small sample sizes for each of the frequencies of the categorical variables, statistical analysis of frequencies by category was not undertaken in the initial examination.

For factor analysis and Principle Components Analysis, and for further development of the scoring system for the full sample analysis, histological variables were standardised by dividing each observation by the overall mean value for that variable. To identify which factors to retain, principle components analysis (PCA) was carried out in R (RCore Team 2016) using parallel analysis with the “paran” package (5000 iterations). Horn’s parallel analysis (PA) is a method used to decide

how many components in a principal component analysis (PCA) drive the variance observed in a data set of “n” observations on “p” variables (Dinno, 2014). Three sets of standardised variables were selected for inclusion in preliminary scoring systems. Scores were checked for normality and the effects of treatment and forage/concentrate on each of the scores were evaluated by one-way ANOVA. Four scoring systems were selected to apply to the full dataset.

Full sample set analysis (n = 194)

Few variables were normally distributed according to Shapiro-Wilk testing. Data was initially analysed before transformation using Kruskal-Wallis to investigate the effects of factors of interest on continuous variables. Variables were then natural log transformed, and re-tested for normality using Shapiro-Wilk test and histograms. In some cases, Shapiro-Wilk testing suggested data were not normally distributed, but visual examination of histograms suggested a near normal distribution that would allow parametric analysis. For all results, parametric and non-parametric analyses were applied and results were consistent. For each of the continuous variables, univariate analysis was carried out with Kruskal-Wallis test. Generalised linear models (GLM) tested and the p-values of the effects reported. For the full sample set, categorical count data was analysed using GLM assuming Poisson family distribution. Data was checked for overdispersion and fit to the Poisson family using package “AER” in R, no data were overdispersed ($c \geq 1$).

3.4 **Results**

3.4.1 **Grid Sampling**

As shown in figure 3-1, histological examination of rumen samples taken pre-blanching from areas of the rumen that were identified post-blanching as either normal (white) or black showed no immediate differences.

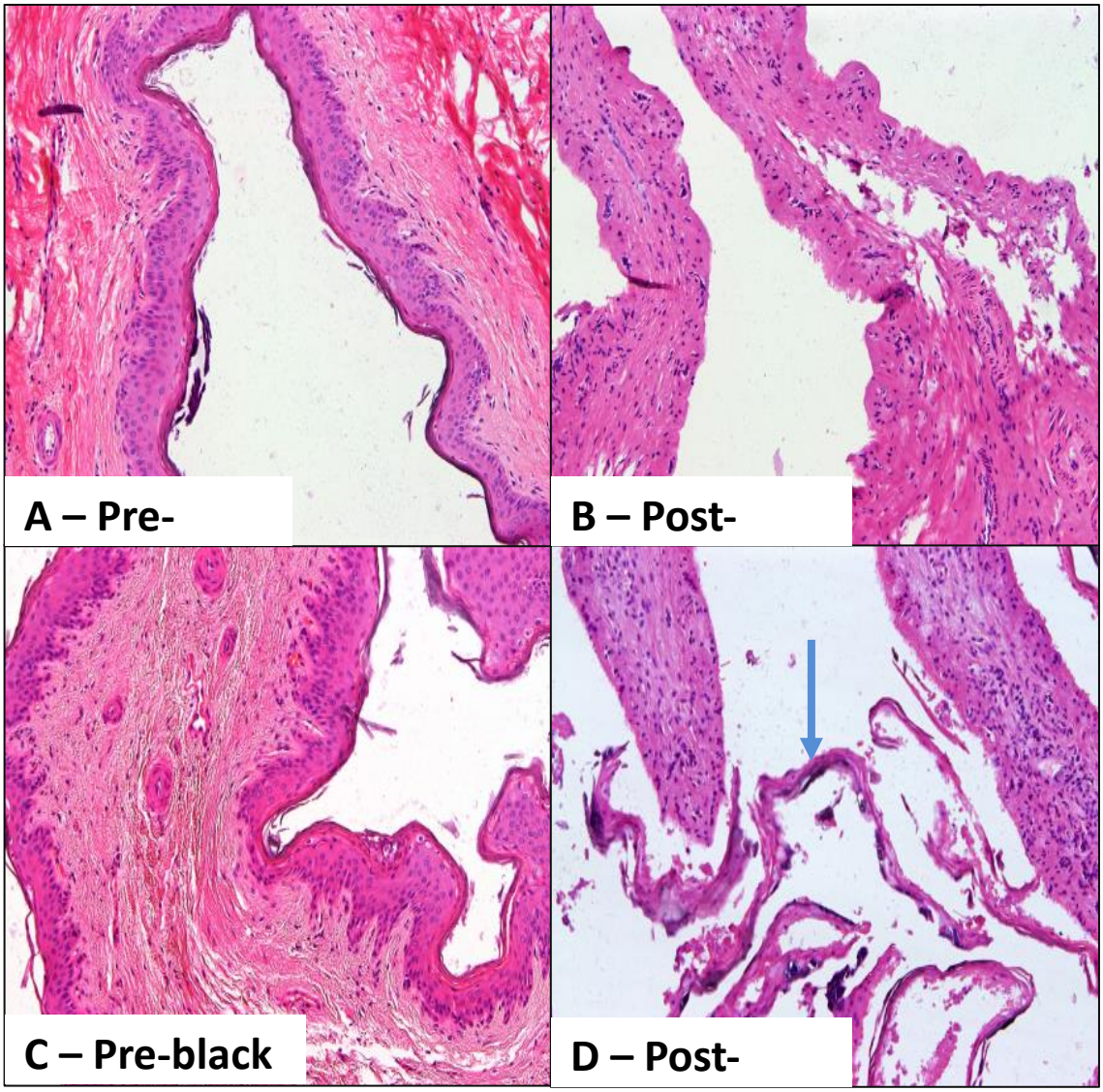

Figure 3-1: H&E (x20) staining of sections from black and white areas of the rumen. "Pre" refers to samples obtained pre-blanching and "post" to samples obtained post-blanching. The post-blanching sample from the white area of the rumen showed the loss of the 3 outermost layers of the rumen wall and papillae. The remaining cell layers and structures were condensed together and lacked cellular definition. The post-blanching sample from the black areas showed similar changes in loss of cellular and structural integrity and the dissociation of the 3 outmost layers. However, in the black areas, the detached layers were retained (arrow).

Samples of the white areas processed for histology after the blanching process showed a clear loss of the 3 outermost layers of the rumen wall and papillae (SC,

SG and SS). The remaining cell layers and structures were condensed and lacked cellular definition. Post-blanching samples from the black areas showed retention of the 3 detached outer layers. The heat of the blanching process also appeared to make any pigmentation present in the SC/SG of the papillae more condensed and obvious.

3.4.2 **Initial observations of tissue structure**

Initial light microscopy observation of the ruminal epithelium was consistent with previous descriptions (Dobson et al., 1956; Graham and Simmons, 2004; Eurell and Frappier, 2013). The stratified squamous epithelium of the ruminal papillae showed 4 distinct layers: the stratum corneum (SC), followed by the stratum granulosum (SG), the stratum spinosum (SS), with the stratum basale (SB) at the deepest level. Underlying this was connective tissue, rich in capillaries and other vasculature. Figure 3-2 shows low magnification images of a single papillae stained with H&E. Figure 3-3 shows low magnification images of a single papillae stained with H&E (Fig 3-3A) and with EMSB (Fig 3-3B). Figures 3-4, 3-5 and 3-6 show representative examples of high and low values, for each of the features considered for inclusion as variables in the scoring system, as described in table 3-1. With the exception of highlighting or confirming the presence of microabscesses (which were otherwise easily detected using H&E stain), myeloperoxidase staining was not informative and extememely few positive cells were noted outside microabscesses. Microabscesses (focal aggregations of neutrophils in the SC) were noted in many samples from animals on all diets. Old or shedding microabscesses were noted but not counted. Figure 3-7 shows representative examples of high and low numbers of MHCII+ and CD3+ staining of ruminal papillae.

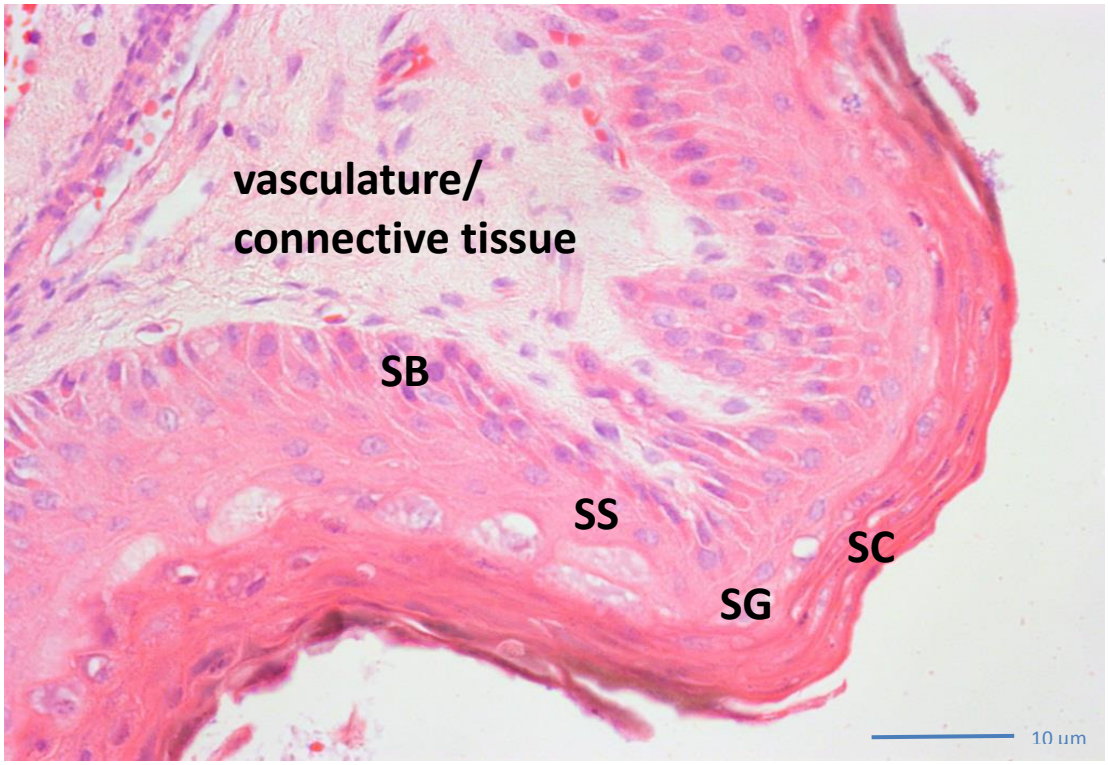

Figure 3-2: H&E (x40) stained papillae section showing the 4 characteristic cell layers (stratum corneum, stratum granulosum, stratum spinosum and stratum basale) and vasculature consistent with previous descriptions in the literature. Image taken from sample from this study.

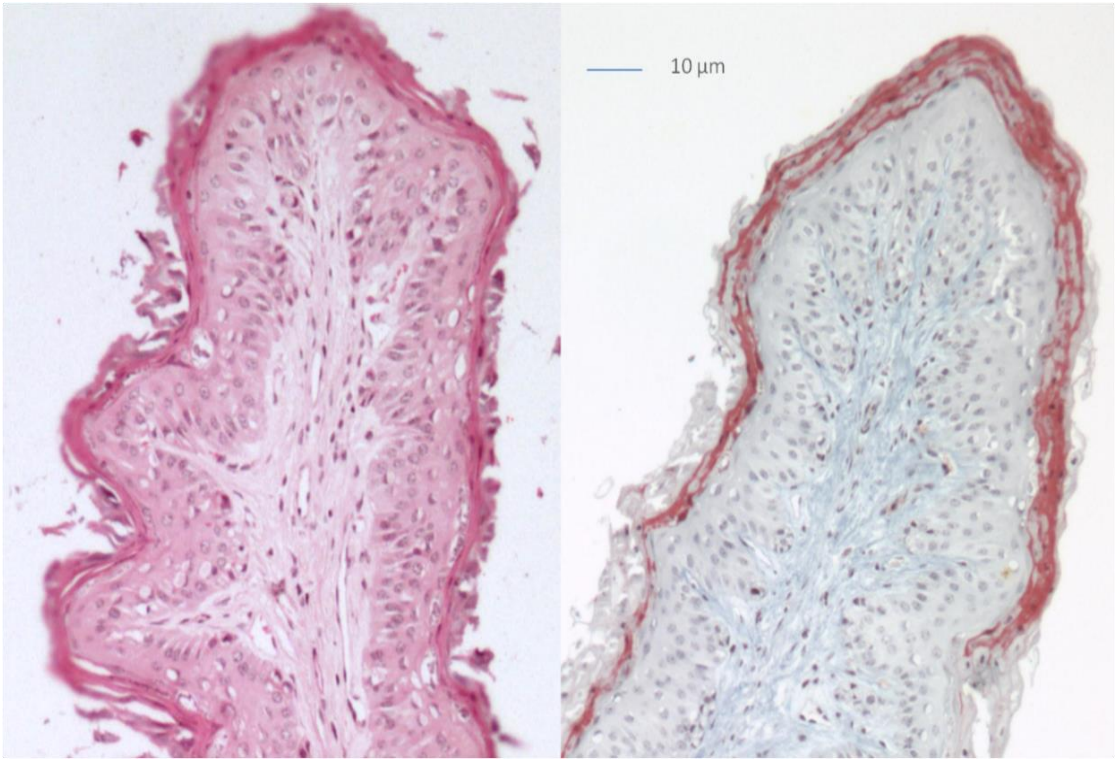

Figure 3-3: Comparative examples of single papillae stained with H&E (2A) and EMSB (2B) stains, showing clearly the characteristic cell layers (stratum corneum, stratum granulosum, stratum spinosum and stratum basale). Image taken from a sample from this study (x10 magnification).

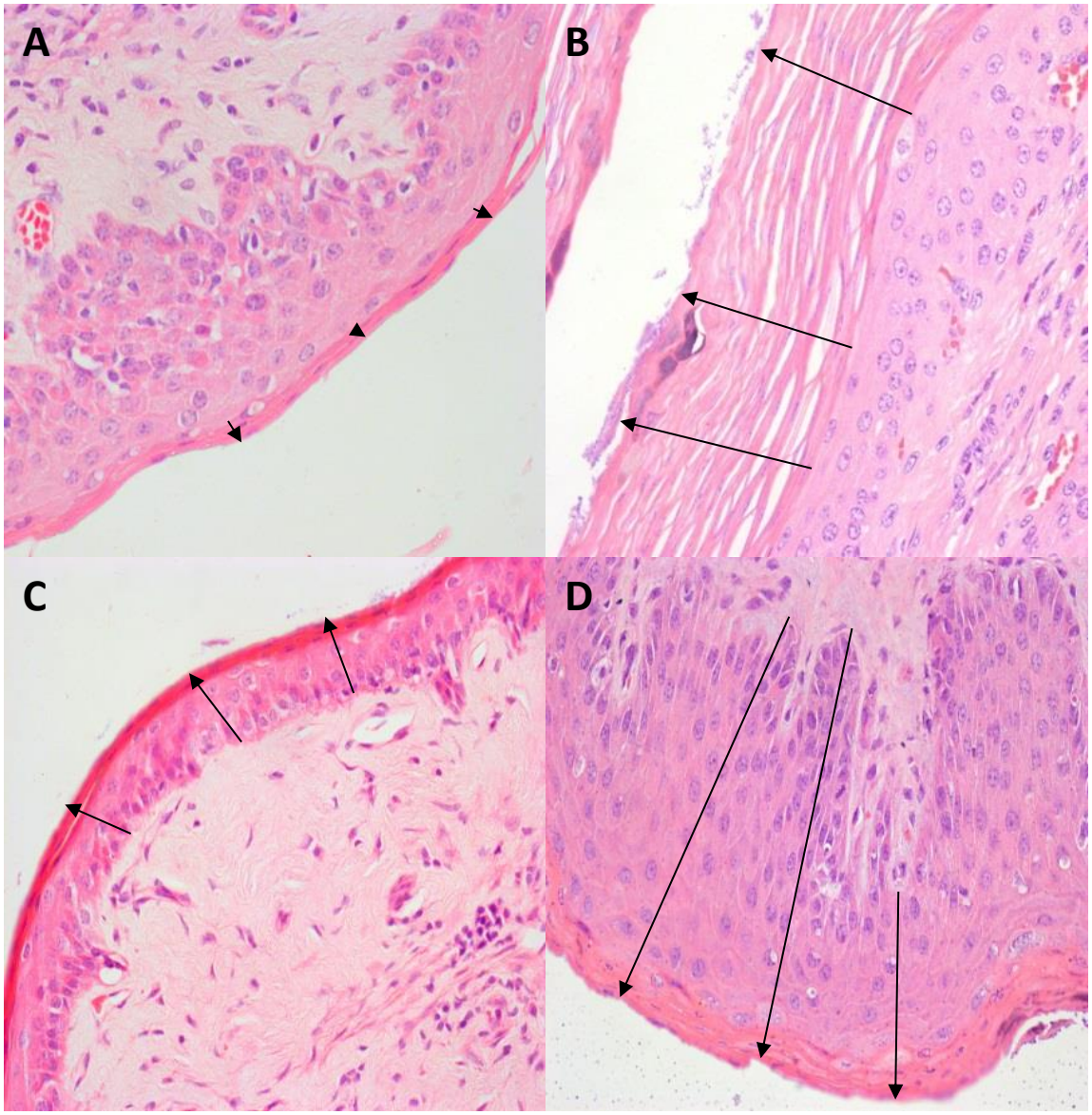

Figure 3-4: H&E (x40) stained sections of ruminal papillae illustrating representative low and high examples of stratum corneum thickness (A – low SC score, B – high SC score) and low and high examples of stratum granulosum thickness (C – low SG score, D – high SG score) as scored using the scoring system in section 3.3.7. Black arrows show examples of the measurements taken using ImageJ. Five representative measurements were taken randomly and averaged, to create a value for average SC and average SG.

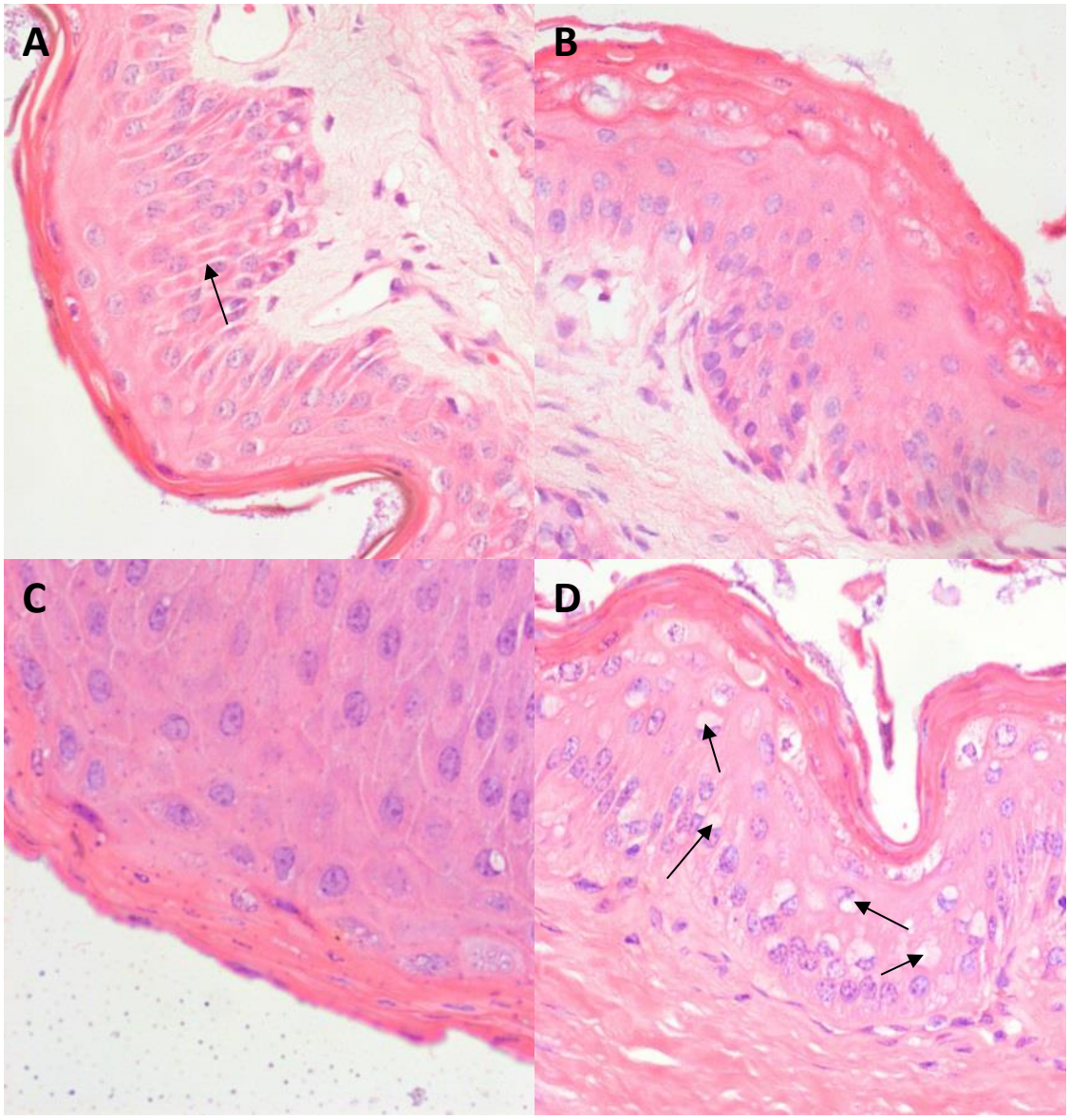

Figure 3-5: H&E (x40) stained sections of ruminal papillae illustrating representative examples of low and high examples of cytoplasmic swell score (A – low swellscore (clear cellular spaces), B – high swellscore (loss of cellular definition)) and perinuclear vacuolation (C- low vacscore, D – high vacscore). Arrows point to A – cellular spaces and D – examples of perinuclear vacuoles.

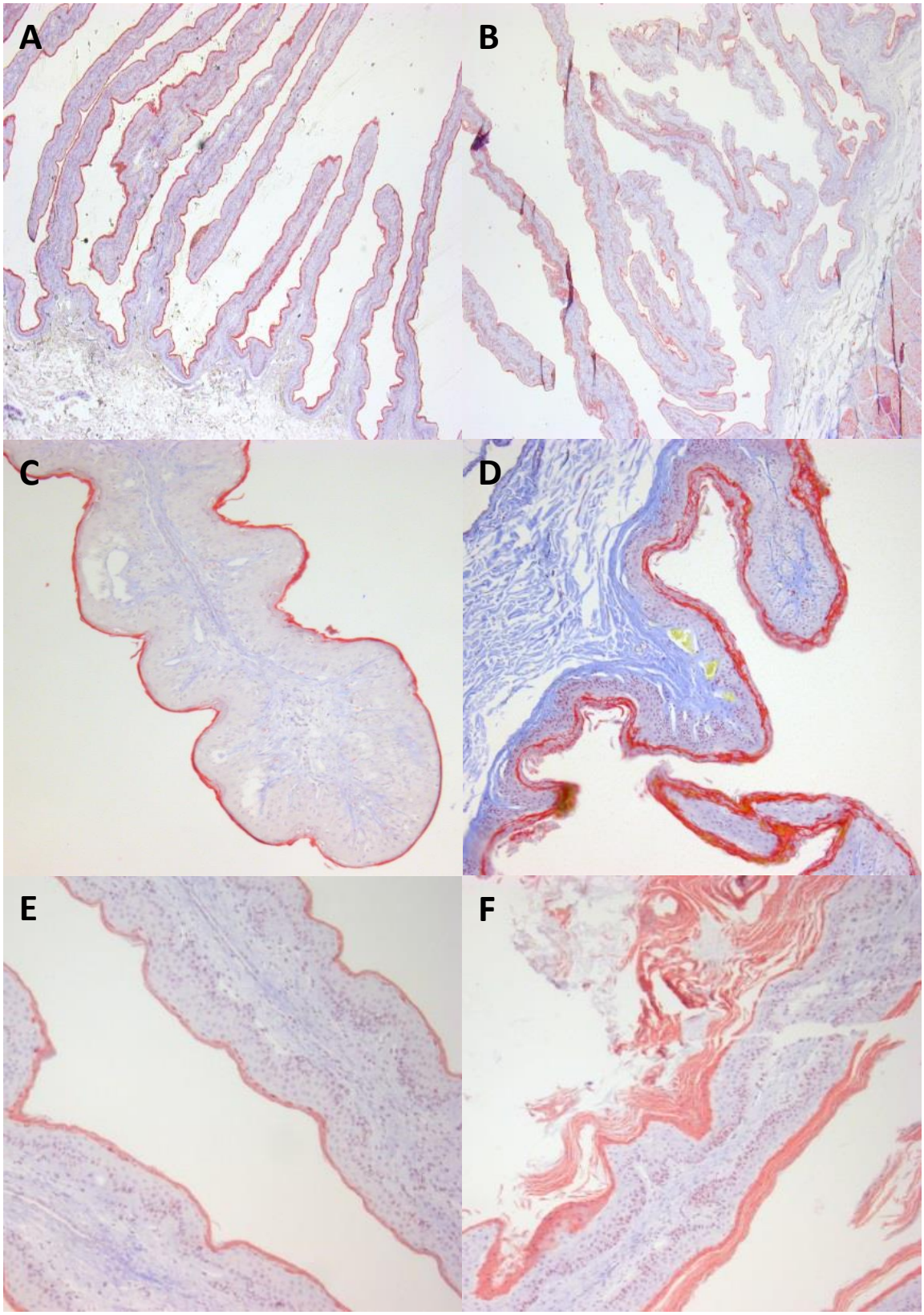

Figure 3-6: EMSB stained sections of ruminal papillae illustrating representative examples of low and high examples of clefting score (A – low clefting, B – high clefting), stratum corneum integrity (C- low SCINT, D – high SCINT) and sloughing score (E – low slough score, F – high slough score) as scored using the scoring system in section 3.3.7.

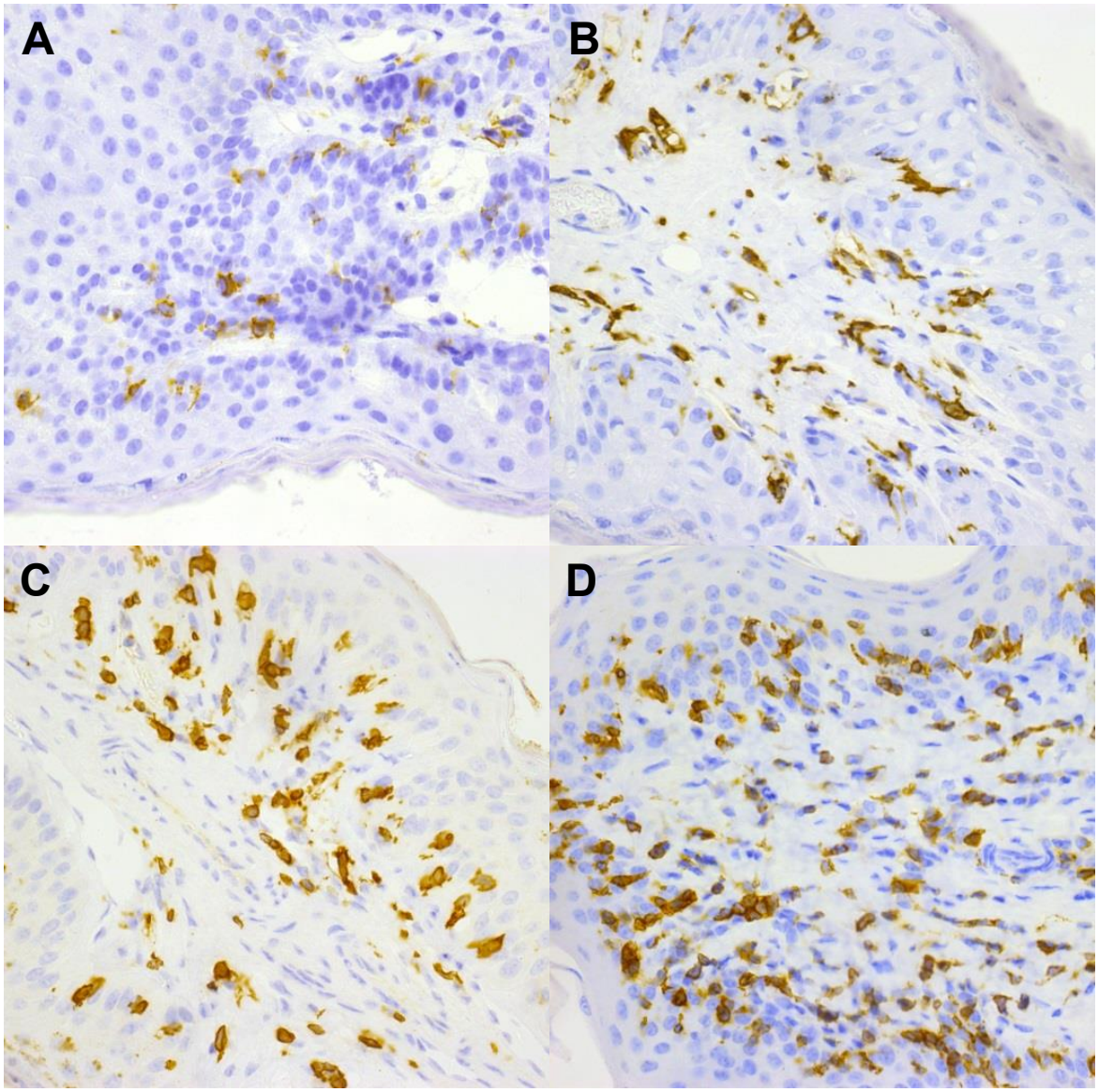

Figure 3-7: (x40) Sections of ruminal papillae illustrating representative examples of low and high examples of CD3 (A – low CD3+ score, B – high CD3+ score) and MHC2 (C – low MHC2+ count, D – high MHC2+ count) as scored using the scoring system in section 3.3.7. Positive cells were counted for the scoring system in a set field of vision on ImageJ.

3.4.3 **Preliminary sample examination (n=25)**

With the exception of the presence of microabscesses on papillae, no obvious pathological changes were noted on histological examination of the ruminal epithelium of any of the animals used in this study. The presence of adult rumen fluke (*Calicophoron daubneyi*) was noted in some animals at slaughter but was not recorded as part of the scoring system.

3.4.3.1 **Continuous scoring variables**

Scores for continuous variables were compared between diets (CGFB, CSFD, CMFB, CCFB and RCFB (A-E)) and between groups (CONCENTRATE (groups C-E) and FORAGE (groups A-B)), as shown in table 3-2. Between diets, average stratum corneum thickness and average vascular diameter were significantly different (p <0.05) but average stratum granulosum thickness, CD3+ and MHC2+ count were not (p >0.05). Between groups (CONCENTRATE and FORAGE), average stratum corneum thickness, average stratum granulosum thickness and average vascular diameter were significantly different (p <0.05) but $CD3^+$ and $MHC2^+$ counts were not (p >0.05).

Table 3-2: Summary of rumen histological observations on 25 samples from animals on diverse diets. Continuous data results expressed as mean value for the group (± SD). Categorical data expressed as the count of each possible level. *nt = not tested because of expected small number of observations per category.

		HIGH FORAGE		HIGH CONCENTRATE				
Variable		CGFB (n=5) (grass-fed beef)	CSFD (n=5) (silage-fed dairy)	CMFB (n=5) (mixed diet beef)	CCFB (n=5) (cereal beef)	RCFB (n=5) (cereal beef)	*P* Value (diet)	*P* Value (forage/conc.)
Thickness of stratum corneum (µm)		13.45 ± 3.44	11.89 ± 2.20	23.06 ± 11.16	16.97 ± 5.13	25.57 ± 8.62	0.012	**0.002**
Thickness of stratum granulosum (µm)		63.73 ± 4.06	67.22 ± 8.48	71.27 ± 10.40	69.97 ± 10.10	85.82 ± 18.47	0.075	0.050
Diameter of largest blood vessel (µm)		21.37 ± 6.65	18.52 ± 4.25	24.84 ± 24.51	10.54 ± 2.82	12.12 ± 2.95	0.046	**0.025**
Count of CD3+ cells		33.40 ± 15.58	26.80 ± 14.17	20.80 ± 8.35	22.00 ± 3.54	35.40 ± 28.52	0.725	0.415
Count of MHCII+ cells		66.40 ± 18.80	55.00 ± 5.79	43.20 ± 33.36	58.20 ± 21.46	39.60 ± 14.52	0.271	0.121
Clefting of papillae	CLEFT 1	0	1	0	3	3	nt*	nt
	CLEFT 2	0	2	2	2	2		
	CLEFT 3	5	2	3	0	0		
Cytoplasmic swelling	SWELL 2	4	3	1	0	0	nt	nt
	SWELL 3	1	2	1	0	0		
	SWELL 4	0	0	1	1	0		
	SWELL 5	0	0	1	2	3		
	SWELL 6	0	0	1	2	2		
Perinuclear vacuolation	VAC 0	3	0	0	1	0	nt	nt
	VAC 1	1	2	2	0	0		
	VAC 2	1	3	3	4	5		
Loss of integrity of stratum corneum	SCINT 1	5	2	0	0	0	nt	nt
	SCINT 2	0	2	2	0	0		
	SCINT 3	0	0	1	3	1		
	SCINT 4	0	1	0	1	1		
	SCINT 5	0	0	2	1	3		
Sloughing of stratum corneum	SLOUGH 1	3	4	3	4	0	nt	nt
	SLOUGH 2	2	1	2	1	3		
	SLOUGH 3	0	0	0	0	2		
Microabscess in epithelium	MICRO 0	5	2	3	5	4	nt	nt
	MICRO 1	0	3	2	0	1		

3.4.3.2 **Categorical scoring variables**

Scores from categorical variables are shown below as composite bar charts in figures 3-8 and 3-9, where the lowest score is represented by the darkest shade of grey. As there was a small number of animals which corresponded to each level for each variable, these data sets could not be analysed statistically. However, it appears that clefting scores were highest in grass fed animals (group A), whereas cytoplasmic swelling, perinuclear vacuolation and loss of stratum corneum integrity were all observed in high concentrate diets (groups C-E).

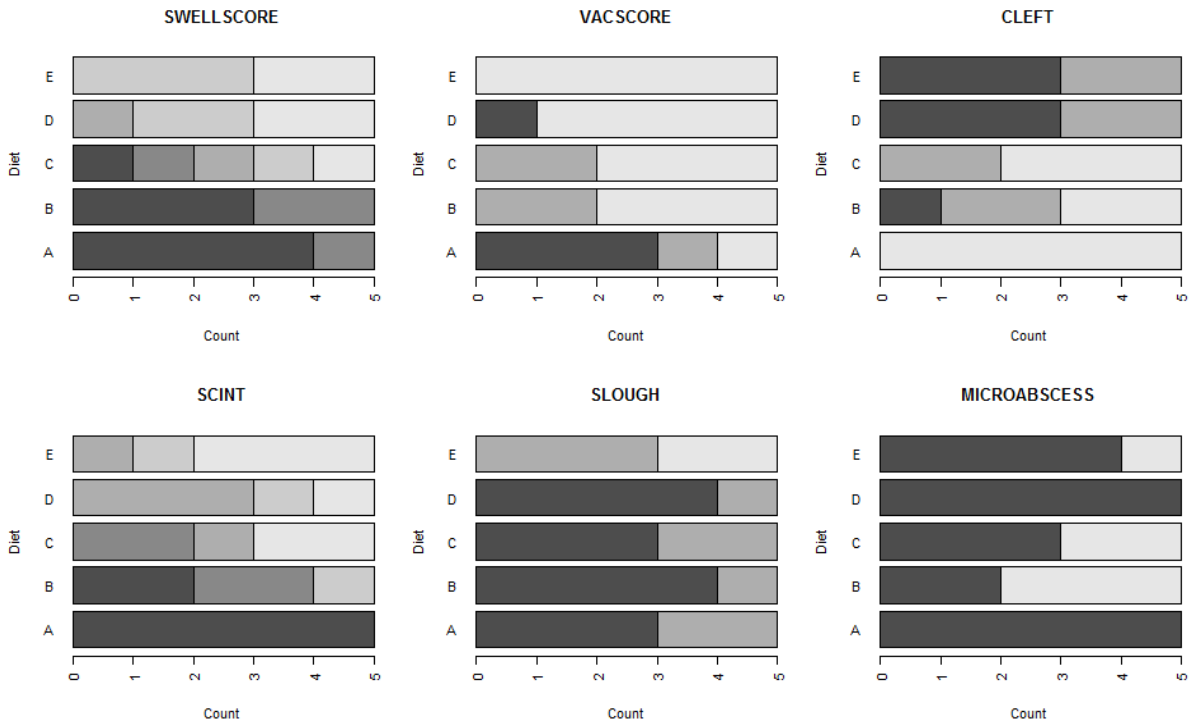

Figure 3-8: Composite bar charts of categorical parameters for each diet, where the lowest score is represented by the darkest shade of grey. Due to the small number of counts for each score, these variables were not statistically analysed. However, it appears that cleft scores were highest in grass fed animals (Diet A) and cytoplasmic swelling (SWELLSCORE), whereas perinuclear vacuolation (VACSCORE) and stratum corneum integrity (SCINT) were all highest in animals fed high concentrate diets (Diets C-E).

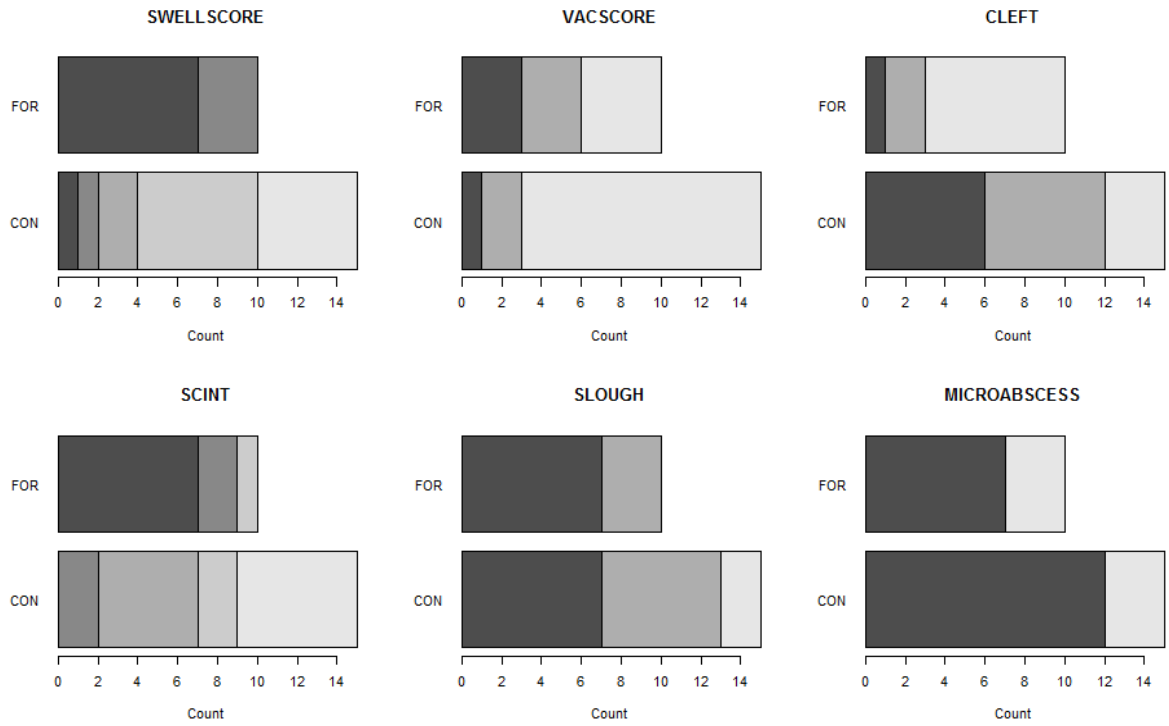

Figure 3-9: Composite bar charts for categorical variables for each group (FORAGE and CONCENTRATE), where the lowest score is represented by the darkest shade of grey. Due to the small number of counts for each score, these variables were not statistically analysed. These graphs suggest that clefting was highest in forage fed animals and cytoplasmic swelling (SWELLSCORE), perinuclear vacuolation (VACSCORE) and stratum corneum integrity (SCINT) were all highest in animals fed high concentrate diets.

3.4.3.3 **Principle components analysis (PCA)**

Horn's parallel analysis (PA) was carried out to investigate which factors should be retained in the histological scoring model for future testing. Table 3-3 lists the Eigenvalues obtained from the PA (where Eigenvalues explain variance among parameters).

The PA suggested that only 2 factors should be retained. The results of the factor analysis, selecting 2 factors, are shown in figure 3-10 and the loadings of each of the variables for the 2 retained factors are listed in Table 3-4. The 2 factors explained 43% of the variance in results.

Table 3-3: Results of Horn's Parallel Analysis for factor retention with 5000 iterations, using the mean estimate (RStudio using Paran package).

Factor	Adjusted Eigenvalue	Unadjusted Eigenvalue	Estimated Bias
1	1.91	3.65	1.73
2	0.13	1.42	1.29
3	-0.07	0.88	0.96
4	-0.25	0.43	0.69
5	-0.10	0.36	0.45
6	-0.01	0.24	0.25
7	0.13	0.21	0.08
8	0.02	-0.04	-0.06
9	0.14	-0.04	-0.18
10	0.11	-0.16	-0.27
11	0.09	-0.24	-0.34

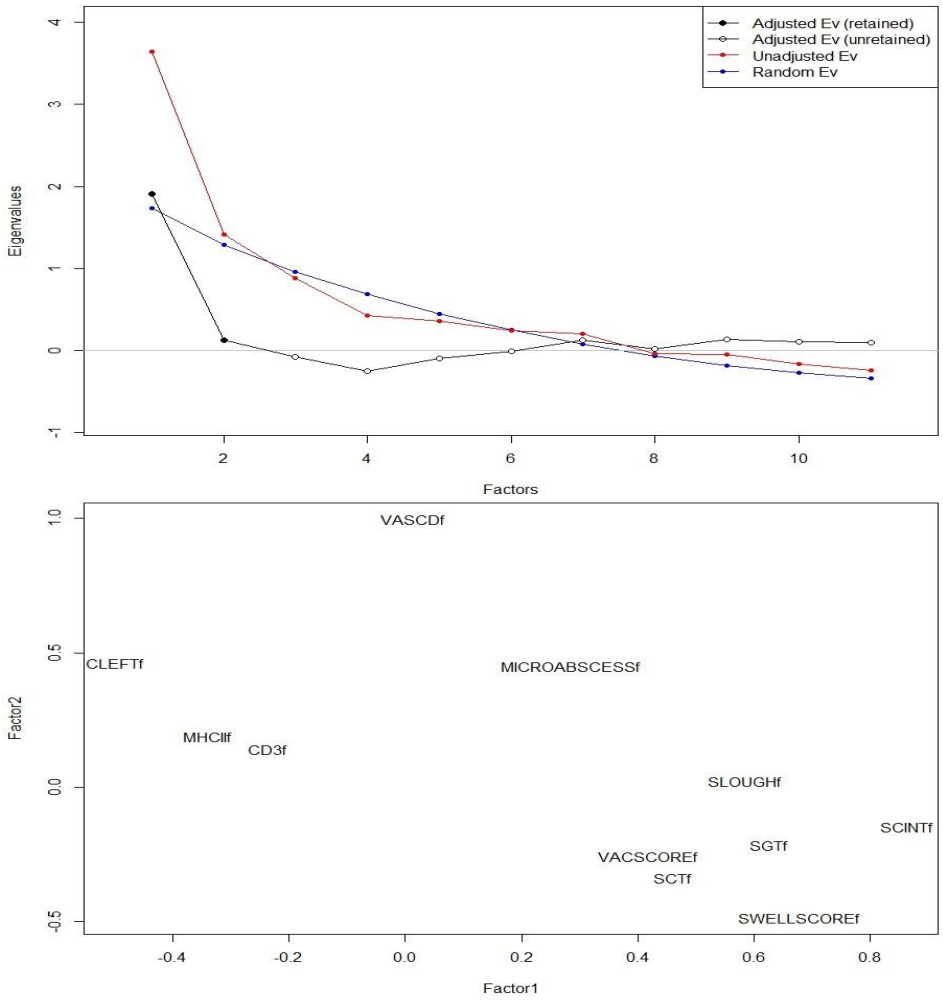

Figure 3-10: Results from Horn's PA and PCA. Horns PA shows only 2 factors should be retained, as shown by the two filled black dots above the 0 line on the adjusted eigenvalues (retained) line.

Table 3-4: Table showing the loading estimates and uniqueness for each of the variables in the two factor model; the test of the hypothesis is that 2 factors are sufficient (chi squared = 36.25, 34 df, p >0.05)

Variable	Factor 1	Factor 2	Uniqueness
SGT	0.63		0.56
SWELLSCORE	0.68	-0.49	0.3
SCINT	0.86		0.23
SLOUGH	0.58		0.66
VASCD		1	0.00
SCT	0.46	-0.34	0.68
VACSCORE	0.42		0.76
CLEFT	-0.5	0.46	0.54
CD3			0.92
MHC2	-0.34		0.85
MICROABSCESS		0.45	0.72
SS Loadings	2.82	1.95	
Proportion of Variation	0.26	0.18	
Cumulative Variation	0.26	0.43	

3.4.3.4 **Scoring system**

Variables tested with the PA were included in the initial scoring model if they had a loading value ≥ 0.3. They were included in the model as detailed in table 3-4 (e.g. loadings on Factor 1 were SGT = 0.63, CLEFT = -0.50, so SGT was assigned a positive value and CLEFT, a negative value). Three other versions of the model were created by excluding variables with lower loadings or low uniqueness values. The scores are shown in table 3-5, together with the results of one-way ANOVA for each score against the factors diet and group. Boxplots of the scores by diet and by group are shown in figure 3-11.

Table 3-5: Scores for histological change: variables and association of each score with diet (CGFB, CSFD, CMFB, CCFB, RCFB and group (FORAGE/CONCENTRATE), based on one-way ANOVA.

	Variables		Diet	Diet	Group	Group
	Positive	Negative	F value	p value	F value	p value
Score 1	SCT, SGT, SCINT, VACSCORE, SLOUGH, SWELLSCORE	CLEFT, VASCD, MICROABSCESS, MHC2	9.75	< 0.001	20.13	< 0.001
Score 2	SCT, SGT, SCINT, VACSCORE, SLOUGH, SWELLSCORE	CLEFT, MICROABSCESS	9.57	< 0.001	17.66	< 0.001
Score 3	SCT, SGT, SCINT, VACSCORE, SWELLSCORE	CLEFT	23.28	< 0.0001	38.2	< 0.0001
Score 4	SCT, SGT, SWELLSCORE		20.83	< 0.0001	60.98	< 0.0001

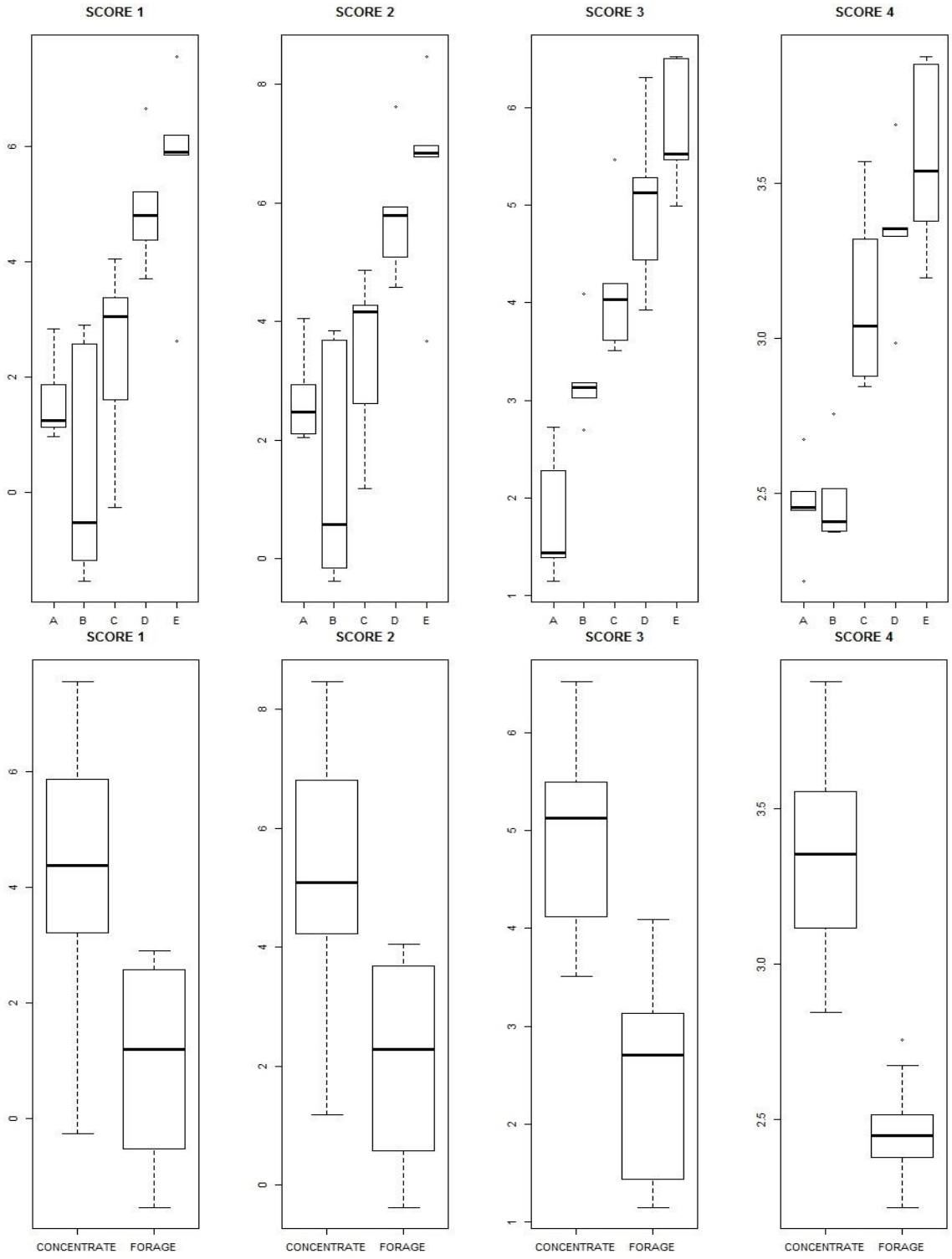

Figure 3-11: Box and whisker plots showing results from each of the 4 scores created from the histological variables for diet (A: CGFB, B: CSFD, C: CMFB, D: CCFB and E: RCFB) and group (Concentrate and Forage). Score 1 = (+) SCT, SGT, SCINT, VACSCORE, SLOUGH, SWELLSCORE and (-) CLEFT, VASCD, MICROABSCESS, MHC2. Score 2 = (+) SCT, SGT, SCINT, VACSCORE, SLOUGH, SWELLSCORE and (-) CLEFT, MICROABSCESS. Score 3 = (+) SCT, SGT, SCINT, VACSCORE, SWELLSCORE and (-) CLEFT. Score 4 = (+) SCT, SGT, SWELLSCORE.

3.4.4 **Farm characteristics**

Table 3-6 is a list of the dietary inputs on each of the 6 beef farms (high (H) or low-risk (L)). A small number of farms were involved in the study and each farm had a wide range of dietary inputs. Due to this small sample size and large range, there was inconsistency between farms in terms of the dietary components. The fact that the vast majority of dietary components were not repeated across farms meant it was not possible to statistically test the majority of the parameters as an explanatory variable for each of the dependent variables. From the parameters that were replicated across farms, those chosen to be further investigated were farm of origin (FARM), initial risk classification (RISK), percentage of DM as barley (BARLEY) and percentage of TMR particles <1.2 mm (FINES).

The effect of farm of origin (FARM) was significant for the majority of parameters, except ruminal fluid (RF) butyrate, CLEFT, SCINT, SWELLSCORE and VACSCORE ($p > 0.05$). The effect of RISK classification was significant on a smaller number of parameters and was not significant for RF HISTAMINE, RF ISOBUTYRATE, RF ACETATE: PROPIONATE, caecal fluid (CF) ACETATE, CF PROPIONAGTE, CF BUTYRATE, CF TOTAL SCFA, CCL11, NHE3, IL2, VASCD, CLEFT, SCINT, SWELLSCORE and VACSCORE. The percentage of DM as BARLEY had a significant effect on the majority of parameters except CF BUTYRATE, CLEFT, SCINT, MICROABSCESS, SWELLSCORE and VACSCORE. The percentage of FINES had a significant effect on the majority of parameters except CF BUTYRATE, CLEFT, SCINT, SWELLSCORE and VACSCORE.

3.4.4.1 **Effects of breed, sex, age and weight**

The effects of breed, sex, age and weight at slaughter were all confounded by the effect of the farm of origin (FARM). As a result, the possible effects of breed, sex, age and weight were not investigated further.

3.4.4.2 **Histamine in reticuloruminal fluid**

RF HISTAMINE concentration was affected significantly by both BARLEY and FINES in the diet ($p < 0.05$) but not by risk category (RISK) ($p > 0.05$).

Table 3-6: List of the dietary components from each of the 6 beef farms. Table shows dietary composition, chemical analysis and particle size. (*values for farm BL7 were verbally confirmed by communication with farmer and could not be verified, thus they are considered estimates only and used only for the initial *a priori* classification of risk).

	Farm					
	BL2	BL3	BL7	BH1	BH6	BH7
Sex						
Male	11	1	10	0	18	20
Female	9	19	9	20	2	0
Age at slaughter ±SD (days)	646±149	689±50	671±65	703±19	702±68	788±85
Coldweight at slaughter ± (kg)	297±27	383±34	356±24	384±30	400±10	403±15
Breed						
Continental	16	19	12	20	20	18
British	4	1	7	0	0	2
Diet (%DM)						
Barley	48.8	43.5	50*	57.3	67.9	70
Straw	18.3	6.5	20*	10.7		
Grass Silage	24.4	50	25*			
Pot ale syrup	8.5		5*	31.5	10.7	15
Dark grains					10.7	5
Sugar beet pulp					10.7	0
Soya hulls						10
Additives						
Minerals	Y	Y	Y	Y	Y	Y
Rumitech	N	N	N	N	Y	Y
Yeast	N	Y	Y	N	Y	Y
Buffer	N	Y	N	N	N	N
TMR Composition (%DM)						
Crude protein	6.62	11.98	13.28	11.06	9.94	12.5
Crude fibre	8.23	5.43	11.48	13.23	5.35	4.84
Starch	24.73	33.26	n/a	37.13	36.93	39.63
NDF	25.85	21.4	n/a	13.81	16.75	19.51
Milled Barley Composition (%DM)						
Crude protein	10.24	10.41	n/a	11.92	11.06	10.51
Crude fibre	5.51	3.63	n/a	4.83	5.15	3.5
Starch	56.86	57.44	n/a	56.26	55.62	46.98
NDF	25.95	15.62	n/a	19.56	21.83	14.13
TMR Particle Size (mm)						
>19	75.6	30.7	55.1	23.6	0.1	0
≤ 19 to ≥ 8	7.1	4.2	12.6	7.5	8.2	3
< 8 to ≥ 1.2	16.5	46.9	31.6	65.1	69.5	74.2
< 1.2	0.4	17.3	0.2	3.5	21.3	22.5
Milled Barley Particle Size (mm)						
>19	0.3	0	n/a	n/a	1.3	2.2
≤ 19 to ≥ 8	1.8	0	n/a	n/a	37.7	42.3
< 8 to ≥ 1.2	95.7	92.1	n/a	n/a	58	60.6
< 1.2	2.7	7.6	n/a	n/a	7.6	1.5

3.4.4.3 **Lipopolysaccharide (LPS) in reticuloruminal and caecal fluid**

Ruminal LPS concentration was significantly affected by RISK category (p <0.01), with LPS concentrations being higher on high-risk farms. Neither the proportion of BARLEY or FINES in the diet significantly affected ruminal LPS concentration (p >0.05). Both RISK and FARM significantly affected caecal LPS concentration, with concentrations lower in the high-risk farms (p <0.05) and BARLEY and FINES in the diet significantly affected caecal LPS concentration (p <0.05).

3.4.4.4 **Reticuloruminal and Caecal SCFA**

Total RF SCFA proportions were significantly lower in high-risk farms and were significantly different between farms (p <0.05). Total RF SCFA decreased significantly as BARLEY and FINES in the diet increased (p <0.05). Total CF SCFA proportions were only significantly different between farms (p <0.05). Table 3-7 shows the mean and SD of reticuloruminal fluid and caecal fluid concentrations of selected variables by FARM.

Of the 3 main SCFA produced (acetate, butyrate and propionate), RF concentrations were consistently affected by a larger number of factors than CF concentrations. RF acetate, butyrate and propionate concentrations were significantly lower in high-risk farms (p <0.005). As the proportion of BARLEY and FINES increased in the diet, the concentrations of RF acetate, butyrate and propionate decreased significantly (p <0.005). CF acetate was only significantly different between farms (p <0.05), CF butyrate was not significantly affected by any factor (p >0.05) and CF propionate was significantly affected by farm and barley in the diet, decreasing with increasing barley concentrations (p <0.005).

RF isobutyrate was significantly different between farms and was significantly affected by FINES, although there was no clear pattern of response with a range of high and low concentrations noted on different farms (p <0.005). CF isobutyrate was significantly higher in high-risk farms, significantly different between farms and increased as BARLEY increased (p <0.05). RF isovalerate was significantly higher in high-risk farms, was significantly different between farms, increased significantly as BARLEY increased and was significantly affected by FINES in the diet (p <0.05). CF isovalerate was significantly higher in high-risk farms, was significantly different between farms and increased significantly as BARLEY increased

(p <0.05). RF Valerate was significantly lower on high-risk farms, was significantly different between farms, and was significantly lower with higher BARLEY and FINES in the diet (p <0.001). CF valerate was significantly different between farms and was significantly affected by BARLEY, increasing as barley increased (p < 0.05).

Table 3-7: Mean and SD of reticuloruminal fluid and caecal fluid concentrations of selected variables by FARM. The stated p value is derived from Kruskal-Wallis test on untransformed data. RF = reticuloruminal fluid; CF = caecal fluid. P-values are written as stated unless they fell below 0.0001.

Variable	Farm						
	BH1	BH6	BH7	BL2	BL3	BL7	*p* value
RF acetate (mmol/l)	62.29 (13.46)	40.97 (6.93)	36.93 (17.21)	66.02 (15.96)	54.16 (22.19)	85.33 (13.93)	<0.0001
RF butyrate (mmol/l)	12.68 (7.89)	4.86 (1.40)	4.56 (1.94)	9.79 (3.78)	9.06 (5.55)	16.73 (5.90)	<0.0001
RF propionate (mmol/l)	25.57 (8.56)	14.28 (2.72)	10.90 (6.88)	23.51 (15.44)	29.00 (14.03)	22.68 (4.90)	<0.0001
RF lactate (mmol/l)	3.37 (1.38)	2.31 (1.16)	2.80 (1.19)	1.26 (0.98)	2.02 (1.07)	0.00 (0.00)	<0.0001
RF Total SCFA (mmol/l)	108.17 (25.79)	65.54 (10.12)	57.53 (26.98)	105.71 (26.39)	97.19 (36.09)	128.04 (22.53)	<0.0001
RF Acetate: Propionate	2.59 (0.65)	2.90 (0.32)	3.69 (0.73)	3.30 (1.16)	2.17 (1.03)	3.86 (0.70)	<0.0001
RF LPS (U/l)	68,493 (61,387)	136,221 (66,806)	55,284 (35,212)	111,704 (102,111)	72,077 (85,301)	24,991 (35,043)	<0.0001
RF Histamine (U/l)	7,553 (5,319)	5,432 (4,846)	1,038 (1,858)	2,540 (2,032)	1,734 (2,654)	2,863 (1,113)	<0.0001
CF acetate (mmol/l)	45.39 (9.76)	43.31 (11.74)	55.60 (12.00)	50.50 (14.61)	47.13 (10.74)	54.09 (10.58)	0.014
CF butyrate (mmol/l)	5.35 (4.51)	3.91 (1.12)	3.69 (1.04)	3.54 (0.78)	3.45 (1.35)	4.62 (1.28)	0.10
CF propionate (mmol/l)	12.51 (2.63)	14.15 (3.43)	16.08 (3.23)	13.94 (4.34)	10.67 (2.44)	14.40 (2.43)	<0.0001
CF lactate (mmol/l)	1.31 (0.70)	1.71 (1.16)	(1.31 (0.66)	1.37 (0.75)	0.53 (0.70)	0.57 (0.25)	<0.0001
CF LPS (U/l)	624,286 (531,296)	125,419 (133,316)	871,261 (424,914)	534,684 (455,379)	1,975,886 (1,126,242)	588,463 (380,156)	<0.000-01

RF Lactate was significantly higher in high-risk farms and was significantly different between farms (p <0.05). RF lactate was significantly affected by both BARLEY and FINES in the diet and increased as barley and fines increased (p <0.05). CF lactate was significantly higher on high-risk farms, was significantly different between farms and was significantly affected by BARLEY, with similar values noted across all barley groups except the group fed the lowest amount of barley, which had lower CF lactate (p <0.05).

3.4.4.5 **Gene expression**

Table 3-8 shows the median for the relative expression of each gene overall for all high and low-risk farms and for each individual farm. All genes were significantly affected by FARM (p <0.05). RISK factor significantly affected IL-1β, TLR4 and IFN-γ (p <0.05). Effect of BARLEY and FINES was significant only for IFN-γ and NHE3, with increased BARLEY and FINES increasing expression (p <0.05).

Table 3-8: Median relative expression of each of the studied genes for all high-risk farms and all low-risk farms and for each farm considered alone.

	TLR4RE	IL1BRE	CCL11RE	NHE3RE	IL2RE	IFNGRE
High-risk Median	0.06	0.002	0.04	0.38	0.0002	0.0008
Low-risk Median	0.05	0.002	0.05	0.27	0.0006	0.0012
BH1 Median	0.09	0.002	0.02	0.41	0.0002	0.0005
BH6 Median	0.05	0.002	0.02	0.48	0.0002	0.0006
BH7 Median	0.05	0.002	0.06	0.34	0.0002	0.0012
BL2 Median	0.06	0.0009	0.07	0.27	0.0005	0.0006
BL3 Median	0.07	0.002	0.08	0.32	0.0003	0.0009
BL7 Median	0.03	0.005	0.04	0.27	0.0007	0.0018

3.4.5 **Full sample set examination (n=195)**

3.4.5.1 **Continuous variables**

The full sample set (n=213) was reduced to 195 animals by removing animals that did not have a full set of results for histological score or that had many missing values for other measured variables. Animals retained had full results for all variables, including ruminal histamine, ruminal LPS, ruminal SCFA, ruminal epithelium gene expression and histology. Table 3-9 shows a summary of the histological observations for the 195 animals.

Scores for continuous variables were compared between diets (CGFB, CSFD, CMFB, CCFB and RCFB (A-E)) and between groups (CONCENTRATE and FORAGE). Between diets, SCT, SGT and CD3+ were significantly different (p <0.001) but VASCD and MHC2+ count were not. Between groups (CONCENTRATE and FORAGE), SCT, SGT and CD3+ again were significantly different (p <0.001) but VASCD and $MHC2^+$ counts were not. SCT and SGT were higher in concentrate fed animals (p <0.05) but the number of CD3+ cells and MHC2+ cells were both lower in concentrate fed animals.

Table 3-9: Summary of rumen histological observations on 195 samples from animals on diverse diets. Continuous data results expressed as mean value for the group (± SD). Categorical data expressed as a proportion (%) of the total counts. P-values are written as stated unless <0.0001.

		Percentage of Concentrate in Ration					
Variable (± SD)		0% (n=18)	40% (n=15)	60% (n=47)	90% (n=76)	100% (n=39)	*P* value (conc%)
Thickness of stratum corneum (µm)		3.72 ± 0.88	7.20 ± 2.27	5.81 ± 2.68	6.30 ± 2.33	14.35 ± 7.47	<0.0001
Thickness of stratum granulosum (µm)		25.74 ± 7.69	34.93 ± 6.96	34.52 ± 7.90	32.30 ± 7.16	58.09 ± 24.11	<0.0001
Diameter of largest blood vessel (µm)		23.07 ± 6.28	20.28 ± 6.30	22.14 ± 7.55	22.08 ± 8.45	37.33 ± 25.65	0.00062
Count of CD3+ cells		61.44 ± 16.61	41.07 ± 10.19	51.02 ± 18.36	39.32 ± 15.19	35.08 ± 15.96	<0.0001
Count of MHCII+ cells		70.33 ± 16.31	39.80 ± 22.72	60.96 ± 25.55	48.70 ± 20.30	39.74 ± 14.54	<0.0001
Clefting of papillae	CLEFT 1	0.5	3.1	6.66	9.74	6.15	0.31
	CLEFT 2	2.05	2.05	7.18	13.33	5.12	
	CLEFT 3	6.66	2.56	10.26	15.9	8.72	
Epithelial cytoplasmic swelling	SWELL 2	1.03	0	1.03	0	0.51	0.046
	SWELL 3	2.05	1.03	2.05	3.08	3.59	
	SWELL 4	3.59	5.64	12.3	13.85	9.23	
	SWELL 5	2.05	1.03	7.69	13.85	3.08	
	SWELL 6	0.5	0	1.03	8.21	3.59	
Perinuclear vacuolation	VAC 0	2.05	1.03	3.08	3.62	2.05	0.79
	VAC 1	3.62	2.56	10.26	13.85	7.69	
	VAC 2	2.57	4.1	10.77	20.51	10.26	
Loss of integrity of stratum corneum	SCINT 1	2.56	0.5	4.1	2.56	1.03	<0.0001
	SCINT 2	2.56	1.03	4.1	4.1	2.05	
	SCINT 3	0.5	3.59	5.13	9.74	7.69	
	SCINT 4	3.08	0.5	8.72	5.13	2.05	
	SCINT 5	0.5	2.05	2.05	17.44	7.18	
Sloughing of stratum corneum	SLOUGH 1	2.05	4.62	16.4	11.79	5.13	<0.0001
	SLOUGH 2	3.08	2.05	6.15	20	9.74	
	SLOUGH 3	4.1	1.03	1.54	7.18	5.13	
Microabscess in epithelium	MICRO 0	5.64	4.1	9.74	18.97	14,87	0.25
	MICRO 1	3.59	3.59	14.36	20	5.13	

3.4.5.2 **Categorical variables**

Scores from categorical variables are shown below in composite bar charts in figure 3-12 and 3-13, where the lowest score is represented by the darkest shade of grey. As is clearly shown in the bar charts, as a result of the availability of samples from each management group, there was a much lower number of animals in the forage groups (A and B, n = 27) in comparison to the concentrate fed groups (C-E, n = 167). Diet group was not significant for cytoplasmic swelling (SWELLSCORE), papillary clefting (CLEFT) or presence or absence of microabscesses (p >0.05). Group A was significantly different from other diets for vacuolation (VACSCORE) and sloughing (p <0.05) and group B was significantly different for SC integrity (SCINT) (p <0.05).

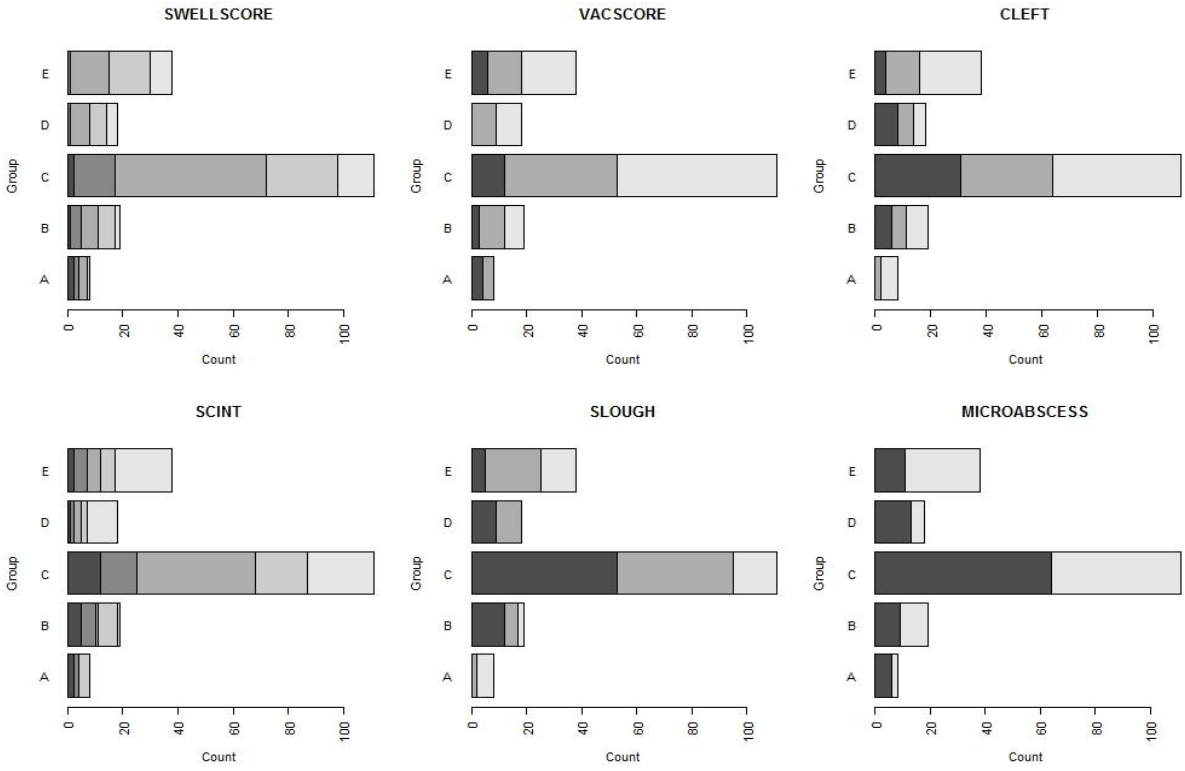

Figure 3-12: Composite bar charts for categorical variables for each diet A – CGFB, B – CSFD, C – CMFB, D – CCFB, E – RCFB for swelling (SWELLSCORE), perinuclear vacuolation (VACSCORE), papillary clefting (CLEFT), stratum corneum integrity (SCINT), retention of sloughed stratum corneum sheaths (SLOUGH) and the presence or absence of microabscesses. Only group A was significantly different from other diets for VACSCORE (p <0.05), group B significantly different for SCINT (p <0.05) and group A significantly different for SLOUGH (p <0.05).

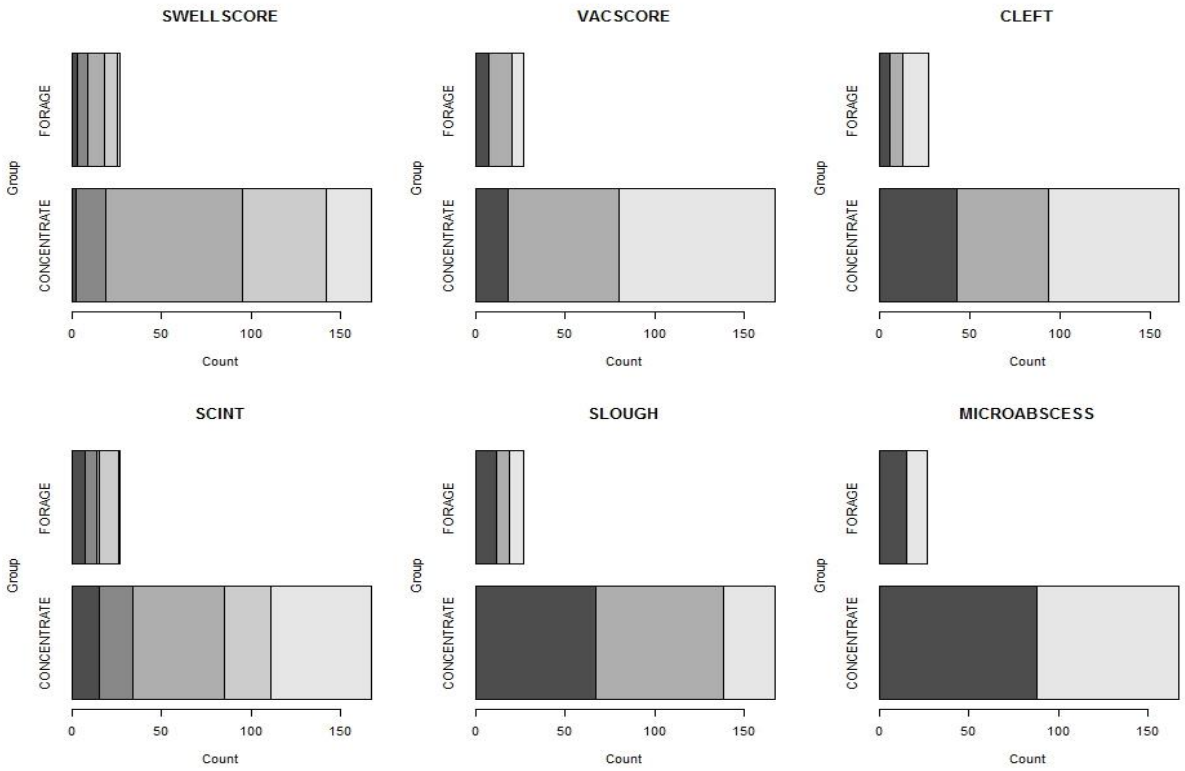

Figure 3-13: Figure 3-14: Composite bar charts for categorical variables for each Group (FORAGE: A – CGFB, B – CSFD and CONCENTRATE: C – CMFB, D – CCFB, E – RCFB) for swelling (SWELLSCORE), perinuclear vacuolation (VACSCORE), papillary clefting (CLEFT), stratum corneum integrity (SCINT), retention of sloughed stratum corneum sheaths (SLOUGH) and the presence or absence of microabscesses. Only group A was significantly different from other diets for VACSCORE (p <0.05), group B significantly different for SCINT (p <0.05) and group A significantly different for SLOUGH (p <0.05).

Using the 4 histological scores detailed in section 3.4.3.4, (Score 1 = (+) SCT, SGT, SCINT, VACSCORE, SLOUGH, SWELLSCORE and (-) CLEFT, VASCD, MICROABSCESS, MHC2, score 2 = (+) SCT, SGT, SCINT, VACSCORE, SLOUGH, SWELLSCORE and (-) CLEFT, MICROABSCESS, score 3 = (+) SCT, SGT, SCINT, VACSCORE, SWELLSCORE and (-) CLEFT and score 4 = (+) SCT, SGT, SWELLSCORE) diet groups were also compared by splitting by the level of concentrate in the diet (%DM basis). From the 4 histological scores, as shown in figure 3-15, score 4 separated the 5 diets the most clearly when considering the percentage of concentrate in the diet and had the most significant effect in comparison to the other 3 scores (p <0.001).

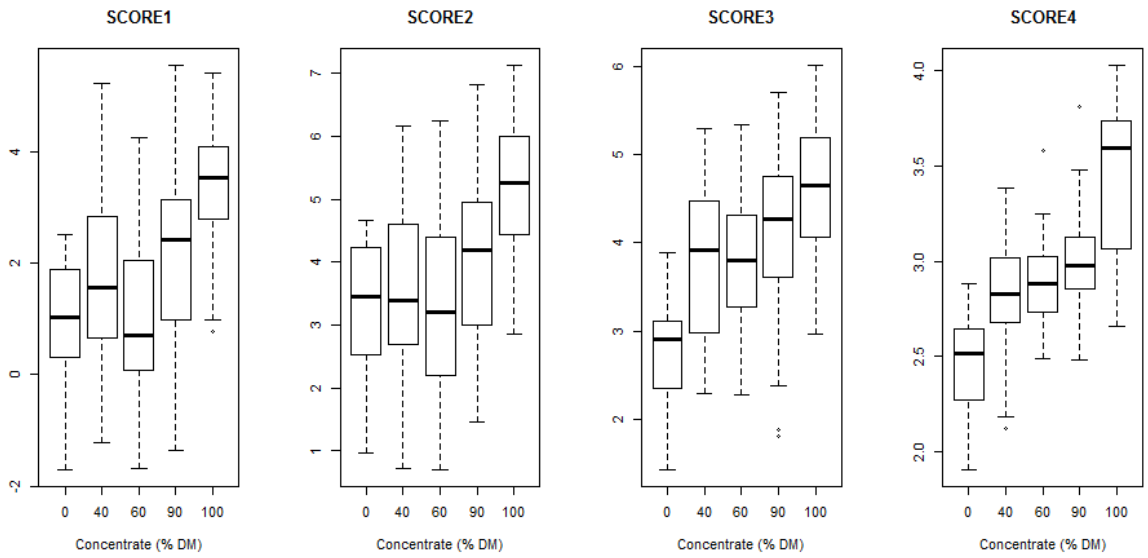

Figure 3-15: Histological score results for scores 1-4 (Score 1 = (+) SCT, SGT, SCINT, VACSCORE, SLOUGH, SWELLSCORE and (-) CLEFT, VASCD, MICROABSCESS, MHC2, score 2 = (+) SCT, SGT, SCINT, VACSCORE, SLOUGH, SWELLSCORE and (-) CLEFT, MICROABSCESS, score 3 = (+) SCT, SGT, SCINT, VACSCORE, SWELLSCORE and (-) CLEFT and score 4 = (+) SCT, SGT, SWELLSCORE) where diets are split by concentrate percentage (0-100%) where 0% concentrate = diet A, CGFB, 40% concentrate = diet B, CSFD, 60% concentrate = diet C, CMFB, 90% concentrate = diet D, CCFB and 100% concentrate = diet E, RCFB.

From the 4 different scores detailed in section 3.4.3.4, score 1 ((+) SCT, SGT, SCINT, VACSCORE, SLOUGH, SWELLSCORE, (-) CLEFT, VASCD, MICROABSCESS, MHC2) was used to analyse the data from the 6 beef farms. Results from this score were normally distributed and provided more information than the others. Score 1 differed between farms and was significantly higher in high-risk groups (p <0.05). BARLEY significantly affected histology score (p <0.05) but FINES did not (p >0.05). SCT and SGT were significantly different between farms and were skewed by high results for farm BH6. CD3+ cell count was significantly affected by RISK and FARM (p <0.05), but was not affected by BARLEY or FINES (p >0.05). MHC2+ cell count was significantly affected by RISK, FARM, BARLEY and FINES (p <0.05). Both CD3+ and MHC2+ counts were lower on high-risk farms. Figure 3-16 shows score 1 results for each of the 6 beef farms and the SG and SG thickness for each farm, showing clearly the high values for farm BH6.

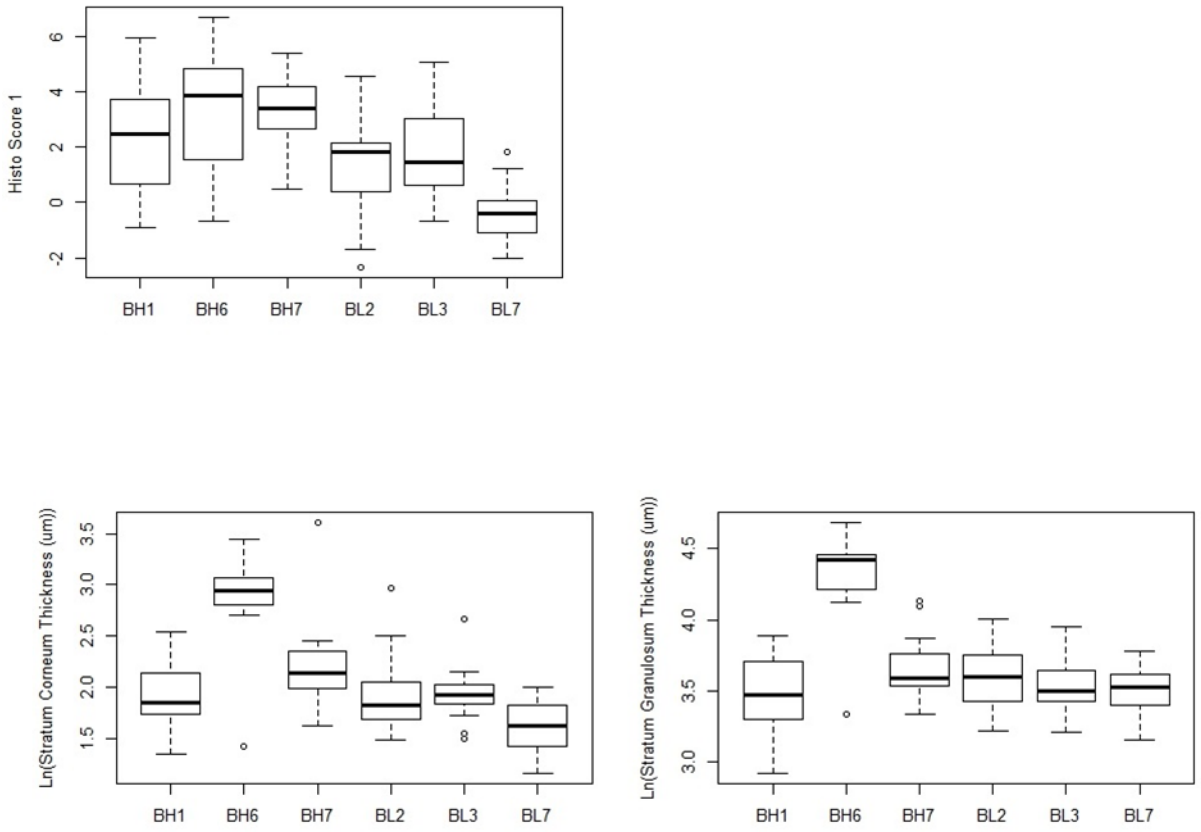

Figure 3-16: box and whisker plots showing score 1 results for each of the 6 beef farms and box and whisker plots showing high SCT and SGT values for farm BH6.

3.5 Discussion

3.5.1 Beef farm analysis

The factor that strongly affected almost every variable in the study was the farm of origin. Farm of origin significantly affected all but 1 of the dependent variables (caecal fluid butyrate). The extent of the variation among farms and in the inputs and dependent variables was not expected; farms were selected for the trial to provide broadly similar high and low-risk profiles. Every farm had a unique set of input variables and the small number of farms involved in the beef study, coupled with the wide range of differing dietary inputs meant that there was a lack of replication of the dietary components between farms. This lack of replication meant it was not possible to test statistically the effect of most of the factors as intended. Four parameters replicated on all farms were chosen for investigation: farm of origin (FARM), initial risk classification (RISK: based on barley, straw and silage in the diet and diet appearance) percentage of DM as barley (BARLEY) and percentage of fine TMR particles <1.2 mm (FINES). In addition to farm of origin, the proportion of barley and fine particles in the diet had a significant effect on the largest number of response variables in comparison to the effect of the initial risk classification.

The risk classification was a significant factor for 22 of 33 continuous dependent variables. Animals on high-risk farms showed increased concentrations of reticuloruminal LPS and lactate, thicker stratum corneum and stratum granulosum and higher relative expression of TLR4. Histological score 1 differed between farms and was significantly higher in high-risk groups, primarily as a result of this increased SC and SG, and was also significantly affected by the proportion of barley in diet. Additionally, animals on high-risk farms showed reduced reticuloruminal SCFA concentrations and reduced caecal LPS concentrations. The relative expression of IL1B and IFN-γ and the positive count of MHC2 and CD3 cells were lower in animals from high-risk farms.

This study investigated the effects of a diet expected to induce acidosis, with the majority of animals being maintained on a ration containing >40% concentrate to forage ratios. However, in comparison to the majority of acidosis trials in the literature that look at an acute effect of diet, animals in this trial had been maintained

on the diet for 90-100 days prior to sampling. This long exposure to a diet high in rapidly fermentable carbohydrates means that results could not be expected to reflect exactly those noted in acute response studies. As the proportion of barley in the ration increased, so did the concentration of reticuloruminal and caecal lactate, reticuloruminal histamine, LPS and the thickness of the stratum corneum and granulosum. Conversely, there was a reduction in the total SCFA concentration in the reticulorumen, the expression of IFN-γ and the count of MHCII+ cells in the ruminal epithelium. Increased LPS, histamine and lactate were expected and consistent with other studies (Emmanuel et al., 2008). Most studies investigating the acute effect of a diet high in rapidly fermentable carbohydrates report increases in reticuloruminal SCFA concentration (Loncke et al., 2009, Aschenbach et al., 2011) and previous histological studies have shown a decrease in the stratum corneum and granulosum with the diet (Steele et al., 2011a). This decrease in the main SCFA and total SCFA concentrations observed in this trial was also observed in the studies carried out in chapters 4 and 5 during expected "high-risk/challenge" feeding times. As animals in this present trial were maintained on the diet for a longer duration, this allowed time for adaptations in the rumen such as proliferation of cells associated with ion pumps and increased SCFA absorption, leading to increased surface area (O'Shea et al., 2016). This adaptation would explain the unexpected decrease in SCFA when barley and proportion of fines increased. The significant effect of barley and fines on NHE3 relative expression suggests epithelial adaptation as a mechanism responsible for decreased SCFA. Expression of NHE3 has been shown to increase in the ruminal epithelium as the papillae surface area increases through cell proliferation (O'Shea et al., 2016). The fact that the fine particles in the diet had a significant effect on a wide range of variables reflects the importance of dietary presentation, in addition to formulation, when considering the risk of acidosis in a herd.

3.5.2 **Histology**

The scoring system created in this chapter was shown to be capable of significantly differentiating animals based on the diet they had been maintained on prior to slaughter. As with the results from the beef study, there was a wide variation shown between animals and between diets, except at the most extreme ends of the spectrum – grass fed and research cereal-fed beef. Contrasting with reports from

acute response studies in the literature (Steele et al., 2009, Steele et al., 2011a, Steele et al., 2012), the most consistent result from the scoring system was an increase in stratum corneum and stratum granulosum thickness and cytoplasmic swelling as the level of rapidly fermentable carbohydrates in the diet increased. This increase has been shown in few studies in the literature in response to a high grain diets (Nocek et al., 1984, Metzler-Zebeli et al., 2013). As with the SCFA results that conflict with the current literature regarding acidosis, it is thought the variation in the histological results occurred due to the length of time the animals were exposed to the diet and the capacity for adaptation this allowed prior to sampling. Despite these contrasting observations, initial histological investigations in this chapter were consistent with previous light microscopic descriptions of the tissue structure and architecture in the literature and showed the characteristic 4 layers followed by connective tissue at the deepest level in the papillae (Dobson et al., 1956, Graham and Simmons, 2004, Eurell and Frappier, 2013). During the initial examination, it was evident that the modified MSB stain was extremely useful for visualising rumen histology, despite there being no previous mention of this use for the stain in the literature that we could find. With the exception of highlighting or confirming the presence of microabscesses (focal aggregations of neutrophils in the SC, which were easily identified via EMSB stain), myeloperoxidase staining of the rumen was not informative and very few myeloperoxidase-positive cells were noted outside microabscesses. It was not possible to distinguish with the stains used between vasculature and lymphatics, so all were considered as one – “vasculature”. The increase in vasculature diameter or increase in the amount of vasculature noted was significantly different for both diet and group, and fitted with the increase in vasculature noted by Steele et al (2011a) in animals fed a high grain diet. An increase in blood supply to the rumen has previously been associated with increasing SCFA absorption when feeding a high concentrate diet (Dobson, 1984), fitting with these results and reflecting the decreased SCFA noted in high-risk farms. Commercial grass-fed beef was significantly different from the others for perinuclear vacuolation and sloughing. This was unexpected, because as shown by Steele et al. (2011a), sloughing of the SC is generally associated with higher concentrate diets. Clefting was associated with the grass fed group in the preliminary sample examination, then sloughing, vacuolation and higher CD3 and MHC2 positive cell counts were associated with the grass fed group in the larger group. These are all parameters expected to be associated with higher grain diets. It is possible that

there is another factor influencing the animals in this group, as the diet fed does not appear to fit with the histological results. Group B (commercial silage fed dairy) was significantly different for SC integrity, again another parameter that would generally be associated with sloughing due to a higher carbohydrate diet

With the exception of microabscesses and rumen fluke, no pathological changes were observed in the rumen of any animals. The only change that was considered to be of a pathological nature prior to investigation were the black patches noted post-blanching. However, following investigation there were no obvious morphological or structural changes between the black and white areas of the rumen histologically, other than the retention of 3 outermost layers of the epithelium in the black patches post-blanching, and occasional condensing of pigmentation. The black areas did not have any associated pathologies and additionally, were not considered detrimental to rumen sale by abattoir staff - rumens were sold with black patches intact or simply trimmed. Therefore, post-blanching effects were not investigated further.

The main and unexpected result of this study was the overwhelming effect of farm of origin on all variables studied. Farms that had a similar *a priori* risk classification based on the major recognised criteria nonetheless showed very diverse patterns of response in commonly studied variables. The results suggest that as farm of origin has such a strong effect on results, care is needed when assuming that farms which appear similar superficially would have a similar risk of animals in the herds developing acidosis. With finishing cattle, groups are often formed from animals which originated from many diverse farms and therefore from different neonatal and genetic backgrounds. This variation in the genetic backgrounds and therefore epigenetic and microbiome variation could explain some of this strong effect of farm. Thus, considering the components of a diet and the particle size is not enough when considering the risk of acidosis.

The scoring system created for this thesis was proven in this chapter to be successful at significantly differentiating between animals based on their specific diet groups (such as grass fed beef, barley beef or commercial mixed fed beef) or based on less specific groupings of forage or concentrate diets. This scoring system could be useful to investigate further the histological appearance of the rumen and

the effects of varying diets. As the need for more efficient, precision farming increases in the future, the scoring system could be useful for investigating effects of diet that may not be noticeable clinically or for investigating relationships between score and observable differences. A future study using the scoring system on larger subsets of animals from one farm fed differing diets, to investigate the histological score where farm was not a confounding factor or on a large group of finishing cattle on one farm where the genetic background was known would provide further understanding of the effects of farm and genetic background. If the scoring system could pick up differing results in score from different diets from one farm or differing results on the same diet with the knowledge of different genetic backgrounds, scores could possibly be used to help refine diet or be used to select animals for selective breeding. This refinement or selective breeding could be used to increase production outputs, whilst minimising negative changes to the rumen epithelium and the overall rumen environment associated with diet.

4 Effects of a challenge diet high in soluble carbohydrates on production and physiological parameters associated with SARA, with and without direct-fed microbials

4.1 Introduction

It has consistently been shown that some herds are more susceptible to sub-acute ruminal acidosis or SARA than others and that within herds, some animals are more prone to SARA than others (Garrett, 1996, Kleen et al., 2009, Morgante et al., 2007, Penner et al., 2009a). This variation in response creates a challenge for nutritional management, creating the need to balance providing a diet high in fermentable carbohydrates for energy provision with the risk of developing SARA in the herd (Penner and Beauchemin, 2010). Some animals in a herd may not adapt well to a diet high in soluble carbohydrates and the associated reticuloruminal pH decrease, resulting in a decrease in production parameters such as milk yield, milk fat or daily live weight gain (Enemark et al., 2002). In contrast, other animals may be able to maintain their pH or production levels, or both, in response to the same high carbohydrate diet. This natural variation between animals is not only an opportunity for genetic selection for those less likely to suffer from SARA, but for the early recognition of a problem. This variation means that some animals in a herd will show clinical symptoms well before the entire herd is affected.

This study aimed to describe the variation in commonly used acidosis-indicator traits in lactating dairy cattle, in response to a challenge diet high in soluble carbohydrates. The traits investigated in this study have previously been shown repeatedly in the literature to be associated with SARA in cattle and have been shown to vary among individuals. Traits studied include reticuloruminal pH, body condition score (BCS), mobility score, live weight, milk production parameters, blood biochemistry and haematology, reticuloruminal SCFA proportions, gene expression levels, acute phase protein concentrations, ruminal and plasma histamine concentrations and ruminal lipopolysaccharide (LPS) concentrations. For example, movement of SCFA from the rumen via absorption across the ruminal epithelium has previously been stated as a key process involved in the regulation of ruminal pH (Eurell and Frappier, 2013), preventing a build-up of SCFA in the rumen and the consequential pH drop.

It would be expected that increased levels of SCFA in the rumen would be noted with decreasing ruminal pH, and those animals with increased absorption or lower levels of SCFA would show fewer signs associated with acidosis. Ruminal acidosis and the associated leaking of reactive substances through the ruminal epithelium, such as LPS and histamine, have been shown to cause a localised or systemic inflammatory response (Plaizier et al., 2008, Plaizier et al., 2012). Thus, animals that are more affected by ruminal acidosis would be expected to have higher expression of inflammatory cytokine genes in response to translocation of these substances and the corresponding immune response due to ruminal inflammation. By investigating these parameters and associated mechanisms, it was hoped to further understand the effects of high starch supplementation on lactating dairy cattle and to determine the range of individual variation in response to a high starch challenge diet.

Probiotics and yeasts are commonly fed alongside a high starch diet and have been shown to have the ability to modulate and maintain the balance of gastrointestinal microbiota and ruminal pH and increase feed intake and milk production in dairy cows (Robinson and Garrett, 1999, Dann et al., 2000, Nocek et al., 2003). In this context, they are referred to as direct-fed microbial (DFM) agents. Supplementation of diets with yeasts has long been known to stabilise ruminal pH (Bach et al., 2007) through increasing utilisation of lactic acid (via lactate-utilising bacteria), increasing microbial growth and by directly competing with ruminal bacteria for rapidly fermentable carbohydrates. Frequently used DFM in cattle include various strains of yeasts (commonly *Saccharomyces cerevisiae*) and lactate producing bacteria (*Enterococcus* and *Lactobacillus* sp.). This study was intended to investigate the effects of both commonly used, commercially available DFM based on yeast and novel products developed (by consortium partners) via cultures obtained from ruminal and caecal fluid samples *post-mortem*. The novel DFM to be tested in this study included both yeast and bacterial cultures.

4.2 **Aims**

The aims of the work presented in this chapter were to:

1. Quantify the effects of high levels of starch supplementation on specific physiological parameters that characterise adaptation to soluble carbohydrate challenges in lactating Holstein-Friesian dairy cows
2. Determine the variation in individual variation in response to high starch supplementation
3. Determine the efficacy of new DFM for the amelioration of effects related to high starch supplementation in lactating dairy cows.
4. Contribute to the understanding of the pathogenesis of digestive disturbances in dairy cattle.

4.3 Materials and methods

This experiment was conducted during October and November 2015 at the University of Glasgow's Cochno Farm and Research Centre under Home Office Licence project number 60/4156 and Food Standards Agency (FSA) project number 80296.

4.3.1 Experimental design

Forty lactating Holstein-Friesian cattle were divided evenly into 5 treatment groups via a formal stratified randomisation method. Live weight (kg), milk yield (kg), body condition score and days in milk were equally represented in each group. There were no significant differences between groups in any parameters (p >0.05). Cattle were housed together in the same environment and exposed to the same management system throughout, so the unit of interest for the study was the individual cow. To facilitate treatment administration, individual treatment group was identified by colour coded tail tape. "Treatment" refers to specific DFM as follows:

Treatment 1: Negative control (No protective products used unless clinical signs of acidosis warranted appropriate veterinary treatment). Fed as 4 g of granulated sugar/day

Treatment 2: Positive control (BOVAMINE® Complete (Chr.Hansen, Denmark). 1 x 10^9 CFU of 3 *E. faecium* strains, 5 x10^{10} CFU of live *S.cerevisiae,* Torula dried yeast (TDY) and hydrolysed *Pichia jadinii,* supplying mannan-oligosaccharide (MOS), beta-glucans and yeast extract. Fed as 14 g BOVAMINE® Complete + 4 g granulated sugar/day

Treatment 3: Yeast (Vistacell live yeast (AB Vista, UK) + hydrolysed dead yeast) Fed as 4 g Vistacell + 10 g hydrolysed dead yeast + 4 g granulated sugar/day

Treatment 4: Bacteria (1 × 10^{10} CFU/day of *Pediococcus acidilactici* (Chr.Hansen, Denmark)). Fed as 4 g + 4 g granulated sugar/day

Treatment 5: Bacteria (1 × 10^{10} CFU/day of Biomate SF 20 *Enterococcus faecium* Sf 273 (Chr.Hansen, Denmark)). Fed as 4 g + 4 g granulated sugar/day

Treatments were top-dressed onto supplemental parlour concentrate during the afternoon milking on each day. Following milking, consumption of treatments was noted and recorded for individual cows as “yes”, “no” or “partial”, dependent on the amount left in individual troughs. Weight of leftover parlour feed was negligible throughout the trial and was not recorded.

Cattle were fed a basal ration for 3 weeks (D-1 to D-21), fed a basal ration plus group specific DFM for 3 weeks (D-22 to D-43) then challenged with a diet intended to induce SARA in addition to their group-specific DFM for 2 weeks (D-45 to D-58). All animals were fitted with motion-sensing collars (Afimilk Silent Herdsman, UK, data not presented) and indwelling pH monitoring boluses (smaXtec, Austria). Samples were collected at 3 time-points: commencement of study (TP-A), immediately after treatment + basal (TP-B) and immediately after the challenge diet (TP-C). At each of these 3 sample collections, rumen fluid and blood samples were taken. Samples were collected from an additional 3 animals at the end of the first 3-week period (also denoted TP-A), to replace animals removed from the trial for health reasons. Replacement animals were maintained on the same basal ration as experimental animals. Live weight, BCS and mobility scoring was carried out weekly by the same person.

4.3.2 **Rations**

Cows were given *ad libitum* access to a partial mixed ration (PMR), fed out in a single feed bunk and supplementary parlour concentrate (according to existing farm algorithms for production, lactation stage and BCS). Rations were formulated from only 4 ingredients: grass silage, straw, parlour concentrate blend and PMR blend. Representative samples were taken from each of these ingredients and the analysis is shown in table 4-1.

Table 4-1: Feed ingredient analysis (as fed % except where specified)

	Parlour Mix	Blend for TMR	Silage	Straw
ME (DM basis)	13.1	13.2	9.7	5.6
NDF	21.9	21.6	9.8	78.7
CP	16.9	16.2	3.2	3.6
CF	5.9	7.4	5.5	41.1
Moisture	13	12.5	81.2	10.9
Sucrose	6.03	3.33	0.1	0.88
Starch	23.3	30.5	0.2	0.2
Soluble CHO	9.55	5.73	0.11	1.8
Cellulose	7.8	8	5	37.1

Table 4-2 and 4-3 show the components of the partial mixed ration (PMR) and the daily dairy blend parlour allocation for each of the diets. The basal ration was fed to achieve daily milk production levels consistent with a 300-day lactation yield of 9,000-10,000 litres. Cows were fed at 4 different levels during the period on the basal diet: “low”, “medium”, “high” and “top”, dependent on milk yield and liveweight. The challenge ration consisted of an increase in the proportion of concentrate in the PMR, an increase in concentrate fed in the parlour and a reduction in silage and straw in the PMR. The objective of the challenge was to provide an increase in soluble carbohydrates to a level where one would expect the onset of subclinical to clinical signs of an induced ruminal fermentation disorder. Signs could include diarrhoea, reduced appetite, reduced milk production and reduced milk fat percentage. Cows were fed at 3 different levels during the challenge diet: “medium”, “high” and “top”, dependent on milk yield and live weight. The challenge ration was designed to fall within a range of values observed on UK dairy farms, result in a statistically significant reduction in rumen pH to levels previously associated with SARA, to significantly decrease milk yield/components in the negative control group or to significantly decrease both pH and milk parameters. It was intended that the diet would not be so extreme that it caused unacceptably severe welfare outcomes for cattle or excessive financial loss to the farm. It was also necessary to ensure that the challenge was approximately equivalent for the late lactation and the low yielding cows in the herd. Hence, there was unavoidable variation among individuals in the extent of the challenge.

Table 4-2: Partial mixed ration (PMR) composition: dietary components in the PMR for each period: BASAL (time-point A), CHALLENGE 1 and CHALLENGE 2 (time-point C).

Period	Grass Silage	Dairy Blend	Barley Straw
BASAL	42	10	0.5
CHALLENGE 1	38	12	0.5
CHALLENGE 2	35	13	0

Table 4-3: Daily dairy blend parlour concentrate allocations for individual cattle for each period; BASAL (time-point A), CHALLENGE 1 and CHALLENGE 2 (time-point C).

	Mean (kg)	SEM	Minimum	Median	Maximum
Basal	3	0.44	0	2.3	9.8
Challenge 1	5.1	0.45	2	4	10
Challenge 2	5.7	0.36	4	4	10
Difference (CH2-Basal)	2.5	0.19	0	3	6

Following the introduction of the initially programmed challenge diet (D-45) there was a transient, slight reduction in mean pH in a small number of cows with rumen boluses and overall milk yield increased. There was no obvious change in cow activity or comfort. Therefore, it was decided to increase the challenge ration a second time (D-51), giving 2 weeks prior to the end of the study on an extra high ration (CHALLENGE 2). Table 4-4 shows the predicted diets for a cow on the median level of parlour supplementation during the BASAL and CHALLENGE 2 periods. For a cow typical of the average production level for the herd, the Challenge 2 diet provided a 1kg (DM) increase in starch (14% of DM to 18% of DM) and a reduction in NDF from forage from 16% to 12%.

Table 4-4: Composition of the predicted diets for a cow on the median level of parlour supplementation during the BASAL and CHALLENGE 2 periods

Component	Basal DM (kg)	Basal (% of DM)	Challenge 2 DM (kg)	Challenge 2 (% of DM)
NDF	3.18	27	3.64	26
NDF from Forage	1.95	16	1.67	12
CP	1.51	13	2.03	14
CF	1.49	13	1.57	11
Sucrose	0.24	2	0.39	3
Starch	1.66	14	2.60	18
Soluble CHO	0.40	3	0.64	5
Cellulose	1.44	12	1.57	11
Total	11.87	100	14.12	100

4.3.3 Blood collection and laboratory analysis

Blood samples were collected by venepuncture of the coccygeal vein into EDTA, heparin and serum vacutainers (Becton Dickinson, BD Vacutainers, USA) and

stored in a passive cool box on ice blocks (<4˚C) until processing. Bloods were processed in the laboratory as detailed in section 2.5. Bloods were analysed for standard bovine biochemistry and haematology profiles by Veterinary Diagnostic Services (VDS) of the University Of Glasgow School Of Veterinary Medicine, using the Dimension Xpand Plus (Siemens) and ADVIA 20.120 Haematology System (Siemens) as detailed in sections 2.5.2 and 2.5.3 respectively.

4.3.4 **Plasma histamine**

Circulating plasma histamine concentration was determined using the Abnova Histamine ELISA Kit (Cat. No. KA2589) intended for determination of histamine in EDTA plasma, using a LabTech LT-4500 microplate reader, as detailed in section 2.8. Duplicate measurements were used for all standards, controls and samples. Quantification of unknown samples was calculated by creating standard curves of known standards and calculating from the regression.

4.3.5 **Acute phase proteins**

Serum, collected after centrifugation of coagulated blood samples, was assayed for haptoglobin (Hp) using the haemoglobin-binding assay method described by Eckersall et al. (1999). The concentration of serum albumin-A (SAA) was determined using a commercial ELISA kit (Tridelta Development plc, Dublin, Ireland) according to manufacturer's instructions.

4.3.6 **Semi-Quantitative PCR**

EDTA RNA was extracted from buffy coat stored in Qiazol Lysis Reagent (Qiagen, Ref. 79306) for *CCL11, IFN-γ, TLR-4, IL1-β* and *IL2* expression analysis by semi-quantitative, real-time PCR (qPCR), as detailed in section 2.6. In brief, RNA was extracted from Qiazol/buffy coat homogenates following an in-house standard phenol-chloroform method (In-house method, Koh-Tan, 2015). Extracted RNA was converted to cDNA using the TaqMan Universal Master Mix kit (Thermo Fisher, Paisley, UK). qPCR was carried out using TaqMan Gene Expression Master Mix assay kit (Applied Biosystems) with the extracted cDNA according to manufacturer's instructions. The relative quantification of each gene was calculated utilising the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) of relative quantification (Livak and

Schmittgen, 2001, Steibel et al., 2009) using *GAPDH* as the reference gene. Samples were compared between time-points, each animal at time-point A was its own calibrator.

4.3.7 **Rumen fluid collection**

Rumen fluid was collected using a SELEKT pump and SELEKT collector (Nimrod). The collector was connected to the pump, placed down the oesophagus into the rumen and pumped into a bucket. Ruminal fluid was pumped through for several pumps to reduce saliva contamination prior to collection. Fluid was collected into 2 x 15 ml tubes, 1 plain and 1 with 20% glycerol (cryoprotectant). Fluid was transported on ice blocks in passive cool box (<4˚C) and stored at -20˚C before processing for SCFA, LPS and histamine concentrations. Fluid with glycerol was stored at -20˚C for future microbiome work.

4.3.8 **SCFA and ethanol in reticuloruminal fluid**

SCFA analysis was carried out by Sciantec Analytical Ltd. using gas chromatography (GC) as detailed in section 2.10. Reticuloruminal fluid was tested for the presence of ethanol, propan-1-ol, acetic acid, propionic acid, isobutyric acid, propane-1,2-diol, butyric acid, isovaleric acid, valeric acid, hexanoic acid, heptanoic acid and lactic acid. Any concentration < 25 mg/kg fell below the detection level of the GC and could not be quantified. Molar concentrations of the SCFA were obtained by dividing the measured mass of each SCFA in mg/kg by its molecular weight: acetate - 60.05; butyrate - 88.11; propionate - 74.08; valerate - 102.13; isobutyrate - 88.11; isovalerate - 102.13 (g/mol).

4.3.9 **Histamine in reticuloruminal fluid**

Analysis of histamine concentration in reticuloruminal fluid was carried out using the Histamine ELISA Kit (Abnova, cat no. KA1888), designed for analysis of histamine in human faeces, as detailed in section 2.8. Duplicate measurements were used for all standards, controls and samples. Quantification of unknown samples was calculated by creating standard curves of known standards and calculating from the regression.

4.3.10 **LPS in reticuloruminal fluid**

Lipopolysaccharide (LPS) concentration in reticuloruminal fluid was determined using the EndoLISA endpoint fluorescence microplate kit (Hyglos), with a Varioskan Fluorescent microplate reader, as detailed in section 2.9. Serial dilutions were used to plot a standard curve of log (endotoxin units or EU/ml) against log (relative fluorescence units or RFU/ml), and the unknown concentrations calculated from the regression.

4.3.11 **Milk production and composition**

Milk production was measured by pre-existing in-house systems (ALPRO, DeLaval) and by National Milk Recording (NMR) at morning and afternoon milking. Milk weight (kg) was obtained by recording the values for total volume of milk collected at the morning and afternoon milking. For milk composition, individual cow samples were tested for butterfat percentage (%) protein percentage (%), lactose percentage (%) and somatic cell count (1000 cells/ml) by NMR.

4.3.12 **Body condition scoring (BCS), liveweight (LWT) and mobility scoring**

Weekly BCS, LWT change and mobility scores were recorded by one individual. Standard BCS scoring was used (scoring 1-5 in increments of 0.5) based on the Department for Environment, Food and Rural Affairs (DEFRA) guidelines. Cattle were split into 5 categories – poor, moderate, good, fat, grossly fat – dependent on the appearance and feel of their tail head, loin and overall body. Live weight was measured using Gallagher W210 scales with Gallagher load-bars, fitted to the crush. Mobility was scored using 4 point (0-3) scale, based on the Agriculture and Horticulture Development Board (AHDB) mobility scoring guidelines. Cattle were followed at their natural pace ensuring a minimum of 6-10 uninterrupted strides were observed from the rear and side and ensuring cattle were observed turning a corner. Abnormalities like overgrown claws, which could affect mobility, were noted. Scoring schemes are detailed tables 4-5 and 4-6.

Table 4-5: Guide to body condition scoring (BCS) as detailed in "Condition scoring of dairy cows" by the Department for Environment, Food and Rural Affairs (DEFRA).

Score	Condition	Description
1	Poor	Tail head – deep cavity with no fatty tissue under skin. Skin is fairly supple but coat condition often rough. Loin – spine prominent and horizontal processes sharp.
2	Moderate	Tail head – shallow cavity but pin bones prominent; some fat under skin. Skin supple. Loin – horizontal processes can be identified individually with ends rounded.
3	Good	Tail head – fat cover over whole area and skin smooth but pelvis can be felt. Loin – end of horizontal process can only be felt with pressure; only slight depression in loin.
4	Fat	Tail head – completely filled and folds and patches of fat evident. Loin – cannot feel processes and has completely rounded appearance.
5	Grossly Fat	Tail head – buried in fatty tissue, pelvis impalpable even with firm pressure.

Table 4-6: Mobility scoring as detailed in the Agriculture and Horticulture Development Board (AHDB) guidelines for dairy mobility scoring in their Healthy Feet programme

Score	Category	Description
0	Good mobility	Walks with even weight bearing rhythm on all four feet with a flat back. Long fluid strides possible.
1	Imperfect mobility	Steps uneven (rhythm or weight bearing) or strides shortened; affected limb or limbs not immediately identifiable.
2	Impaired mobility	Uneven weight bearing on a limb that is immediately identifiable and or obviously shortened strides (usually with an arch to the centre of the back).
3	Severely impaired mobility	Unable to walk as fast as a brisk human pace (cannot keep up with healthy herd). Lame leg easy to identify – limping; may barely stand on lame leg/s; back arched when standing and walking. Very lame.

4.3.13 Feed analysis

Samples of grass silage, straw, PMR and parlour concentrate mix were sent to Sciantec Analytical for analysis of their components. Determination of neutral detergent fibre (NDF), nitrogen and crude protein, crude fibre, moisture, ash, oil/fat, starch, cellulose and hemicellulose, total sugars, water-soluble carbohydrates, acid detergent lignin (ADL) and biogenic amines in the feed were carried out. Protocols, as given by Sciantec, are detailed in Appendix 1.

4.3.14 Statistical analyses

All data were analysed using R (RCore Team, 2016). Parameters investigated in the trial, abbreviations, units and normality of results is detailed in Appendix 2. For

each parameter, Shapiro-Wilk test, Q-Q plots and histograms were carried out to test and visualise distributions for normality. If data were not normal (i.e. SW $p < 0.05$), data were log transformed and re-tested. If normal, or transformed normal, data were visualised in boxplots to show changes in the parameter over time-points and between groups. Normal or near-normal data were tested using ANOVA and t-tests (milk yield, butterfat, milk protein, milk lactose, RBC, Hb, PCV, WBC, MONO, PLT, CHO, TGs, K+, Ca, PO4, Mg, plasma protein, albumin, acetate, butyrate, propionate, valerate, isobutyrate, isovalerate). Data considered not normal were tested using Kruskal-Wallis rank sum test (SCC, NEUT, LYMPH, EOS, BASO, BHB, urea, creatinine, bilirubin, ALP, AST, GGT, GLDH, globulins, Hp,). Significance thresholds were set at $p < 0.05$. BCS and mobility score are ordinal, categorical variables and should be analysed by frequency rather than as a continuous variables. However, group sizes were not compatible with effective chi-squared tests, so data were presented in tabular form. Changes in BCS between time-points were assessed for each animal and classified into three categories: increase, decrease or no change (<0, 0,>0), which were subsequently used as a categorical variable in Kruskal-Wallis rank sum test and t-tests.

4.4 **Results**

The study commenced with 40 cows. Three were substituted out early in the study (D-16, 20/10/15) and replaced with equivalent cows that had been maintained on the same diet. Upon conclusion of the trial, a further 5 cows had been removed, leaving 35 cows (group 1 n=8, group 2 n=7, group 3 n=7, group 4 n=6, group 5 n=7), representing 20% loss over the trial. Reasons for loss included sudden death, chronic lameness, mastitis and early drying off (due to misinformation when recruiting animals for the trial).

4.4.1 **Test product and ration consumption**

Throughout the trial, 1,500 observations of treatment consumption were noted in parlour. Of these observations, DFM treatment was considered to have been consumed completely 1,346 times (90%), partially consumed on 103 occasions (7%) and not consumed on 51 occasions (3%). Figure 4-1 shows the proportion of the test product consumed at each milking. The proportion of DFM consumed completely increased in the latter half of the study. By the time of the dietary challenges (D-46 (18/11/15) and D-51 (24/11/15)) there were no complete rejections and few partial rejections.

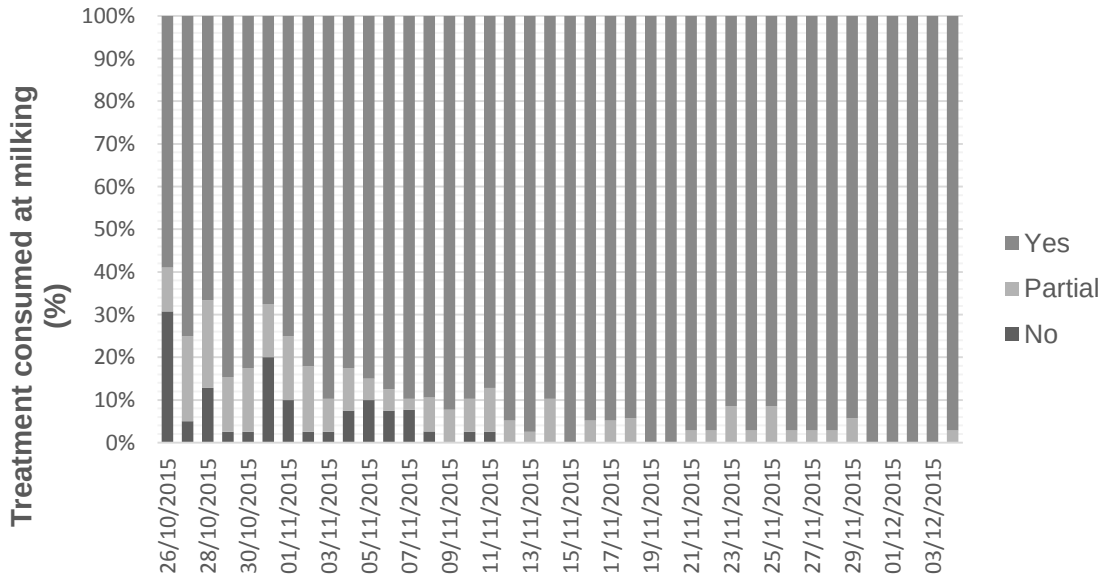

Figure 4-1: Stacked bar chart showing the percentage of treatment consumed considered as Yes, No or Partial at each afternoon milking for all animals in all groups (1: negative Control (sugar), 2: positive control (BOVAMINE® Complete), 3: Vistacell Live Yeast/Hydrolysed yeast, 4: Pediococcus acidilactici, 5: Enterococcus faecium) across the duration of the trial.

The proportion of treatment consumed was higher in group 1 (negative control, 99% eaten) and groups 4 and 5 (bacterial DFM, 99% and 95% eaten respectively) in comparison to group 2 (positive control BOVAMINE® Complete, 80% eaten) and group 3 (Vistacell yeast, 76% eaten), both of which contained yeasts. Difference in consumption of treatment between groups was statistically significant (p <0.001, table 4-7).

Table 4-7: Consumption of treatments for each group (1: negative Control (sugar), 2: positive control (BOVAMINE® Complete), 3: Vistacell Live Yeast/Hydrolysed yeast, 4: *Pediococcus acidilactici*, 5: *Enterococcus faecium*), where "Yes" refers to all of the treatment eaten, "No" to a complete refusal and "Partial" to partial consumption. The difference in consumption between groups was statistically significant (Pearson's chi squared test on proportions of treatment eaten: X-squared = 152 p < 0.001).

Group	Yes	No	Partial	Sum
1: Negative control	317	2	1	320
2: Positive control	243	19	41	303
3: Vistacell Yeast	231	20	51	302
4*: P. acidilactici*	270	1	3	274
5*: E. faecium*	285	9	7	301

4.4.2 **Reticuloruminal pH**

Upon conclusion of the trial, 3 animals with boluses had been removed; 2 dried off early (group 4) and 1 euthanased due to a dislocated shoulder (group 5). Data were complete from all boluses from remaining groups and consisted of 156,400 readings (6,800 readings/cow). As shown in figure 4-2, reticuloruminal pH showed a significant diurnal pattern of variation across the trial, peaking between 04:00-05:00 h with a nadir between 16:00-17:00h (p <0.001). Daily mean pH varied across the trial and showed a wider range of values during the period of time after the challenge diet, shown in figure 4-3A. Daily minimum pH tended towards decline during the challenge period, shown in figure 4-3B and standard deviation in pH values increased, shown in figure 4-3C. Across all readings, there was a significant effect of individual animal ID, time-point and treatment group on pH (p <0.001). Time-point and treatment group had a significant effect on the number of observations noted below pH 6 (p <0.001). As shown in figure 4-4, Group 2 (BOVAMINE® Complete) showed the lowest number of instances of pH below 6.

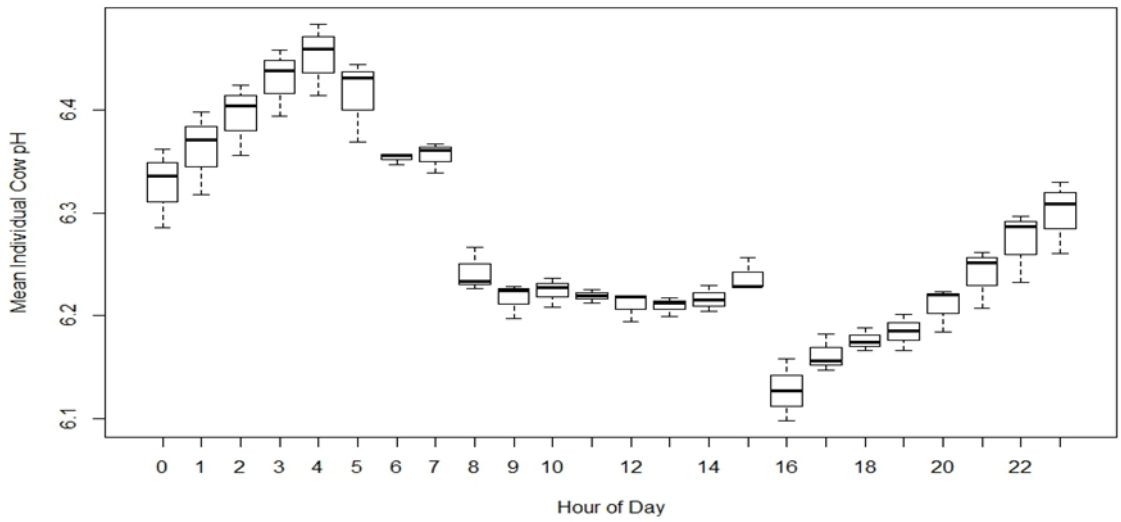

Figure 4-2: Diurnal variation in mean individual cow reticuloruminal pH over all time-points and treatments. Bolus traces showed a peak at 04:00h and a nadir at 16:00h. Effect of hour of day on mean pH was significant ($p < 0.001$).

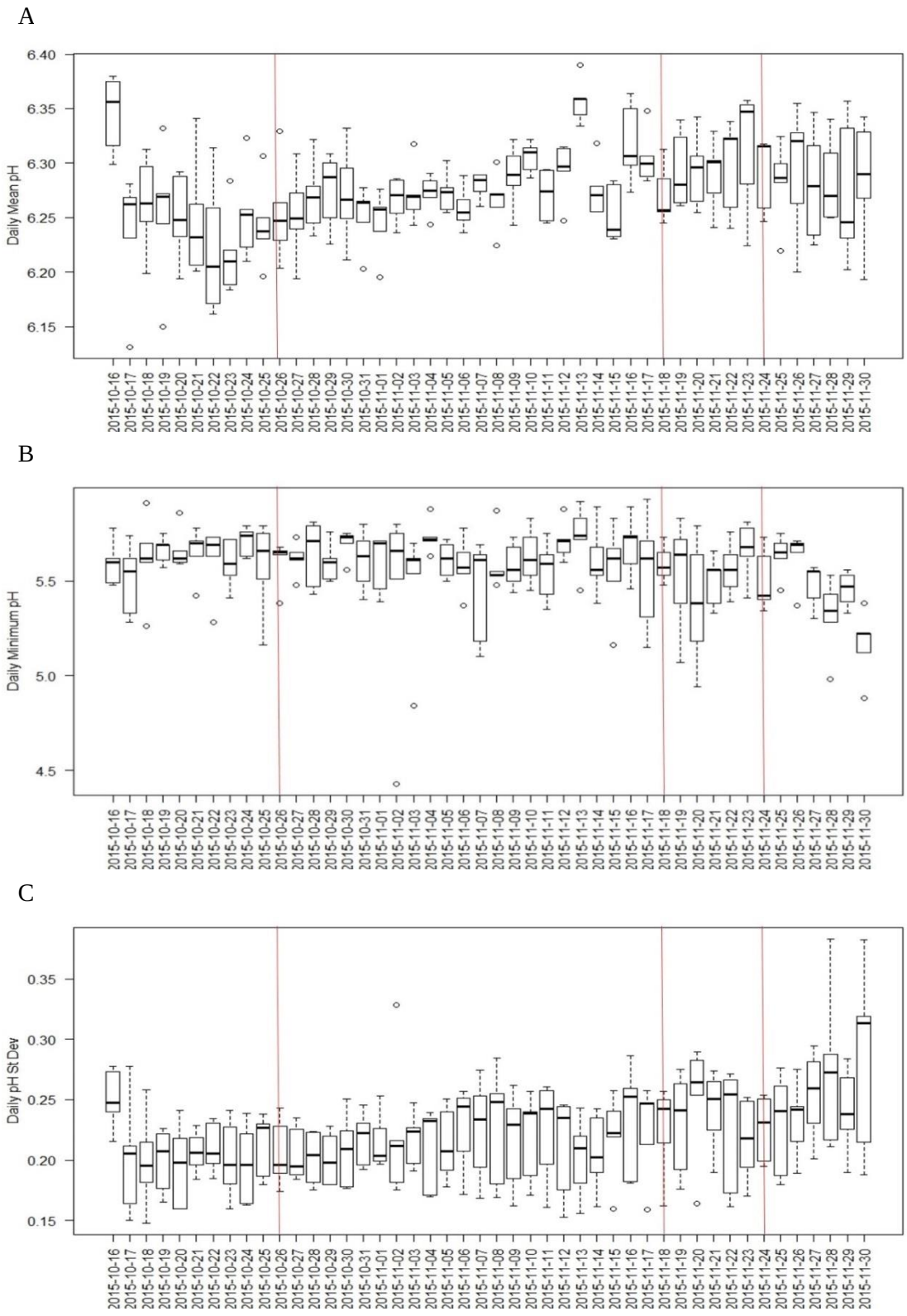

Figure 4-3: (4-3A) Daily mean pH observations (4-3B) daily minimum pH observations (4-3C) Daily standard deviation in pH. Shown for individual cows across the whole trial for all groups. Red lines indicate the introduction of DFM (D-22) and introduction of CHALLENGE 1 and CHALLENGE 2 (initial and revised challenge diets, D-45 and D-51).

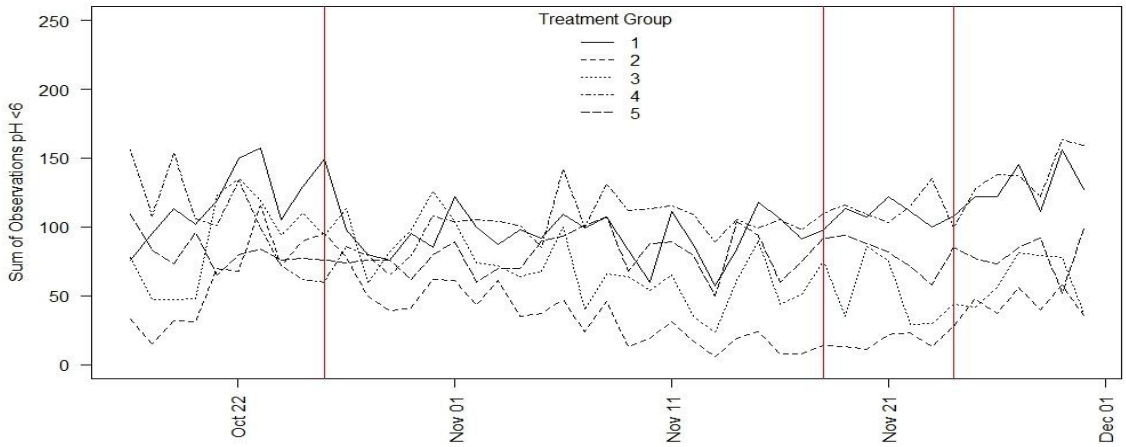

Figure 4-4: Sum of the observations for each group, where pH was below pH 6 across all time-points. Red lines indicate the introduction of the DFM (D-22) and introduction of CHALLENGE 1 and CHALLENGE 2 (initial and revised challenge diets, D-45 and D-51).

4.4.3 **Body condition score (BCS)**

There was no clear effect of time on BCS during the trial. As shown in table 4-8, between time-points A and B, 14 cows lost condition (-0.5 to -2.0 BCS), 10 cows did not change and 11 cows gained condition (0.5 to 1.5 BCS). Between time-points B and C, 12 cows lost condition (-0.5 to -1.5 BCS), 11 cows did not change and 12 cows gained condition (0.5 to 1.5 BCS). As was expected, BCS was significantly higher in pregnant cows (p <0.05) and those in later lactation (p <0.001).

Table 4-8: Body condition score by time-point as scored using DEFRA guidelines detailed in section 4.3.12.

	Body Condition Score (BCS)					
Time-point	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**
A	0	1	6	20	5	3
B	0	1	6	20	5	3
C	1	2	8	14	8	2

4.4.4 **Mobility score**

There was no clear effect of time on mobility score during the trial. As shown in table 4-9, between time-points A and B, 10 cows decreased mobility score (improved mobility), 16 did not change, and 9 cows increased mobility score (impaired mobility). An equal number of cows increased, decreased or remained the same from time-point B to time-point C as from A to B.

Table 4-9: Mobility score by time-point as scored using AHDB guidelines detailed in section 4.3.12.

	Mobility Score (MOB)			
Time-point	**0**	**1**	**2**	**3**
A	21	12	2	0
B	23	9	2	1
C	23	10	2	0

4.4.5 **Production parameters**

Table 4-10 shows the values for each production related parameter across the trial for each group and shows the significance of the change in values between time-points and the significance of the treatment effect between time-points. Treatment effect was not significant for any parameter between any time-points. Live weight only changed significantly between TP-A and B (p <0.05), increasing in all treatment groups. Live weight was not significantly associated with pregnancy (p >0.05), but was significantly higher in cattle in late lactation (p <0.001), as was expected.

Change in milk yield was significantly different between TP B and TP-C (p <0.01), increasing in all groups. Butterfat change was significant between TP-B and TP-C (p <0.001), decreasing in all treatment groups. Lactose percentage changed significantly between TP-A and B and between TP-B and C, however a clear pattern of change could not be identified across group means. Change in milk protein and SCC was not significant between TP-A and B or B and C (p >0.05).

Table 4-10: Mean (above, for each variable) and median (below for each variable) values for selected production-related variables for each time-point and treatment group. Time effects are expressed here as the p-value of single-sample t-tests on normally distributed variables or Kruskal-Wallis rank sum tests for non-normally distributed variables applied to the individual animal differences for each variable from time-points A to B and B to C. Treatment effect is expressed here as the p-value estimated from either a one-way analysis of variance of the normally distributed individual animal differences with treatment as the factor, or a Kruskal-Wallis rank sum test applied to non-normally distributed individual animal differences from time-points A to B and B to C. Variables analysed by Kruskal-Wallis rank sum test = liveweight, milk yield, milk protein, milk somatic cell count.

TP ->	A					B					C					Time Effect		Treatment Effect	
Treatment ->	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	AB	BC	AB	BC
Liveweight (kg)	681	679	681	693	687	686	684	691	702	695	688	686	683	710	690	**0.015**	0.94	0.97	0.43
median	703	688	668	704	696	710	692	684	708	706	703	686	668	713	692				
Milk yield (kg/d)	36.61	35.80	34.26	33.68	35.24	27.48	33.92	32.73	36.50	34.07	39.63	40.33	34.59	38.43	35.26	0.11	**0.0025**	0.45	0.10
median	32.90	35.05	31.30	33.80	33.60	31.20	29.60	31.10	35.90	37.40	38.40	36.20	35.40	36.20	36.30				
Butterfat (%)	4.37	4.74	4.25	4.03	4.28	4.22	4.33	3.84	4.09	4.07	3.78	3.94	3.54	3.74	3.65	0.12	**0.00096**	0.90	0.99
median	4.39	4.92	4.14	3.89	4.43	4.26	4.53	3.79	4.03	3.98	3.44	3.97	3.41	3.66	3.43				
Protein (%)	3.28	3.27	3.24	3.19	3.23	3.28	3.13	3.36	3.35	3.15	3.23	3.25	3.26	2.91	3.30	0.90	0.38	0.49	0.097
median	3.29	3.30	3.19	3.19	3.30	3.28	3.07	3.26	3.29	3.10	3.23	3.20	3.30	3.03	3.29				
Lactose (%)	4.54	4.60	4.56	4.51	4.51	4.62	4.57	4.57	4.56	4.54	4.61	4.63	4.54	4.62	4.54	**0.038**	**0.0091**	0.33	0.48
median	4.60	4.57	4.63	4.55	4.51	4.65	4.55	4.66	4.53	4.51	4.62	4.66	4.61	4.65	4.54				
SCC (×1000/ml)	37.25	59.43	61.14	107.60	76.14	48.63	56.83	144.71	78.83	48.14	21.25	52.71	78.00	48.67	74.86	0.38	0.20	0.087	0.40
median	33.50	40.00	25.00	63.00	63.00	30.00	40.50	50.00	38.00	36.00	20.00	26.00	23.00	19.00	42.00				

4.4.6 **Biochemistry, haematology and acute phase proteins**

As shown in tables 4-11 and 4-12, the majority of biochemistry, haematology and acute phase protein parameters were significantly affected by time-point as a factor, and showed a significant change between time-points but no parameters were significantly affected by treatment effect. Many parameters showed similar patterns of change between time-points. Cholesterol increased significantly in all groups between TP-A and TP-B. Triglycerides (TGs) increased significantly between TP-A and TP-B and decreased significantly between TP-B and TP-C, as did calcium. βHB, albumin and creatinine also decreased significantly between TP-B and TP-C.

Conversely, globulins and urea significantly decreased between TP-A and TP-B and significantly increased between TP-B and TP-C. AST and haptoglobin followed a similar response, decreasing significantly between TP-A and TP-B. Magnesium, GLDH and SAA also increased significantly between TP-B and TP-C.

As with biochemistry parameters, the vast majority of haematological parameters were significantly affected by time-point as a factor and significantly changed between time-points A and B and B and C. No haematological parameters were affected by treatment between time-points. Red cell count, WBC and lymphocyte count decreased significantly between TP-A and TP-B then increased significantly between TP-B and TP-C. Neutrophil count decreased significantly between TP-A and TP-B and monocyte count increased significantly between TP-B and TP-C.

Table 4-11 Mean (above, for each variable) and median (below for each variable) values for selected plasma biochemical variables for each time-point and treatment group. Time effects are expressed here as the p-value of single-sample t-tests on normally distributed variables or Kruskal-Wallis rank sum tests for non-normally distributed variables applied to the individual animal differences for each variable from time-points A to B and B to C. Treatment effect is expressed here as the p-value estimated from either a one-way analysis of variance of the normally distributed individual animal differences with treatment as the factor, or a Kruskal-Wallis rank sum test applied to non-normally distributed individual animal differences from time-points A to B and B to C. Variables analysed by Kruskal-Wallis rank sum test = Ca, ALP, AST, GGT, GLDH, globulin, Hp, SAA, urea, creatinine

Time-point	A					B					C					Time Effect		Treatment Effect	
Treatment	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	AB	BC	AB	BC
Cholesterol (mmol/l)	5.59	5.43	5.92	5.00	6.13	6.60	6.40	6.97	7.09	6.67	7.13	7.08	6.82	6.75	7.68	**0.0035**	0.24	0.77	0.642
median	6.23	5.03	5.98	4.93	6.35	6.98	5.61	6.53	7.59	6.33	7.40	7.02	6.60	6.58	8.23				
Triglycerides (mmol/l)	0.10	0.09	0.11	0.08	0.12	0.14	0.15	0.16	0.14	0.14	0.09	0.09	0.09	0.08	0.10	**<0.0001**	**<0.0001**	0.63	0.83
median	0.10	0.09	0.11	0.08	0.11	0.14	0.14	0.15	0.15	0.14	0.09	0.09	0.09	0.09	0.11				
BHB (mmol/l)	0.84	0.80	0.63	0.70	0.60	0.69	0.64	0.56	0.57	0.74	0.45	0.56	0.57	0.53	0.43	0.10	**0.0095**	0.16	0.18
median	0.90	0.80	0.60	0.70	0.50	0.75	0.60	0.60	0.60	0.80	0.40	0.50	0.50	0.40	0.40				
Ca (mmol/l)	2.28	2.32	2.29	2.26	2.35	2.40	2.42	2.40	2.43	2.40	2.36	2.34	2.32	2.34	2.33	**<0.0001**	**<0.0001**	0.58	0.84
median	2.27	2.37	2.31	2.22	2.38	2.42	2.44	2.41	2.46	2.35	2.39	2.32	2.32	2.31	2.31				
Mg (mmol/l)	1.07	1.00	1.10	0.98	1.05	1.05	0.98	1.10	1.00	1.04	1.08	1.09	1.13	1.05	1.08	0.50	**0.0012**	0.87	0.57
median	1.04	1.00	1.13	0.99	1.04	1.04	0.96	1.12	1.01	1.04	1.08	1.01	1.12	1.07	1.11				
Bilirubin (mmol/l)	8.38	7.43	8.71	7.50	10.14	8.13	8.71	7.29	7.17	8.57	8.25	8.29	6.71	7.50	9.00	0.29	0.95	0.22	0.95
median	9.00	7.00	11.00	7.00	11.00	8.00	7.00	7.00	7.00	9.00	7.00	8.00	7.00	7.00	9.00				
ALP (U/l)	76.38	82.29	85.71	78.33	99.14	71.75	84.43	80.71	93.00	95.57	83.50	92.43	87.14	94.83	113.71	0.81	0.21	0.67	0.24
median	68.50	66.00	85.00	77.00	83.00	71.00	79.00	77.00	71.50	88.00	81.50	86.00	76.00	78.00	107.00				
AST (U/l)	66.38	77.14	74.71	90.33	92.71	58.88	65.57	59.00	68.33	65.14	64.50	69.14	69.86	69.67	73.43	**<0.0001**	0.051	0.32	0.69
median	69.50	73.00	76.00	84.50	70.00	57.00	60.00	56.00	63.50	60.00	66.00	66.00	60.00	71.00	75.00				
GGT (U/l)	20.25	23.00	20.57	17.83	21.43	22.25	21.43	21.14	24.33	22.14	19.88	17.86	18.86	25.00	15.00	0.58	0.10	0.98	0.65
median	18.50	19.00	21.00	15.50	21.00	21.50	25.00	18.00	19.00	20.00	19.00	17.00	19.00	14.00	15.00				
GLDH (U/l)	14.63	13.43	14.71	18.83	28.29	16.75	24.00	20.14	27.17	18.86	23.50	48.57	43.14	23.83	32.14	0.21	**0.0097**	0.34	0.25
median	13.50	11.00	12.00	12.50	17.00	16.00	17.00	14.00	11.50	17.00	20.00	23.00	22.00	18.50	24.00				
Globulin (g/l)	33.75	36.00	33.00	36.17	34.29	29.50	31.00	28.71	33.33	29.86	36.75	38.14	37.43	39.67	38.00	**<0.0001**	**<0.0001**	0.93	0.65

median	34.00	35.00	34.00	35.50	35.00	28.50	30.00	29.00	34.00	29.00	37.50	38.00	36.00	40.50	39.00				
Alb (g/l)	36.25	34.86	37.14	34.00	36.00	36.25	35.00	36.43	34.50	35.57	31.75	30.57	31.14	30.50	31.29	0.77	**<0.0001**	0.90	0.59
median	36.00	34.00	38.00	33.50	37.00	36.00	35.00	36.00	34.50	36.00	31.50	31.00	31.00	30.00	31.00				
Hp (U/l)	0.02	0.14	0.03	0.02	0.02	0.02	0.06	0.02	0.02	0.02	0.04	0.02	0.19	0.01	0.59	**0.010**	0.080	0.36	0.58
median	0.02	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01				
SAA (U/l)	3.86	18.17	7.66	8.30	5.96	5.19	24.99	8.50	5.53	16.80	25.85	12.36	35.01	6.42	54.41	0.30	**0.039**	0.74	0.27
median	3.20	5.70	5.10	5.05	4.00	4.70	6.20	9.80	3.45	7.40	18.55	7.70	15.90	5.60	26.70				
Urea (mmol/l)	5.53	5.06	5.10	5.17	5.33	3.91	3.83	3.96	4.05	4.06	4.36	4.33	4.50	4.08	4.57	**<0.0001**	**0.0028**	0.69	0.93
median	5.80	4.90	5.50	4.90	5.50	4.00	3.60	4.20	4.05	3.70	4.30	3.90	4.60	3.65	4.60				
Creatinine (µmol/l)	94.75	86.00	91.86	92.17	86.00	91.75	84.00	89.29	86.67	85.00	86.00	77.14	84.00	81.33	75.57	0.14	**0.0031**	0.60	0.93
median	92.50	85.00	88.00	92.00	89.00	89.50	86.00	89.00	86.50	83.00	81.00	73.00	78.00	79.50	75.00				

Table 4-12: Mean (above, for each variable) and median (below for each variable) values for selected haematological variables for each time-point and treatment group. Time effects are expressed here as the p-value of single-sample t-tests on normally distributed variables or Kruskal-Wallis rank sum tests for non-normally distributed variables applied to the individual animal differences for each variable from time-points A to B and B to C. Treatment effect is expressed here as the p-value estimated from either a one-way analysis of variance of the normally distributed individual animal differences with treatment as the factor, or a Kruskal-Wallis rank sum test applied to non-normally distributed individual animal differences from time-points A to B and B to C. All variables were analysed by Kruskal-Wallis rank sum test with the exception of RBC.

TP	A					B					C					TimeEffect		Treatment	
Treatment	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	AB	BC	AB	BC
Red cell count (10^9 cells/l)	6.06	5.90	5.97	6.25	6.08	5.62	5.53	5.56	5.85	5.63	5.99	5.71	5.68	6.05	5.83	**<0.0001**	**<0.0001**	0.99	0.51
median	6.14	5.92	5.79	6.18	6.06	5.65	5.55	5.45	5.77	5.62	5.91	5.77	5.73	6.42	5.71				
White cell count (10^9 cells/l)	5.98	6.47	6.78	6.37	6.88	5.34	4.82	5.79	4.98	5.22	6.10	6.34	6.48	6.01	7.88	**0.00072**	**0.00081**	0.30	0.60
median	5.90	6.81	7.25	6.79	6.23	5.37	4.79	5.66	4.57	5.25	6.31	5.73	6.43	5.94	6.75				
Neutrophils (10^9 cells/l)	2.55	3.13	3.03	3.00	3.60	2.32	1.91	2.58	2.21	2.31	2.82	2.64	2.44	2.54	3.62	**0.0020**	0.076	0.45	0.76
median	2.40	3.57	3.04	2.82	3.05	2.06	2.01	2.51	1.86	2.59	2.83	2.12	2.38	2.49	2.70				
Lymphocytes (10^9 cells/l)	2.82	2.93	3.18	2.85	2.55	2.47	2.47	2.51	2.34	2.40	2.48	2.91	3.02	2.65	2.60	**0.032**	**0.046**	0.81	0.99
median	2.95	2.65	3.57	2.90	2.68	2.59	2.21	2.55	2.27	2.31	2.63	2.76	2.78	2.71	2.63				
Monocyte (10^9 cells/l)	0.34	0.26	0.38	0.33	0.38	0.37	0.31	0.38	0.25	0.31	0.44	0.48	0.53	0.46	0.50	0.79	**0.0015**	0.33	0.89
median	0.29	0.30	0.36	0.32	0.39	0.37	0.31	0.33	0.15	0.29	0.45	0.34	0.58	0.48	0.49				
Eosinophils (10^9 cells/l)	0.22	0.11	0.14	0.13	0.28	0.15	0.10	0.27	0.13	0.16	0.13	0.08	0.12	0.19	0.20	0.56	0.88	0.26	0.20
median	0.18	0.09	0.15	0.13	0.21	0.14	0.13	0.17	0.11	0.16	0.13	0.08	0.13	0.19	0.24				
PLASMAHIST (U/l)	0.04	0.01	0.06	0.02	0.07	0.03	0.02	0.07	0.02	0.01	0.03	0.01	0.01	0.01	0.01	0.074	0.67	0.62	0.97
median	0.01	0.01	0.06	0.00	0.01	0.02	0.01	0.00	0.01	0.01	0.02	0.02	0.00	0.00	0.00				

4.4.7 **Reticuloruminal SCFA**

As shown in table 4-13, all SCFA, with the exception of propionate, decreased following the introduction of DFM, though change was only significant between TP-A and TP-B for butyrate, total SCFA, valerate, isovalerate and isobutyrate. Following introduction of the challenge (change between B and C), change was significant for butyrate, acetate and total SCFA, again showing a mean decrease in the majority of groups. Only the minor SCFA, isovalerate increased significantly between TP-B and TP-C.

4.4.8 **Histamine concentrations**

It was not possible to consistently obtain repeatable measurements of histamine in EDTA plasma and thus data were not analysed. Measurement of histamine concentration in reticuloruminal fluid showed consistent and repeatable results. Histamine concentration was significantly affected by time-point as a factor (p <0.001) and change in histamine concentration was significant between TP-A and TP-B showing a decrease in the mean. There was no significant effect of treatment on the change in reticuloruminal histamine between any time-points or for all time-points combined, as shown in table 4-13.

4.4.9 **Lipopolysaccharide (LPS) concentration**

As shown in table 4-13, reticuloruminal PS concentration was significantly affected by time-point as a factor (p <0.001) and change in LPS concentration was significant between TP-A and TP-B showing an increase in the mean across all groups. There was no significant effect of treatment on the change in LPS concentration between any time-points or for all time-points combined (p >0.05).

Table 4-13: Mean (above, for each variable) and median (below, for each variable) values for concentrations of selected compounds in ruminal fluid for each time-point and treatment group. Time effects are expressed as the p-value of single-sample t-tests on normally distributed variables or Kruskal-Wallis rank sum tests for non-normally distributed variables applied to the individual animal differences for each variable from time-points A to B and B to C. Treatment effect is expressed as the p-value estimated from either a one-way analysis of variance of the normally distributed individual animal differences with treatment as the factor, or a Kruskal-Wallis rank sum test applied to non-normally distributed individual animal differences from time-points A to B and B to C. Variables analysed by Kruskal-Wallis rank sum test = acetate, rumen fluid LPS, rumen fluid histamine.

TP	A					B					C					Time Effect		Treatment Effect	
Treatment	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	AB	BC	AB	BC
Butyrate (mmol/l)	10.39	10.47	9.69	9.82	9.15	8.41	9.32	9.07	8.04	8.86	6.44	7.26	7.52	7.38	6.01	**0.0014**	**<0.0001**	0.46	0.49
median	10.83	9.68	10.10	9.73	9.17	8.47	9.26	9.15	7.93	9.07	5.97	7.37	7.58	6.50	4.98				
Acetate (mmol/l)	61.59	59.57	59.38	59.62	54.43	54.00	55.18	55.11	50.61	53.21	45.22	44.68	46.84	46.28	40.59	0.064	**<0.0001**	0.86	0.67
median	62.32	59.93	59.57	58.26	55.24	55.00	57.47	53.76	50.68	55.60	42.23	42.11	44.86	45.65	38.03				
Propionate (mmol/l)	19.84	17.85	20.59	18.60	16.18	17.60	18.52	16.84	14.50	16.89	18.90	18.43	17.62	19.68	14.85	0.13	0.37	0.48	0.29
median	21.73	16.68	19.51	18.21	15.27	16.21	18.45	17.49	14.98	17.05	18.45	18.71	15.67	20.30	14.61				
Total SCFA (mmol/l)	96.03	91.72	93.47	91.70	82.80	82.84	86.42	84.08	75.79	81.91	73.73	73.68	75.30	76.65	64.32	**0.022**	**0.0025**	0.75	0.46
median	97.85	91.38	92.22	88.99	87.45	80.99	93.59	79.14	76.85	89.50	70.76	71.65	71.45	78.29	58.81				
Valerate (mmol/l)	1.55	1.43	1.45	1.37	1.15	1.00	1.31	1.17	0.95	1.07	1.17	1.21	1.29	1.21	0.99	**0.00039**	0.36	0.21	0.54
median	1.58	1.35	1.44	1.33	1.25	0.96	1.34	1.16	0.97	1.14	1.11	1.28	1.17	1.24	1.03				
Isovalerate (mmol/l)	1.79	1.51	1.54	1.50	1.22	1.19	1.39	1.24	1.10	1.28	1.36	1.47	1.40	1.48	1.30	**0.0060**	**0.038**	0.19	0.66
median	1.77	1.40	1.60	1.41	1.10	1.19	1.42	1.15	1.05	1.24	1.29	1.45	1.23	1.37	1.31				
Isobutyrate (mmol/l)	0.88	0.88	0.83	0.79	0.67	0.65	0.67	0.65	0.59	0.60	0.65	0.64	0.64	0.62	0.57	**<0.0001**	0.62	0.40	0.83
median	0.90	0.89	0.83	0.77	0.68	0.64	0.72	0.65	0.58	0.59	0.62	0.70	0.62	0.62	0.54				
Rumen Fluid LPS (U/l)	32000	2800	2800	55000	46000	250000	960000	370000	670000	530000	400000	400000	730000	1200000	960000	**<0.0001**	0.98	0.54	0.60
median	27000	1200	3300	2200	2800	290000	460000	270000	280000	390000	66000	240000	110000	600000	410000				
Rumen Fluid Histamine (U/l)	2700	2100	3100	3000	130	100	240	130	120	380	390	1500	840	180	160	**0.0039**	0.45	0.23	0.69
median	1200	210	160	1000	120	77	180	110	81	110	140	110	92	210	110				

4.4.10 **Gene expression**

As shown in table 4-14, there was no significant effect of treatment group on the gene expression level between any time-point, or when considering time-point as a factor. Expression levels varied between genes and with the different housekeeping genes. For *TLR4*, correlation between the relative expression values derived from the 2 house-keeping genes *GAPDH* and *β-actin* was very low (-0.015) and the directions of change at each time-point conflicted. Change in *TLR4* with *bAct* was significant between both TP-A and TP-B and TP-B and TP-C, with expression levels increasing and decreasing respectively. When considered with *GAPDH* as the housekeeping gene, change in expression level was significant between TP-A and TP-B and showed a decrease. Change in *IL1-β* was significant between time-points A and B and B and C when derived from both housekeeping genes. *IL1-β* relative expression response varied between time-points A and B with each housekeeping gene but showed a significant and large increase between time-points B and C when derived from both housekeeping genes. Change in *IFN-γ* relative expression between TP-A and TP-B and between TP-B and TP-C was significant when considered with *GAPDH* as the housekeeping gene, and expression levels decreased then increased respectively.

Table 4-14: Mean (above, for each variable) and median (below for each variable) values for relative gene expression for each time-point and treatment group. Values are expressed as the expression of each gene relative to the house-keeping gene used (ie GAP-DH or β-actin). Time effects are expressed here as the p-value of single-sample t-tests on normally distributed variables or Kruskal-Wallis rank sum tests for non-normally distributed variables applied to the individual animal differences for each variable from time-points A to B and B to C. Treatment effect is expressed here as the p-value estimated from either a one-way analysis of variance of the normally distributed individual animal differences with treatment as the factor, or a Kruskal-Wallis rank sum test applied to non-normally distributed individual animal differences from time-points A to B and B to C. All variables were analysed by Kruskal-Wallis rank sum test.

TP	A					B					C					Time Effect		Treatment Effect	
Treatment	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	AB	BC	AB	BC
TLR4bAct	2.29	1.29	4.71	0.73	0.48	3.16	3.67	3.22	2.24	2.77	1.18	5.07	0.88	1.38	2.32	**0.0014**	**0.0056**	0.63	0.83
median	1.04	0.66	1.03	0.63	0.50	1.83	1.61	2.21	2.38	1.58	1.12	1.06	0.41	0.66	1.74				
TLR4GAP	1.94	1.04	1.25	1.48	0.92	0.46	0.32	0.26	0.11	0.16	0.84	0.95	0.35	1.03	0.86	**<0.0001**	**0.0001**	0.40	0.66
median	1.44	0.67	1.07	1.08	0.97	0.21	0.18	0.15	0.04	0.07	0.48	0.54	0.30	0.78	0.62				
IL1bbAct	4.56	7.40	5.14	48.48	2.26	9.85	4.32	12.78	4.14	10.59	67.73	213.38	23.09	334.28	46.93	**0.00020**	**0.0065**	0.46	0.32
median	0.77	0.54	1.31	0.56	1.15	6.05	2.85	2.41	3.92	6.80	8.50	29.26	7.85	238.79	26.14				
IL1bGAP	4.77	10.38	1.33	48.38	5.13	6.06	0.95	5.89	0.29	2.26	180.74	844.51	19.92	1101.86	23.09	**0.053**	**0.00019**	0.30	0.36
median	2.03	0.13	1.72	1.83	2.01	0.59	0.20	0.28	0.13	0.25	4.38	24.75	10.82	238.11	7.25				
IFNgbAct	0.78	1.55	4.96	1.61	0.52	13.73	0.86	1.20	2.11	2.22	1.12	1.06	0.45	6.36	0.70	1.0	0.16	0.19	0.59
median	0.70	1.70	0.40	1.41	0.39	0.68	0.71	0.34	0.71	1.38	0.61	0.36	0.27	1.36	0.52				
IFNgGAP	2.01	1.08	2.37	3.37	1.19	0.27	0.22	0.07	0.06	0.09	0.34	0.45	0.45	3.55	0.24	**<0.0001**	**0.0049**	0.47	0.087
median	0.68	0.85	0.61	2.12	0.46	0.09	0.16	0.06	0.03	0.08	0.31	0.17	0.13	0.93	0.13				

4.5 **Discussion**

In this study, the administration of a challenge diet, higher in starch and lower in NDF than the diet to which the animals were accustomed, resulted in a substantial increase in production performance in the face of a systemic inflammatory response. Following introduction of the challenge diet, the changes in WBC, monocyte count, lymphocyte count and RBC were significant and all increased. Additionally, positive acute phase proteins, haptoglobin and SAA, and the relative expression levels of *IL1-β* also increased following the challenge diet. An increase in these variables has been associated with an inflammatory response. For example, an increase in monocyte count has previously been shown to be associated with an increase in pro-inflammatory cytokine expression (Pomeroy *et al.,* 2017), which is thought to occur as a result of decreased reticuloruminal pH, leading to translocation of LPS of ruminal bacterial origin (Pomeroy *et al.,* 2017). Additonally, Hp levels have been shown to increase with ruminal acidosis in dairy cattle (Mount et al., 2009), sheep (Minato et al., 1992) and goats (Mertens, 1994).

In addition to investigating circulating gene expression levels in the PBMC in this study, relative expressions of inflammatory genes was investigated in the rumen epithelial tissue in chapters 3 and 5, and they were shown to increase in response to a challenge. *IL1β* is a pro-inflammatory cytokine that plays an important role in acute and chronic inflammation (Ren & Torres 2009). After the introduction of the DFM, *IL1β* expression changed significantly between time-points A and B and B and C but the pattern of change was not clear. However, following the challenge diet, the change in expression was significant and relative expression derived from both housekeeping genes showed a significant increase, suggesting an inflammatory effect of the challenge diet and fitting with the inflammatory response noted in the biochemistry. *TLR4* is a toll-like receptor responsible for activating the innate immune system and it recognises LPS via a high specificity for the lipid-A portion of LPS (Kim et al. 2007). If the rumen epithelial barrier is affected by dietary induced pH change, LPS of ruminal bacterial origin may be released into circulation. Therefore, increased *TLR4* expression in circulating blood can indicate initiation of innate responses due to the presence of LPS. In this study, *TLR4* results were difficult to interpret, the correlation between the relative expression values derived from the 2 house-keeping genes *GAPDH* and *β-actin* was very low (-0.015) and the

directions of change at each time-point conflicted, suggesting that the treatment affected the expression of at least 1 of the house-keeping genes. As with *TLR4, IFNγ* relative expression results were difficult to interpret as the 2 housekeeping genes resulted in differing results, though results tended to suggest an increase in relative expression following the challenge diet.

The increase in variables associated with inflammation preceded and were more pronounced that those changes observed in variables commonly monitored as a symptom of reticuloruminal acidosis, such as reticuloruminal pH. Although the response was less pronounced, pH was significantly different between time-points. The introduction of the challenge ration resulted in no change to the mean pH, but an increase in the range of pH values observed. Daily mean pH showed a wide range of values, which narrowed during the treatment period and widened during the challenge diet, suggesting that the treatments were buffering the reticuloruminal pH as intended. Daily minimum pH decreased during the challenge, particularly after the second dietary challenge, suggesting that the diet was providing a challenge to the rumen function as intended. The challenge ration did not affect mean or median reticuloruminal pH values but did increase the number of observations below pH 6.0 and increased the range of pH values noted. However, as there were few pH observations below 5.5, it is unlikely that the herd would have been classified as affected by SARA according to classical definitions. This lack of effect on mean pH suggests that the challenge diet was not severe enough to induce SARA in the majority of animals, but was having an overall negative effect on rumen function. It is also suggestive of the variation between animals, with the wider range of values observed showing some animals were more affected by the diet and showed a larger pH decrease than others. There was also a significant effect of animal ID, again reflecting the individual animal variation in reticuloruminal pH. Reticuloruminal pH showed a significant diurnal pattern previously described in cattle (Mani et al., 2012, Mandebvu and Galbraith, 1999), with a peak at 04:00-05:00h and a nadir at 16:00-17:00h. It is hypothesised that the reason for this diurnal cycle is the period at night with a high rate of rumination combined with reduced feeding, likely increasing saliva production and allowing removal of SCFA from the rumen (Denwood *et al*., 2018). Denwood *et al.* (2018) suggest an alternative method of analysing pH variation using the absolute deviation from an individual cow model, consisting of a double or single sine wave (representing diurnal cycles with or

without an additional cycle for milking frequency) fitted iteratively to a generalized additive mode. They found that this approach enabled the identification of periods when milk yield was depressed, whereas periods of depression of pH below any arbitrary pH threshold was not effective. The relative lack of a reticuloruminal pH response to the challenge diet contrasts markedly with the clear evidence of mild systemic inflammation in response to the challenge diet.

Despite the presence of indicators of an inflammatory syndrome being apparent in the cattle, production parameters and energy balance increased following the introduction of the challenge diet. Energy metabolism variables, plasma triglycerides (TGs) and β-hydroxybutyrate (βHB), both changed significantly with the challenge diet, both showing a decrease. This decrease in energy metabolism variables was expected, as the challenge diet was high in energy, leading to improved energy balance and a decrease in these variables reflects this (Doepel *et al*., 2002, Butler *et al*., 2006).This improved energy balance was reflected in the increased milk yield noted following the challenge diet. An increase in milk yield when cattle are fed DFM has previously been shown (McLeay and Smith, 2006) but milk yield has previously been shown to decrease in animals induced with SARA (Lan et al., 2008), again suggestive that the challenge was not as severe as intended. Although milk yield increased, milk fat percentage change was significant following the challenge and showed a decrease. As with milk yield, decreased milk fat has been previously associated with SARA (Nocek, 1997), although a decrease in milk fat in conjunction with ruminal acidosis is not always observed (Lardner, 2001). Milk lactose concentration changed significantly following introduction of DFM and following the challenge diet, though a clear pattern of change could not be observed. Increased lactose percentage has been observed in experimentally induced ruminal acidosis. (Lardner, 2001). BCS and mobility score were not significantly affected by time-point or treatment and did not show a clear pattern of change throughout the trial. Live weight significantly increased following introduction of the DFM, but as treatment did not have a significant effect on the liveweight change it is thought that this increase may be partly due to the increased consistency of diets over time after the trial commencement.

Reticuloruminal SCFA varied significantly with time-point but did not follow the pattern expected from the literature. Following the challenge diet, the change in the

concentrations of 2 of the main SCFA produced in the rumen (Penner et al., 2009b), acetic and butyric acid, was significantly different, with both decreasing. In comparison with this, Stefańska et al. (1999) found that in animals from acidotic farms, higher concentrations of acetate, propionate, n-butyrate, n-valerate were noted. In another study Martin et al. (2005) found that in "SARA-positive" cows, fermentation results in more butyrate than propionate production, again something not seen in our results; the percentage contribution of butyrate decreased significantly after the challenge diet was introduced and the propionate percentage contribution increased. Change in total SCFA concentration was significant following introduction of the DFM and the challenge diet, decreasing with DFM introduction and again with the challenge diet. This decrease following the challenge diet was initially unexpected, not following the expected increase in SCFA due to rapid fermentation of carbohydrates and corresponding SCFA accumulation detailed in literature. However, this decrease was observed in both trials described in chapters 3 and 5 and a similar reduction in total SCFA concentrations during a challenge diet has also been noted in sheep following induction of ruminal acidosis (Liu et al., 2015). It is hypothesised that this decrease is a consequence of adaptation, leading to increased rates of SCFA absorption or decreased microbial activity (Liu et al., 2015). The decrease in SCFA in this trial could not be compared to levels of sodium-hydrogen antiporter gene (*NHE3*) as was done in chapters 3 and 5 to confirm the hypothesis that decreased SCFA were due to increased absorption. Although not directly responsible, increased *NHE3* has been associated with increased SCFA absorption. It is thought that this positive correlation occurs as a result of cell proliferation in the rumen epithelium increasing sites for *NHE3*, thus causing an increase in expression and as a result of the proliferation, increasing surface area for absorption of SCFA. As *NHE3* is expressed only in rumen tissue, variation in its expression could therefore not be detected in this trial through plasma gene expression levels. Minor SCFA, valerate, isovalerate and isobutyrate followed a similar response pattern to the total SCFA and acetate and butyrate, decreasing following introduction of the DFM but were not significantly changed following the challenge diet.

The DFM used in the study had little or no effect on most of the changes in response to the challenge diet, although some of them modulated the reticuloruminal pH - though the changes were very small in numerical terms - with mean pH significantly

increasing following introduction of DFM. Treatment group also had a significant effect on the number of observations noted below pH 6. The DFM, BOVAMINE® Complete and *E. faecium* were most effective at buffering the rumen pH and may be useful at preventing the negative effects of a high carbohydrate diet. BOVAMINE® Complete yeast showed the lowest number of instances where of pH below 6, supportive of its use as a commonly used commercial DFM, marketed as a successful ruminal buffer. Test product consumption was high, increasing in the latter part of the study, presumably as cattle became accustomed to the products and route of administration. Proportion of treatment consumed at each milking was significantly different between groups, higher in the negative control (group 1) and the 2 bacterial products (groups 4/5) in comparison to the yeasts (BOVAMINE® Complete group 2 and group 3) which were a larger volume. This higher rejection rate may be due to a difference in palatability of the products, or it is possible that the larger volume of these powders led to a detection bias in leftover doses (larger treatments more likely to be noted if rejected or partially eaten). Time taken to administer the larger dose products was also slightly increased and meant occasionally cattle ate their full concentrate allocation before the dose was fully administered; meaning cattle were more likely to receive treatments into the trough alone. Leftover parlour feed was negligible throughout the trial and was not recorded. This very low feed refusal rate was not as would be expected if significant ruminal acidosis had been induced.

It was critical that the challenge diet in this trial challenged the rumen to an extent where variation in production parameters and an effect of DFM could be assessed. The diet was formulated to provide a challenge and induce SARA but not to cause severe clinical disease. The expected values for all animals for NDF from forage (%) were well below recommendations and the expected intake of starch and sugar and overall concentrate to roughage ratio were above standard recommendations. However, as animals on farm were parlour fed partly according to yield, it is unlikely that all animals were challenged to the same level. Higher producing animals would have experienced a more severe challenge. In future, standardising the amount of parlour feed would provide a challenge to more animals in the herd, but would perhaps not be typical of standard feeding on farms, as the majority of animals on farms will be fed partly to yield.

In conclusion, the key finding from the results detailed in this chapter is that feeding a challenge diet high in rapidly fermentable carbohydrates induced changes consistent with an inflammatory response. It appears that an inflammatory type response in cattle may be a pre-cursor to SARA and may become evident before clinical symptoms arise in individuals. It is possible that by identifying early indicators of inflammation, fermentation disorders could be identified early, prevented before symptoms arise and preventing both welfare issues and financial losses.

5 A direct comparison of physiological responses in cattle and sheep to diets intended to induce acidosis

5.1 Introduction and background

The majority of studies investigating reticuloruminal function and the effect of a high carbohydrate diet in ruminants primarily use cattle, often dairy cattle, as the subject. However, cattle are expensive to purchase, can be expensive to maintain, and require access to a relatively large amount of space. Conversely, sheep are cheaper to purchase, easier to maintain and work with and require less space than cattle. The use of sheep as a model organism for ruminant studies into SARA and ruminal acidosis would be supported if their responses to a diet expected to induce SARA were comparable with those of cattle.

Sheep have been shown to be an effective and more economical model for ruminants and have been used as a model organism in studies investigating diseases and infections affecting both cattle and sheep, such as *Escherichia coli* O157:H7 (Kudva et al., 1995) and comparative prion diseases such as scrapie and bovine spongiform encephalopathy (BSE) (Hunter, 2003). Both cattle and sheep have been the subject of extensive nutritional studies and both species have previously and independently been shown to be affected by ruminal acidosis as a result of feeding a diet high in rapidly fermentable carbohydrates (Patra et al., 1996, Aschenbach and Gabel, 2000, Keunen et al., 2002, Penner et al., 2009a, Marchesini et al., 2013). However, the comparative effects of a diet intended to induce ruminal acidosis have not previously been reported.

The variables investigated in this study had previously been shown to be affected by feeding a diet intended to induce SARA in beef and dairy cattle in extensive trials in the literature (Owens et al., 1998, Aschenbach and Gabel, 2000, Gozho et al., 2007). It would be expected that if responses in variables previously associated with SARA, such as reticuloruminal pH (Mulligan et al., 2016), SCFA levels (Penner et al., 2009b), reticuloruminal LPS (Khafipour et al., 2009) and reticuloruminal histamine (Wang et al., 2013) were similar in both species then the use of sheep as a model organism for cattle would be supported.

The expression of inflammatory cytokines including C-C motif chemokines, and interleukins have been shown to increase in response to a challenge diet intended to induce ruminal acidosis in both the rumen epithelium (Zhang et al., 2016) and peripheral blood leukocytes (Danscher, 2011). Investigating metabolic, biochemical, haematological, histological and immune response variables in both species should enable more complete characterisation of the response to a challenge diet in the two species. Many variables are already known to differ between sheep and cattle, including haematology and biochemistry (Jackson and Cockcroft, 2007), so there is a need to consider the between-species variation in the responses.

The objective of the study was to determine the extent to which sheep and cattle differ in their reticuloruminal pH, ruminal microbiome, ruminal histology and gene expression, ruminal SCFA, histamine and LPS concentrations, and haematological and biochemical profiles, in response to challenge with a diet intended to induce ruminal acidosis

5.2 Aims

There were 3 main objectives for this study:

1. Determine to what extent sheep should be used as a model for dietary manipulation in cattle
2. Determine to what extent sheep and cattle vary in their response to a diet intended to induce ruminal acidosis, in their reticuloruminal pH, ruminal histology, SCFA proportions, LPS concentration, rumen fluid histamine levels, plasma histamine levels and blood haematology and biochemistry profiles
3. Provide baseline data on the comparative rumen microbiome of sheep and cattle and their overall gut biology using 16S sequencing

5.3 Materials and methods

5.3.1 Study design

Samples were collected from 6 ruminally fistulated cattle and 4 ruminally fistulated sheep when animals were maintained on a basal forage ration and after challenge with a high carbohydrate ration formulated with the intention of inducing ruminal acidosis. Each animal was administered an indwelling pH/temperature monitoring bolus (Well Cow™, Roslin, Midlothian) prior to the beginning of the trial. Blood, reticuloruminal fluid and ruminal papillae biopsy samples were collected at 3 time-points during the study: D-28 (basal), D-30 (1 day post challenge) and D-36 (1 week post challenge). Figure 5-1 shows a schematic of the time line for the study, detailing sample collection days and the analysis carried out from the samples collected at each point.

Cattle used in this study were adult, female, purebred Jersey cows, ranging from 6 to 7 years and 466 kg to 624 kg. Cattle were designated FC (fistulated cow) 1-6. Cattle were previously fistulated animals owned by and kept at the University of Glasgow's Cochno Farm and Research Centre.

Sheep used in this study were 4 Scotch Mule ewes aged 11 months and ranging in weight from 55-79 kg. Sheep were sourced from the Cochno flock and fistulated specifically for this study, 4 months prior to the commencement, to give an appropriate surgical recovery time. Six sheep were intended to undergo fistulation, however 2 failed preclinical tests and thus only 4 were used. Sheep were designated FS (fistulated sheep) 2, 4, 5 and 6. Sheep were fistulated using a modified Bar-Diamond cannulation protocol, the same used in the cattle, detailed in full in Appendix 3.

5.3.2 **Timeline**

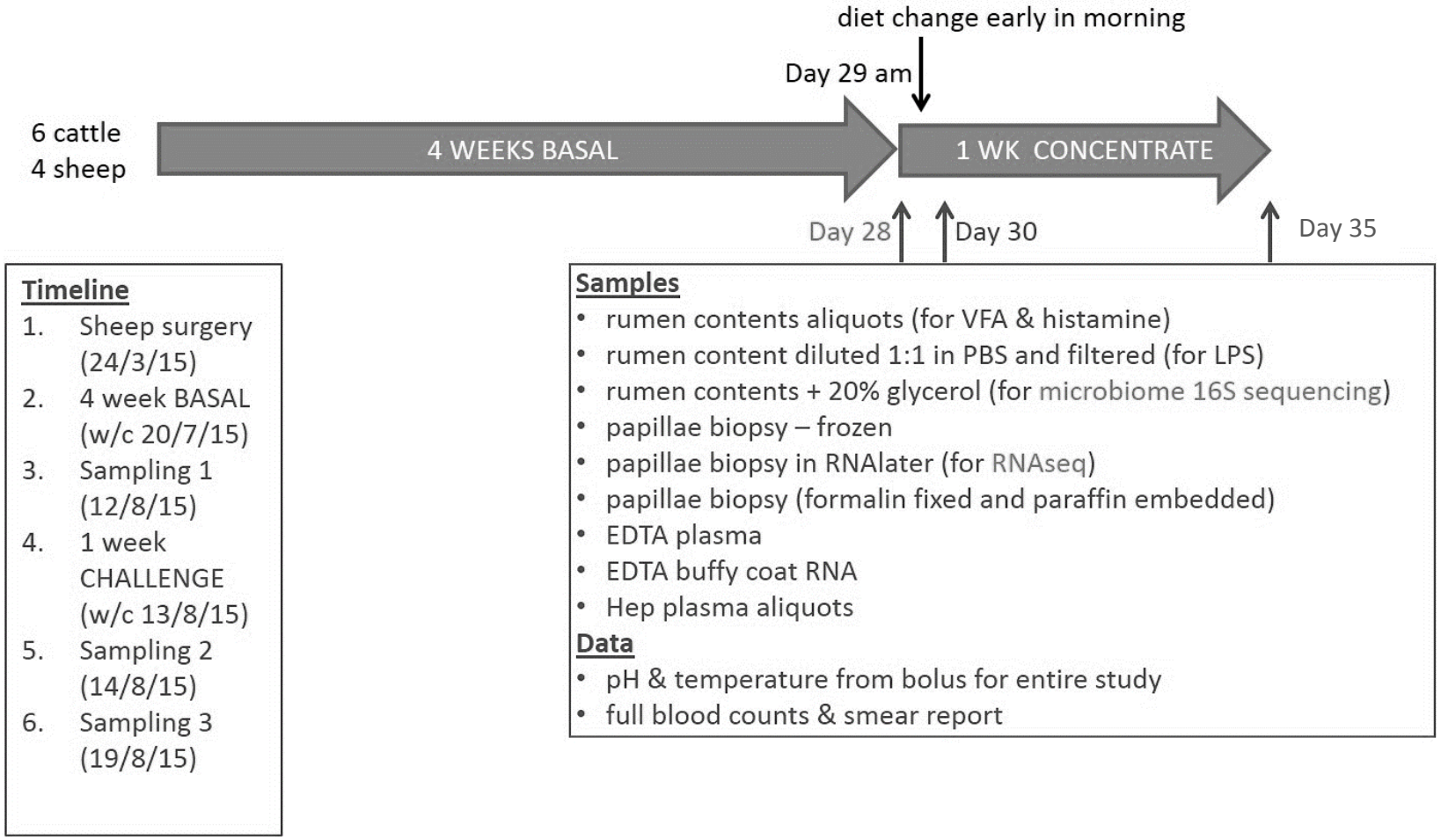

Figure 5-1: Schematic of the study timeline, detailing the samples collected at each time-point and the analysis they were used for at a later date. Microbiome analysis and RNAseq were carried out only on samples collected on D-28 and D-30.

Animals were housed in conspecific groups in open sided, straw-bedded sheds, with *ad libitum* access to water and individual troughs with locking head gates for feeding (bucket fed in the absence of individual troughs).

Table 5-1: Table showing cattle and sheep information. The table shows age, bolus ID and weights across the trial. No pre-surgery weight was available for cattle as they were fistulated prior to the beginning of the trial.

Species	ID	Age	Bolus ID	Pre surgery weight (kg)	Start weight (kg)	End weight (kg)
Cow	FC1	92 months	664	-	466	462
Cow	FC2	89 months	665	-	538	532
Cow	FC3	89 months	666	-	584	574
Cow	FC4	88 months	667	-	624	618
Cow	FC5	74 months	658	-	540	518
Cow	FC6	73 months	659	-	500	500
Sheep	FS2	11 months	660	66	64	61.5
Sheep	FS4	11 months	661	56	56	54
Sheep	FS5	11 months	662	60	55	54.5
Sheep	FS6	11 months	663	83	79	69

5.3.3 Rations

Before the trial, both species were fed some barley each day as per the farm's normal feeding regime. This was standardised and treated as their basal diet (150 g/sheep/day bruised barley or 600 g/cow/day bruised barley) with *ad libitum* haylage and water. Both species were fed in shared troughs for the first week of the trial, after which animals were individually bucket fed or fed in individual troughs to prevent any feed dominance and ensure an accurate record of feed residues could be maintained. Leftover feed was weighed using household digital scales (Salter, maximum capacity 5 kg, measured in 1 g increments).

Prior to the study, sheep received approximately twice the amount of barley per kg of liveweight as the cattle (2.3 g/kg liveweight compared with 1.1 g/kg liveweight in cattle). We scaled the increase in barley content allometrically according to the equation of Sharma and McNeill (2009):

$$Sheep\ Increase = \frac{Cow\ Increase}{(\frac{Sheep\ Weight}{Cow\ Weight})\text{^}\ 1/3}$$

This resulted in approximately 5-fold increase per kg for cattle (0.6 kg to 3.0 kg) and 10-fold increase for sheep (0.15 to 1.5 kg), thereby compensating for the relatively

higher surface area to volume and weight of the sheep. Hence, the basal ration of cattle was *ad libitum* haylage plus 600 g/cow/d barley and of sheep was a*d libitum* haylage plus 150 g/sheep/d barley. The challenge diet for cattle was *ad lib*itum haylage plus 3.0 kg/cow/d of barley, and that of sheep was *ad libitum* haylage and 1.5 kg/sheep/d of barley.

5.3.4 **Boluses**

One Well Cow™ (Roslin, Midlothian) pH and temperature monitoring bolus was placed in the reticulum of each animal prior to the commencement of the trial. The boluses measure pH and temperature every 15 minutes and store data for up to 120 days, until collected via a Bluetooth receiver and Android tablet and sent to a Cloud-based storage system. Each bolus measures 32 mm x 145 mm and weighs 240 g. It has a deployed target life of 80-100 d, a range of pH 4-7, and a reported "accuracy" of +/- 0.3 pH units according to Well Cow (Roslin, Midlothian).

Boluses were calibrated before insertion using 2 pH standard solutions (pH 4 and pH 10). The bolus is intended to be administered orally using a standard bolus gun. However, for this study the bolus was placed through the fistula.

Continuously monitored reticuloruminal pH data were collected every 3 days by restraining the animal and using the handheld Bluetooth reader to collect data stored on the indwelling bolus. The reader was placed on the animal's abdomen and an Android tablet was used to synchronise the bolus and reader, allowing upload of data.

5.3.5 **Rumen cannulation of sheep**

Sheep were cannulated on-site at the University of Glasgow's Cochno Farm and Research Centre using a protocol adapted from "Rumen cannulation of adult cows using the Bar – Diamond plastic cannula" (see Appendix 3). Candidates for cannulation were healthy adults, with no history of chronic conditions such as lameness. The para-lumbar fossa was checked prior to surgery using a template to ensure sufficient size to take the cannula. A full clinical examination was performed and blood was taken from each candidate for standard haematology and

biochemistry panels. Two animals were excluded because of elevated white blood cell counts, leaving 4 animals to be submitted to surgery.

5.3.6 **Weights**

Cattle and sheep body weights were recorded at each sampling time-point and throughout the trial. In cattle, liveweight was measured using Gallagher W210 scales with Gallagher load-bars fitted onto a crush. The W210 scales operate accurately ± 1% between -20 ºC to 50 ºC. The scales have a "zero tracking" feature, which automatically zero the scales to compensate for accumulation of mud and faeces. Sheep were weighed using Polden Vale pen-side scales (accuracy of ±0.5kg). Scale calibration was checked by using calibration weights.

5.3.7 **Reticuloruminal fluid**

Reticuloruminal fluid was collected via the cannula in both species. Ruminal contents were collected by hand from the ventral sac, close to the reticulum and strained through muslin. The strained fluid was collected into 2 × 50 ml tubes; either plain (for SCFA/LPS/histamine analysis), or with 20% glycerol as a cryoprotectant (16S rRNA microbiome sequencing). Rumen contents were stored in a passive cool-box with ice blocks (< 4˚C) until they could be processed in the laboratory as per section 2.1.3.

5.3.8 **Reticuloruminal lipopolysaccharide (LPS)**

Lipopolysaccharide (LPS) concentration in reticuloruminal fluid was determined using the EndoLISA endpoint fluorescence microplate kit (Hyglos, France), with a Varioskan LUX Fluorescent microplate reader (Thermo Fisher, UK) as detailed in section 2.9. Serial dilutions were used to plot a standard curve of log (endotoxin units or EU/ml) against log (relative fluorescence units or RFU/ml), and the unknown sample concentrations calculated from the regression.

5.3.9 **Reticuloruminal SCFA and ethanol**

Measurement of SCFA concentration in reticuloruminal fluid was carried out by Sciantec Analytical Ltd. using gas chromatography (GC) as detailed in section 2.10. Reticuloruminal fluid was tested for the presence of ethanol, propan-1-ol, acetic acid,

propionic acid, isobutyric acid, propane-1, 2-diol, butyric acid, isovaleric acid, valeric acid, hexanoic acid, heptanoic acid and lactic acid. Any concentration < 25 mg/kg fell below the detection level of the GC and could not be quantified. Molar concentrations of SCFA were obtained by dividing the measured mass of each SCFA in mg/kg by its molecular weight: acetate - 60.05; butyrate - 88.11; propionate - 74.08; valerate - 102.13; isobutyrate - 88.11; isovalerate - 102.13 (g/mol).

5.3.10 **Histamine**

Histamine concentration in reticuloruminal fluid was measured using the Histamine ELISA Kit (Abnova, catalogue no. KA1888), designed for analysis of histamine in human faeces and a LabTech LT-4500 microplate reader, as detailed in section 2.8.1. Duplicate measurements were used for all standards, controls and samples. The concentration of unknown samples was calculated from standard curves from samples of known concentrations.

Circulating plasma histamine concentration was measured using the Abnova Histamine ELISA Kit (Cat. No. KA2589) intended for determination of histamine in plasma from blood tubes containing EDTA, using a LabTech LT-4500 microplate reader, as detailed in section 2.8.2. Duplicate measurements were used for all standards, controls and samples. Concentrations of unknown samples were estimated from standard curves using samples of known concentrations.

5.3.11 **Microbiome analysis**

16S ribosomal RNA (rRNA) microbiome analysis was carried out by Glasgow Polyomics (University of Glasgow) following DNA extracted from rumen fluid using a protocol adapted from Grahn *et al.* (2003), as summarised below. Due to budgetary limitations, full bacterial sequencing was not carried out and the 16S sequencing in this study sequenced the V3 and V4 regions of the 16S.

5.3.11.1 **DNA extraction from rumen fluid**

Cell lysis

Frozen reticuloruminal fluid plus glycerol was thawed and centrifuged at 10,621 x g for 20 min. The majority of the supernatant was removed, leaving ~1 ml, varying with the size of the pellet. The pellet was re-suspended with a plastic Pasteur pipette. The sample was placed into a 2 ml screw-cap tube containing zirconia beads (0.1 mm and 0.5 mm). 1 ml of lysis buffer was added (NaCl 500 mM, Tris-HCl 50 mM, EDTA 50 mM, 4% sodium dodecyl sulfate (SDS)) and the sample was homogenised with a vortex at high speed. It was then incubated at 70˚C for 15 min before being centrifuged at 17,949 x g. Supernatant was removed and placed into a fresh 2 ml microtube.

Precipitation of nucleic acids

260 µL ammonium acetate (10 mM) was added to the supernatant, mixed well and the samples incubated on ice before being centrifuged at 17,949 x g for 10 min. The solution was added to a microtube, 1 volume of isopropanol (molecular grade) was added and the tubes were incubated on ice, before being centrifuged at 17,949 x g for 15 min. The supernatant was removed and the nucleic acid pellet washed with 70% ethanol (molecular grade) before being dried under vacuum in a freeze dryer. The pellet was dissolved in 100 µL TE (Tris-EDTA 1x) buffer and the purity and concentration determined using the NanoDrop Microvolume Spectrophotometer and Fluorometer (Thermo Fisher, UK).

5.3.11.2 **Generation of 16S amplicon libraries**

DNA samples that had been extracted following the protocol detailed above were quantified using the High Sensitivity DNA Qubit system (Thermo-Fisher, Paisley, UK). 16S libraries encompassing the V3 and V4 regions were generated by Glasgow Polyomics (University of Glasgow). The V3 and V4 regions of bacterial 16S were amplified using Kapa HiFi Hotstart readymix (2x) (Kapa Biosystems, Wilmington, MA, USA), with the addition of primers specific for the V3 and V4 regions of 16S, based on standard Illumina 16S primers, containing an overlap sequence which makes the primers compatible with the Nextera XT indexing

reagents (Illumina, San Diego, CA, USA). Samples were amplified using a 5 min 95˚C “Hotstart” followed by 26 cycles of 95˚C for 30 secs and 60˚C for 1 min with a final elongation step of 60˚C for 5 min.

The resulting amplicons were purified using bead extraction (SPRI select beads, Beckman Coulter, Brea, CA, USA) using 0.9x beads followed by 80% Ethanol washes and resuspension in 20 µL of 10 mM Tris buffer. The amplicons were quantified using the High Sensitivity DNA Qubit system and profiles obtained from an Agilent 2100 Bioanalyser using High Sensitivity DNA reagents (Agilent, Santa Clara, CA, USA).

Samples were standardised to 10 ng/reaction and amplified in the presence of Nextera XT v2 indexes using Kapa HiFi Hotstart readymix (2x) for 8 cycles. The resulting indexed libraries were purified using SPRI select beads and quantified using Qubit. Final library profiles were obtained from the Agilent 2100 Bioanalyser.

5.3.11.3 **Sequencing**

Libraries were combined in equimolar ratios and sequenced on a MiSeq (Illumina, San Diego, CA, USA) using a paired end, 2 x 300 base pair, sequencing run. Samples were sequenced with an average of 100,000 reads per sample.

5.3.11.4 **Analysis**

FastQ files were quality-filtered and trimmed using Cutadapt to remove adapter sequences from high throughput sequencing reads, with a minimum length of 250 basepairs/read and a minimum quality score of 25 (Martin, 2011). Paired end reads were combined using pandaseq (Masella et al., 2012), which were combined into a single Fasta file using the Qiime package (Caporaso et al., 2010) to allow analysis of high-throughput community sequencing data. Further processing and analysis was completed using the Qiime wrapper and its packaged software, aligning against the Greengenes database (gg13): uClust (Edgar, 2010), PyNAST (Caporaso *et al.*, 2010), Greengenes (DeSantis *et al.,* 2006), assigning taxonomy (McDonald et al., 2012; Werner et al. 2012, Wang et al., 2007), Fasttree (Price et al., 2012) to analyse alpha rarefaction, Unifrac (Lozupone and Knight, 2005) and Emperor (Vazquez-Baeza, 2013). Data was prepared for use in the

LefSe pipeline using Koeken.py. Samples were analysed as individual samples and as groups.

5.3.12 **Ruminal papillae biopsies**

Biopsies of ruminal papillae were taken using a protocol adapted from Schlau et al. (2012). The rumen was first partially emptied into a bucket and the densely papillated surface of the right wall of the dorsal sac of the rumen was pulled across to the cannula in the left wall, to enable samples to be taken. The biopsy was taken using 3-4 cuts with curved scissors to harvest 20 to 30 papillae. In both species following biopsy, contents were replaced in the rumen. As the study was carried out during summer and the procedure took less than 15 min/animal, it was not considered necessary to add hot water with the contents to prevent a decrease in body temperature. Following closure of the cannula, the external surface was cleaned to remove spilled ingesta. No antibiotics or analgesics were given post biopsy consistent with practice described elsewhere (Oba, 2015).

Papillae were placed in 0.9% saline solution immediately upon collection then counted equally into 3 aliquots and transferred into 3 sterile bijou sample containers (Sigma-Aldrich, UK), each containing one of RNA-later (ThermoFisher), 10% formalin (Sigma Aldrich, F1268), or plain (immediately placed onto dry ice to freeze and frozen at -80˚C for future use).

In the laboratory, samples in RNA-later were frozen at -20˚C before being processed using miRNeasy kit (Qiagen) with BeadBug tubes, detailed in section 5.3.13. Samples were further processed for Taqman gene expression as per section 5.3.14. Samples in formalin (Sigma Aldrich, F1268) were fixed before being processed for histology, as detailed in sections 2.2 and 2.3.

5.3.13 **Ruminal histology**

Papillary biopsies stored in 10% formalin (Sigma Aldrich, F1268) were fixed and moved to 1 x PBS solution for storage. Biopsies were prepared and embedded in paraffin as detailed in section 2.2.1. Sections were stained for both H&E and EMSB as detailed in section 2.2.and 2.2.3. Immunohistochemistry was carried out for both CD3 and MHC2 by Veterinary Diagnostic Services, as detailed in section 2.3.

Images were captured as detailed in section 2.4. Slides were scored using the system detailed in section 3.3.7.

5.3.14 **RNA extraction from rumen papillae**

Five to 10 papillae stored in RNAlater (Qiagen, Ref. 76104) were homogenised in Qiazol Lysis Reagent (Qiagen) with a Polytron homogeniser (VWR). Homogenates were kept on ice throughout the protocol. Homogenates were transferred to prefilled BeadBug™ (Sigma Aldrich, USA) tubes with additional Qiazol and vortexed with short intervals on ice. Homogenates were transferred to PLG-heavy tubes, chloroform was added and tubes were left at room temperature for 5 min before being centrifuged at 20,817 x g for 14 min. The aqueous phase was transferred to a microtube and 100% ethanol added. The sample was then added to a collection tube (kit), centrifuged at 10,621 x g for 15 min and the protocol for the miRNeasy kit was then followed. Resulting RNA was tested for concentration and the A260/280 and A260/230 ratios were determined using a NanoDrop Microvolume Spectrophotometer and Fluorometer (ThermoFisher, UK). If the RNA concentration was high (>1000 µg/µL), RNase-free water was added to the eluent and it was again tested using the NanoDrop Microvolume Spectrophotometer and Fluorometer (Thermo Fisher, UK). RNA was stored at -80˚C.

5.3.15 **Semi-Quantitative PCR**

EDTA RNA was extracted from ruminal papillae biopsies for *CCL11, IL1-β, TLR-4*, and *NHE3* gene expression analysis by semi-quantitative, real-time PCR (qPCR), as detailed in section 2.6. In brief, RNA extracted as detailed in section 5.3.14 was converted to cDNA using the TaqMan Universal Master Mix kit (Thermo Fisher, Paisley, UK). qPCR was carried out using TaqMan Gene Expression Master Mix assay kit (Applied Biosystems) with the extracted cDNA according to manufacturer's instructions. The relative quantification of each gene was calculated utilising the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$) of relative quantification (Livak and Schmittgen, 2001, Steibel et al., 2009) using *GAPDH* and *RPLPO* as the housekeeping genes, as the correlation between the 2 was good. Relative expression (fold change) at each time-point was calculated by comparing the gene of interest (GOI) cycle threshold (Ct) value and the housekeeping gene (HKG) Ct value. Fold changes for each GOI relative to *GAPDH* and *RPLPO* were averaged

to give the fold change for the animals between D-28 and D-30 and D-30 and D-35. *βAct* was excluded from analysis as a HKG because the reported Ct values varied greatly for each sample. All plates were carried out in a 10 µl volume (6 µl MasterMix, 4 µl cDNA) and all samples and standards were run in duplicate.

5.3.16 **Blood collection and laboratory analysis**

Blood samples were collected via venepuncture of the jugular or coccygeal vein and artery, into 7.5 ml EDTA and heparin-containing vacutainers (Becton Dickinson, BD Vacutainer, USA). Samples were stored in a passive cool box with ice blocks (< 4˚C), before direct transport to the laboratory. In the laboratory, blood was processed as detailed in section 2.5. Standard haematology panels were carried out by the University of Glasgow's Veterinary Diagnostic Services (VDS), using the ADVIA 20.120 Haematology System (Siemens) as detailed in section 2.5.3.

5.3.16.1 **RNA extraction from EDTA blood**

RNA was extracted from the EDTA blood for future use using Qiazol Lysis Reagent (Qiagen, Ref. 79306) and the miRNeasy kit (Qiagen), as detailed in section 2.6.2.

5.3.17 **Statistical analysis**

Data were analysed using R (RCore Team, 2016). Data were normality tested using Shapiro-Wilkes test and Q-Q plots and histograms to visualise normality. Variables which were considered not normal following testing were natural-log transformed and retested. All variables were considered to approximate a normal distribution following log transformation. Overall effect of time-point as a factor (D-28, D-30 and D-35 combined), species and the time-point/ species interaction term were identified using a one-way analysis of variance with time-point and species as fixed effects in R (RCore team, 2016), with a significance of $p < 0.05$. For each parameter, mean, median and standard deviation was also calculated for all cattle and all sheep across all time-points and at each individual time-point. Additionally, paired t-tests were used to identify the statistical significance of the change in variables between time-points A and B and time-points B and C, where change in the value was considered significant if it were significantly different to 0.

Data from a 48 h period at each time-point (basal, diet change, 1 week post diet change) were summarised for each animal to provide hourly mean pH values for each 24 h period at D-28, D-30 and D-35. Minimum and maximum pH values were recorded for each time-point and time spent below commonly used normal thresholds was calculated for each animal.

Histological scores created for each sample using the scoring system detailed in section 3.3.7 were analysed using R (RCore Team, 2016). For numeric variables, Shapiro-Wilkes normality testing was carried out. Non-normal parameters were log transformed and retested; all being considered normal following transformation. Paired t-tests were used to identify significant changes in scores between time-points. Mean, median and SD was calculated for all cattle and all sheep across all time-points and at each individual time-point for each variable. Categorical data were presented in tables with no attempt to determine the significance of frequencies in each category because the expected numbers of observations in each cell were low.

16S rRNA outputs were analysed using Qiime and LefSe. Qiime is used to analyse, process and plot 16S data (Caporaso et al., 2010). LefSe (Segata et al., 2011) was used to build a Linear Discriminant Analysis (LDA) models for samples, where relative difference in abundance of differing phyla between groups was analysed and created “biomarker” lists. Biomarkers, as designated by LefSe, refer to “specific organisms, clades, operational taxonomic units, or pathways whose relative abundances differ between 2 or more groups of samples” (Segata et al., 2011). Bar charts and cladograms were generated of the biomarkers. Phylogenetic diversity (PD) was looked at for all animals, and examined the differences in bacterial species present in each sample, using a Newick format phylogenetic tree. Larger PD values can be inferred as corresponding to greater expected diversity (Vellend, 2010). PD is defined as the minimum total length (number of nodes) of all the phylogenetic branches required to span a given set of differences in bacterial species on the phylogenetic tree (Vellend, 2010). PD was examined for 5,000 reads/sample 10 times. UniFrac (EMPeror (Vazquez-Baeza, 2013)) plots were used to determine whether communities in a sample were significantly different from other samples and to compare communities simultaneously using clustering and ordination techniques (Turner et al., 2012).

5.4 Results

5.4.1 Diet and weights

The amount of leftover feed recorded for each animal at the morning and afternoon feeds over the course of the challenge week is shown in table 5-2. No cattle left any feed at any point in the trial. All sheep refused the majority of their feed throughout the trial, with sheep FS6 showing the largest refusal from the outset.

Table 5-2: Feed residues at AM and PM feedings, for both cattle and sheep over the challenge period and percentage of ration refused at each feed for each sheep. Cattle were fed 1500 g and sheep were fed 750 g at each feed (AM and PM).

	Thurs		Fri		Sat		Sun		Mon		Tues		Wed
	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM	PM	AM
FS2	363	280	326	379	444	721	715	610	607	565	589	660	724
% refused	48.4	37.3	43.4	50.5	59.2	96	95.3	81.3	80.9	75.3	78.5	88	96.5
FS4	0	416	730	691	722	709	523	380	698	473	506	601	730
% refused		55.4	97.3	92.1	96.2	94.5	69.7	50.6	93	63	67.5	80.1	97.3
FS5	0	0	0	720	728	730	730	729	730	711	711	684	724
% refused				96	97	97.6	97.6	99.8	97.6	94.8	94.8	91.2	96.5
FS6	671	683	701	728	720	680	705	664	719	696	696	698	707
% refused	89.5	90.8	93.4	97	96	90.6	94	88.5	95.9	92.8	92.8	93.1	94.3
FC1	0	0	0	0	0	0	0	0	0	0	0	0	0
FC2	0	0	0	0	0	0	0	0	0	0	0	0	0
FC3	0	0	0	0	0	0	0	0	0	0	0	0	0
FC4	0	0	0	0	0	0	0	0	0	0	0	0	0
FC5	0	0	0	0	0	0	0	0	0	0	0	0	0
FC6	0	0	0	0	0	0	0	0	0	0	0	0	0

Pre-surgery weights of cattle were unknown, surgery having been carried out years prior to the commencement of this trial. Weights taken pre-surgery for sheep and throughout the trial for both species are detailed in table 5-3. In cattle, weight changed significantly only between D-28 and D-35 ($t=-2.58$, $p < 0.05$). In sheep, weight changed significantly only between pre-surgery and D-28 ($t = -3.6$, $p < 0.05$).

Table 5-3: Animal weights (kg) pre surgery and across the whole trial for all individuals for both species. Weight was measured pre-surgery for sheep, at two points during the basal diet and on the first (A) and last sampling date (C).

Animal	ID	*Eartag*	Pre-Surgery	27/07/2015	10/08/2015	12/08/2015 (A)	19/08/2015 (C)
Cow	FC1	765			466		462
Cow	FC2	776			538		532
Cow	FC3	809			584		574
Cow	FC4	826			624		618
Cow	FC5	978			540		518
Cow	FC6	994			500		500
Sheep	FS2	337	66	64		61	61.5
Sheep	FS4	482	56	56		50	54
Sheep	FS5	310	60	55		53	54.5
Sheep	FS6	321	83	79		68	69

5.4.2 **Reticuloruminal pH**

From the beginning and throughout the study, it was apparent that the boluses and the bolus relaying system were inefficient and often failed. Final bolus data and corresponding hand held readings were sent to Well Cow™ to allow them to classify the remaining boluses as functioning or not. Three boluses were classified as definite failures by Well Cow™; FC4 failed 23/6/15, FC5 failed immediately and FS2 failed 13/8/15. According to checks by Well Cow™ (checking the drift in data, range observed and repetition of results), the remaining data was considered acceptable and a true reflection of the reticuloruminal pH at that time. Reticuloruminal pH was significantly different between time-points and between species, and was significantly different between individual animals (p <0.001), shown in figure 5-2. Sheep showed a wider range of pH values at D-30 in comparison with cattle and consistently had a lower mean pH across the trial.

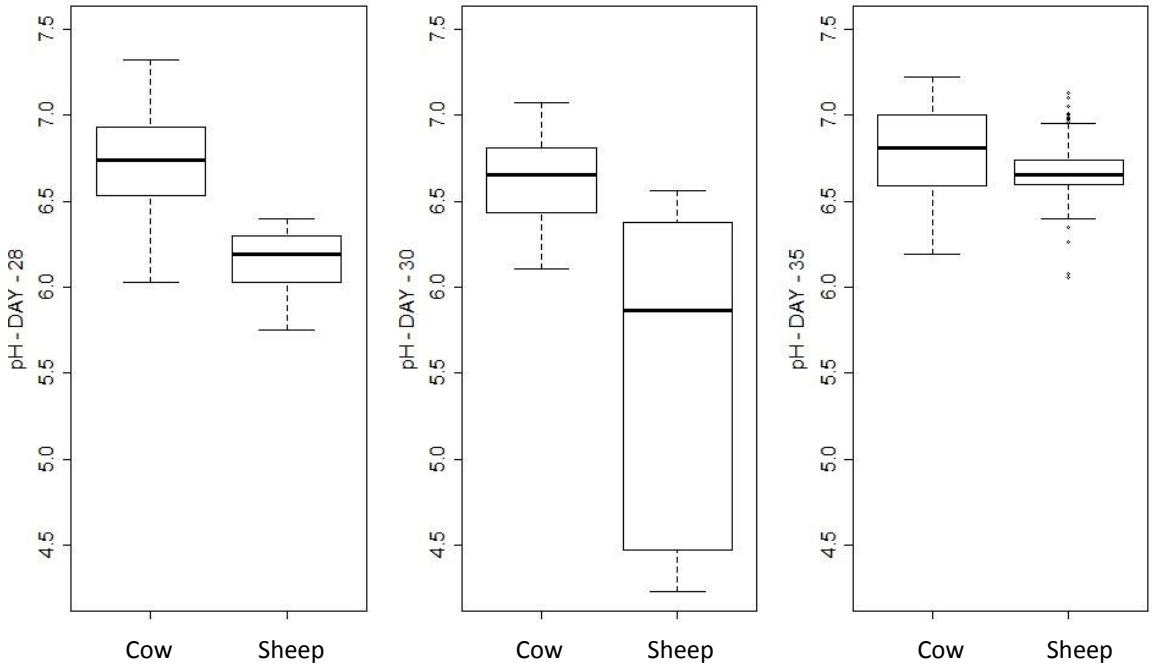

Figure 5-2: Reticuloruminal pH at each time-point: basal (D-28), 1 day post challenge (D-30) and 1 week post challenge (D35) for each species. Reticuloruminal pH was statistically significantly different between time-points, species and individual animals ($p < 0.001$).

Effect of hour of day on reticuloruminal pH was significant for cattle (p <0.001), but was not significant in sheep. Change between time-points was significant for both species and both species followed the same pattern. Figure 5-3 shows that hourly reticuloruminal pH did not vary greatly between D-28 and D-30 in cattle (figures 5-3A and 5-3B). However, despite the change being small in absolute terms (average -0.08 units), the pH decreased significantly between D-28 and D-30 in cattle (t=-9.43, p <0.0001) and increased significantly between D-30 and D-35 (t = 24.3474, p <0.0001). In sheep, a larger, significant reduction was noted in pH between D-28 and D-30 (t=-10.89, p <0.0001) and again a larger increase was noted at between D-30 and D-35 (t = 17.4354, p <0.0001).

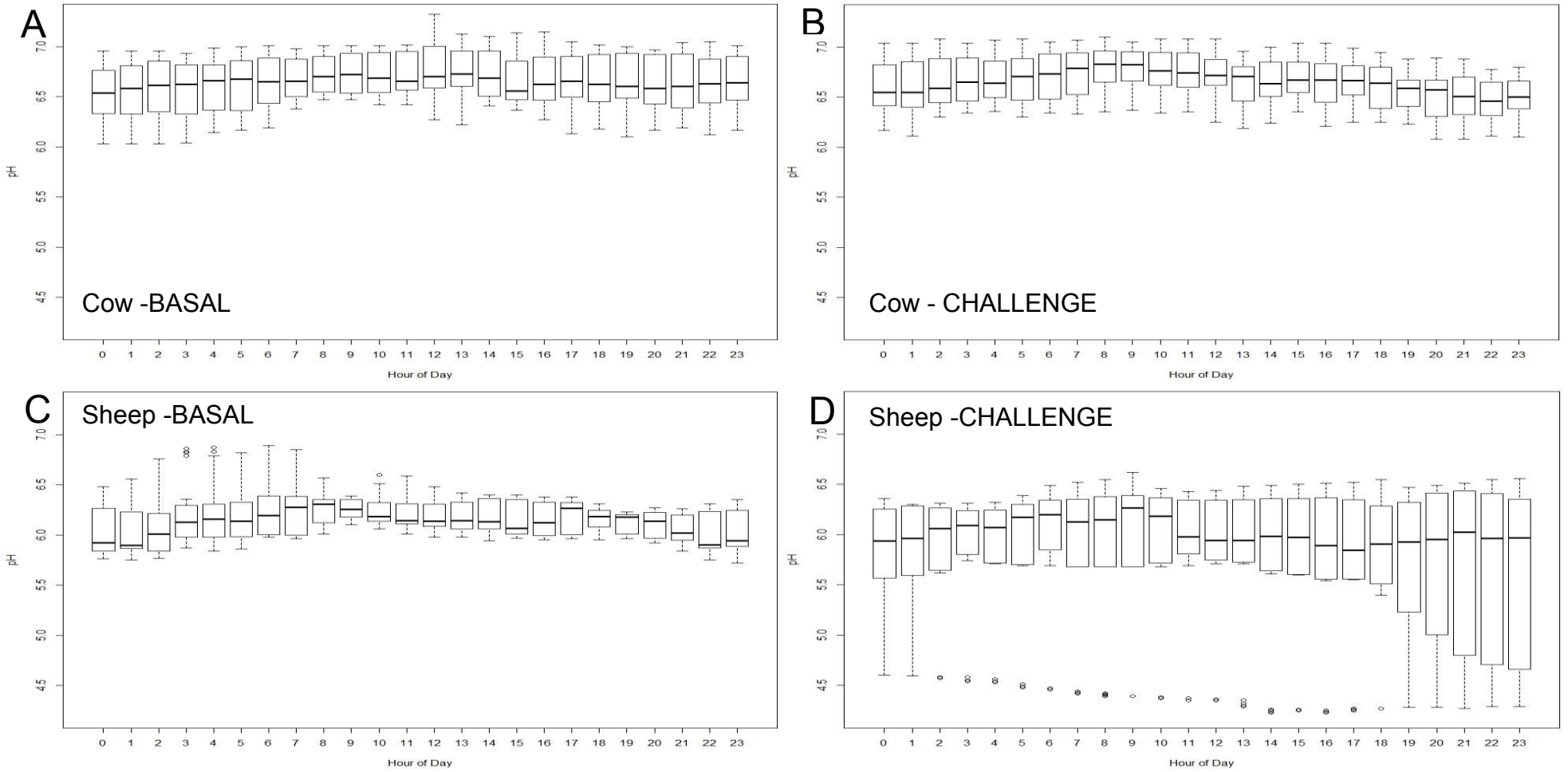

Figure 5-3: Box and whisker plots showing daily reticuloruminal pH for all cattle (3A/B) and all sheep (3C/D) at time-points A (3A and 3C) and time-point B (3B and 3D). Time-point A refers to D-27 and D-28 (basal) and time-point B to D-29 (diet change) and D-30 (1 day post challenge). Reticuloruminal pH was significantly affected by hour of day only in cattle (*p* <0.001).

Table 5-4 shows the time (hours) spent below commonly reported pH thresholds for each animal at each time-point. All cattle maintained their pH above pH 6 for the duration of the trial. Sheep FS6, who refused the majority of her feed throughout the trial, also maintained her pH above pH 6 for the entire trial. Only sheep FS4 and FS5 showed a large variation in their observed pH, with sheep FS4 showing a minimum pH of 5.51 at D-30 and FS5 showing a pH below 5 for all of D-30, with a minimum pH of 4.23.

Table 5-4: Table showing the time spent below commonly reported pH thresholds. No cattle were below pH 6 at any point. Sheep FS4 and FS5 showed the largest changes in pH.

	D-28 Hours spent at:			D-30 Hours spent at:			D-35 Hours spent at:		
ID	≤ pH 5	pH 5.1– 6	> pH 6	≤ pH 5	pH 5.1 – 6	> pH 6	≤ pH 5	pH 5.1 - 6	> pH 6
FC1	0	0	24	0	0	24	0	0	24
FC2	0	0	24	0	0	24	0	0	24
FC3	0	0	24	0	0	24	0	0	24
FC6	0	0	24	0	0	24	0	0	24
FS4	0	12.75	11.25	0	15.5	8.5	0	0	24
FS5	0	3.5	20.5	24	0	0	0	0	24
FS6	0	0	24	0	0	24	0	0	24

5.4.3 **Haematology**

As shown in table 5-5, species had a significant effect on the majority of haematological variables (p <0.05). Time-point as a factor had a significant effect only on neutrophil count and monocyte count and the interaction term of overall effect of time-point and species was only significant for neutrophil count and plateletcrit (PCT). Table 5-5 shows the significance of the effects of species, overall time-point and their interaction term on the change in haematological variables and the change in variables from D-28 to D-30 and from D-30 to D-35. The mean, median and SD for haematological variables at each of the time-points and for each individual time-point, for each species, is shown in table 5-6. Only white blood cell count (WBC), neutrophil count and monocyte count followed the same pattern over the three time-points in both species. WBC and neutrophil count decreased at both D-30 and D-35 and monocyte count increased at D-30 and decreased at D-35. For both species, red blood cell count (RBC), haemoglobin (Hb) and haematocrit (HCT) increased at D-30 but decreased in cattle at D-35 and increased in sheep.

Table 5-5: The effect of time-point, species and their interaction term on haematological parameters across all time-points (one way ANOVA with time-point and species as fixed effects) and the significance of the change between D-38 and D-30 and between D-28 and D-35 (t-test, mu = 0). The p-values are shown as calculated, except where values were below 0.0001.

	Significance (p-value)			T-Test significance (p-value)		
Variable	Time-point	Species	T-P*Species	Species	D-28 and D-30	D-30 and D-35
RBC (x10E12/l)	0.133	**<0.0001**	0.382	Cow	**0.02**	**0.04**
				Sheep	0.57	0.2
Hb (g/dl)	0.109	0.189	0.455	Cow	**0.01**	**0.02**
				Sheep	0.56	0.7
Haematocrit (%)	0.144	0.073	0.409	Cow	**0.02**	**0.03**
				Sheep	0.7	0.88
Mean corpuscular volume (fl)	0.992	**<0.0001**	0.987	Cow	0.36	0.88
				Sheep	0.57	0.37
WBC (x10E9/l)	0.192	**0.003**	0.362	Cow	0.07	**0.05**
				Sheep	0.07	0.06
Neutrophil count (x10E9/l)	**0.002**	**0.0004**	**0.019**	Cow	**0.01**	**0.01**
				Sheep	0.58	**0.045**
Lymphocyte count (x10E9/l)	0.256	**0.015**	0.238	Cow	0.76	0.56
				Sheep	0.15	0.51
Monocyte count (x10E9/l)	**0.007**	**0.002**	0.467	Cow	**0.049**	0.42
				Sheep	0.9	0.21
Eosinophil count (x10E9/l)	0.289	**<0.0001**	0.714	Cow	0.95	0.74
				Sheep	0.55	0.9
Mean platelet volume (fl)	0.473	**<0.0001**	0.827	Cow	0.83	0.4
				Sheep	0.67	0.1
Plateletcrit (%)	0.653	**<0.0001**	**0.033**	Cow	0.13	0.19
				Sheep	0.07	0.33
Platelet distribution width (%)	0.272	0.543	0.779	Cow	0.7	**0.04**
				Sheep	0.71	0.26

Table 5-6: Mean, median and SD for each haematological variable across all time-points for cattle and sheep and the mean, median and SD for each individual time-point (D-28, D-30 and D-35).

Time-point	Cow All T-P	Cow D-28	Cow D-30	Cow D-35	Sheep All T-P	Sheep D-28	Sheep D-30	Sheep D-35
RBC mean	7.27	6.82	7.46	7.53	12.07	11.94	12.28	12.00
RBC median	7.27	6.82	7.43	7.54	11.95	11.84	12.34	12.09
RBC SD	0.60	0.44	0.67	0.46	0.89	0.60	1.15	1.08
Hb mean	13.13	12.27	13.53	13.60	12.61	12.43	12.80	12.60
Hb median	13.30	12.15	13.95	13.80	12.60	12.50	12.85	12.40
Hb SD	1.29	1.35	1.43	0.69	0.65	0.99	0.32	0.62
HCT mean	37.42	35.17	38.32	38.77	35.47	35.15	35.85	35.40
HCT median	37.85	34.85	38.85	39.05	35.60	35.35	35.60	35.40
HCT SD	3.48	3.46	3.83	2.30	1.60	2.60	1.02	1.09
MCV mean	51.46	51.48	51.43	51.45	29.49	29.48	29.35	29.65
MCV median	51.60	51.70	51.55	51.90	29.65	29.55	29.50	29.95
MCV SD	2.76	2.80	2.81	3.18	2.21	2.62	2.25	2.43
WBC mean	6.58	6.84	6.52	6.38	8.61	10.39	8.28	7.18
WBC median	6.09	6.49	5.90	5.93	7.76	10.42	7.71	6.97
WBC SD	1.42	1.65	1.45	1.40	2.32	2.73	2.17	0.73
Neut mean	1.57	1.88	1.47	1.36	3.30	4.61	4.08	1.23
Neut median	1.63	1.79	1.50	1.37	2.91	4.45	3.33	1.24
Neut SD	0.60	0.63	0.60	0.52	2.21	1.99	2.25	0.05
Lymph mean	3.85	3.84	3.78	3.95	4.96	5.41	3.85	5.64
Lymph median	3.47	3.73	3.45	3.42	5.07	5.73	4.17	5.44
Lymph SD	1.18	1.36	1.12	1.29	1.17	0.95	0.92	0.85
Mono mean	0.33	0.30	0.42	0.26	0.20	0.24	0.25	0.11
Mono median	0.30	0.29	0.43	0.25	0.17	0.21	0.24	0.08
Mono SD	0.11	0.07	0.13	0.07	0.12	0.13	0.13	0.06
Eos mean	0.75	0.76	0.76	0.73	0.13	0.15	0.10	0.14
Eos median	0.59	0.61	0.56	0.70	0.14	0.14	0.07	0.14
Eos SD	0.37	0.36	0.47	0.33	0.08	0.03	0.12	0.08

Figures 5-4 and 5-5 show box and whisker plots for those variables affected by both time-point and species (neutrophil and monocyte count). Although the significant species difference is clear in both graphs, both species can be seen to follow similar response patterns at each time-point.

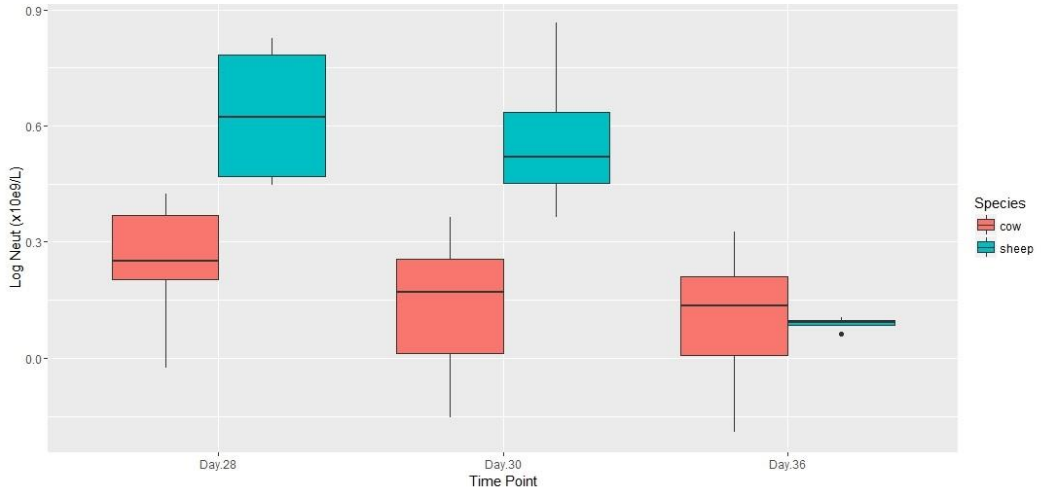

Figure 5-4: Box and whisker plot showing the neutrophil count at D-28, D-30 and D-35 for both species. Neutrophil count was significantly affected by both overall time-point and species (p <0.05). Change in neutrophil count was significant between D-28 and D-30 and D-30 and D-35 in cattle and between D-30 and D-35 in sheep.

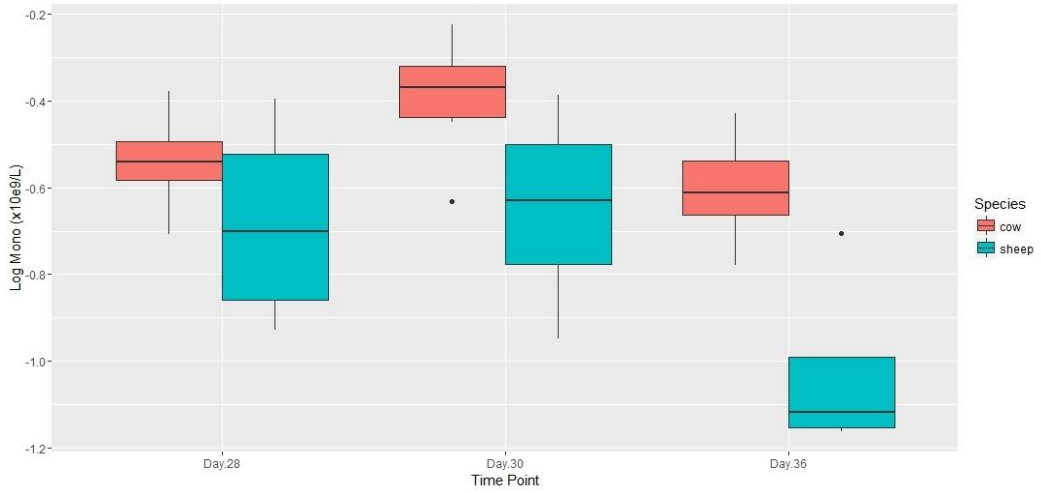

Figure 5-5: Box and whisker plot showing monocyte count at D-28, D-30 and D-35 for both species. Monocyte count was significantly affected by both overall time-point and species (p <0.05). Change in monocyte count was only significantly between D-28 and D-30 for cattle.

5.4.4 **Semi-Quantitative PCR**

As shown in table 5-7, the effect of overall time-point on the fold change, relative to the housekeeping genes *GAPDH* and *RPLPO*, was significant only for *NHE3* expression (*p* <0.05). The effect of species or the overall time-point/species interaction term on gene expression levels was not significant for any gene. Change in expression between D-28 and D-30 was significant for *NHE3* for cattle. Between

D-28 and D-35, change in expression was significant for both species for *IL1β* and was significant in cattle for *NHE3* expression.

Table 5-7: Table showing the effect of time-point, species and their interaction term (one way ANOVA with time-point and species as fixed effects) on gene expression levels for each of the 4 genes studied (CCL11, IL1B, NHE3, TLR4) and the significance of the change in gene expression levels between D-28 and D-30 and between D-28 and D-35 (t-test, mu = 0).

	Significance				T-Test significance	
Variable	Time-point	Species	T-P*Species	Species	D-28 and D-30	D-28 and D-35
CCL11 fold change	0.38	0.46	0.54	Cow	0.35	0.29
				Sheep	0.36	0.20
IL1B fold change	0.20	0.55	0.05	Cow	0.07	**0.03**
				Sheep	0.07	**0.02**
NHE3 fold change	**0.001**	0.67	0.82	Cow	**0.05**	**0.05**
				Sheep	0.14	0.07
TLR4 fold change	0.20	0.63	0.64	Cow	0.24	0.59
				Sheep	0.22	0.10

Figures 5-6 and 5-7 show the change in the relative expression levels for each gene, for each individual animal, between D-28 and D-30 and between D-30 and D-35 respectively. The majority of animals tended towards increase in the levels of expression of all genes between D-28 and D-30 and tended towards decrease in expression of all genes between D-30 and D-35.

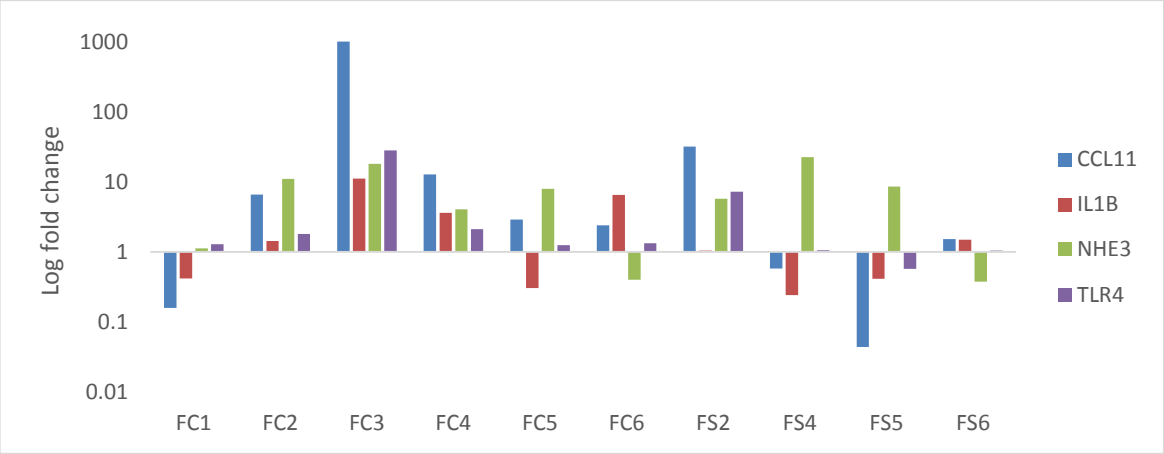

Figure 5-6: Bar chart showing the fold change for each gene of interest between D-28 (basal diet) and D-30 (1 day post challenge) relative to the housekeeping genes GAPDH and RPLPO for each individual animal.

Figure 5-7: Bar chart showing the fold change for each gene of interest between D-30 (1 day post challenge) and D-35 (1 week on the challenge diet) relative to the two housekeeping genes GAPDH and RPLPO, for each individual animal.

5.4.5 **SCFA**

SCFA concentrations were only significantly different between species for acetate (p <0.05). The effect of time-point as a factor and the overall time-point/species interaction was not significant for any SCFA, as shown in table 5-8. However in cattle, the change in propionate concentration between D-28 and D-30 was significant, and between D-28 and D-35 there were significant changes in butyrate, isovalerate and valerate. For sheep, there was no significant change in SCFA between any time-points. The mean SCFA concentration for both species followed similar patterns in acetate, butyrate and total SCFA but other SCFA differed between species.

Table 5-8: Table showing the effect of time-point, species and their interaction term on SCFA proportions across time-points (one way ANOVA with time-point and species as fixed effects) and the significance of the change in SCFA proportions between D-28 and D-30 and between D-28 and D-35 (t-test, mu = 0).

	Significance				T-Test significance	
Variable	Time-point	Species	T-P* Species	Species	TP-A and TP-B	TP-A and TP-C
Total SCFA (mmol/L)	0.964	0.190	0.435	Cow	0.09	0.1
				Sheep	0.07	0.88
Acetate (mmol/L)	0.509	0.044	0.325	Cow	0.12	0.36
				Sheep	0.1	0.56
Propionate (mmol/L)	0.398	0.689	0.199	Cow	0.005	0.23
				Sheep	0.71	0.44
Isobutyrate (mmol/L)	0.366	0.877	0.164	Cow	0.9	0.15
				Sheep	0.41	0.75
Butyrate (mmol/L)	0.426	0.238	0.926	Cow	0.55	0.0009
				Sheep	0.22	0.26
Isovalerate (mmol/L)	0.124	0.441	0.010	Cow	0.13	0.0001
				Sheep	0.43	0.45
Valerate (mmol/L)	0.294	0.265	0.340	Cow	0.67	0.0003
				Sheep	0.37	0.4

Table 5-9: Table showing the mean, median and SD for SCFA concentrations for cattle and sheep across all time-points and at each individual time-point (D-28, D-30, D-35).

	Cow All T-P	Cow D-28	Cow D-30	Cow D-35	Sheep All T-P	Sheep D-28	Sheep D-30	Sheep D-35
Total SCFA mean	86.45	91.09	83.04	85.24	79.04	87.31	63.34	86.46
Total SCFA median	87.18	88.94	82.81	85.47	79.28	91.44	64.2	95.15
Total SCFA SD	9.21	6.83	9.76	10.25	19.98	21.33	12.33	19.07
Acetate mean	61.12	63.59	58.28	61.47	53.11	55.8	43.55	59.98
Acetate median	61.06	62.14	58.09	60.96	49.84	55.29	42.99	62.22
Acetate SD	6.64	4.91	7.74	6.98	13.74	17.29	7.34	12.02
Propionate mean	13.58	14.86	12.1	13.78	13.07	11.32	12.81	15.09
Propionate median	13.45	14.52	12.37	13.85	12.59	10.77	12.84	14.3
Propionate SD	1.8	1.2	1.22	1.86	4.97	4.96	4.45	6.05
Butyrate mean	9.32	9.95	9.58	8.44	9.52	14.6	5.7	8.26
Butyrate median	9.27	10.27	9.4	8.69	6.41	13.89	5.27	8.11
Butyrate SD	1.39	1.46	0.9	1.46	7.75	12.16	1.41	3.79
Isobutyrate mean	0.55	0.58	0.58	0.49	0.64	0.74	0.5	0.67
Isobutyrate median	0.57	0.55	0.58	0.54	0.62	0.6	0.51	0.65
Isobutyrate SD	0.08	0.07	0.06	0.11	0.17	0.28	0.08	0.05
Isovalerate mean	0.81	0.87	1.03	0.54	0.95	1.03	0.53	1.2
Isovalerate median	0.82	0.82	0.99	0.56	0.73	1.03	0.49	1.01
Isovalerate SD	0.25	0.15	0.14	0.13	0.51	0.28	0.12	0.66
Valerate mean	0.67	0.73	0.76	0.51	1.57	3.52	0.45	0.73
Valerate median	0.66	0.74	0.76	0.48	0.53	0.72	0.45	0.84
Valerate SD	0.15	0.1	0.1	0.09	3.38	5.85	0.1	0.28

Figures 5-8 and 5-9 show the acetate concentration and total SCFA concentration at D-28, D-30 and D-35 in each species.

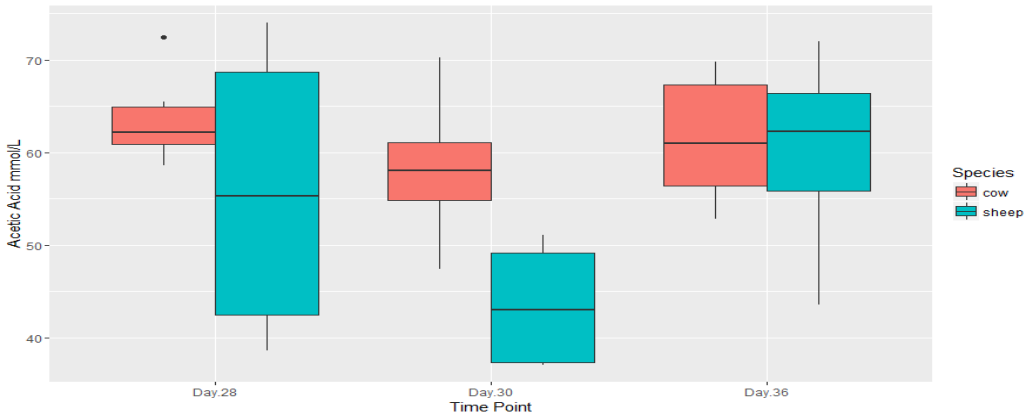

Figure 5-8: Box and whisker plot showing the acetate concentration at D-28, D-30 and D-35 for each species. Although significantly different between species, both species showed similar response patterns.

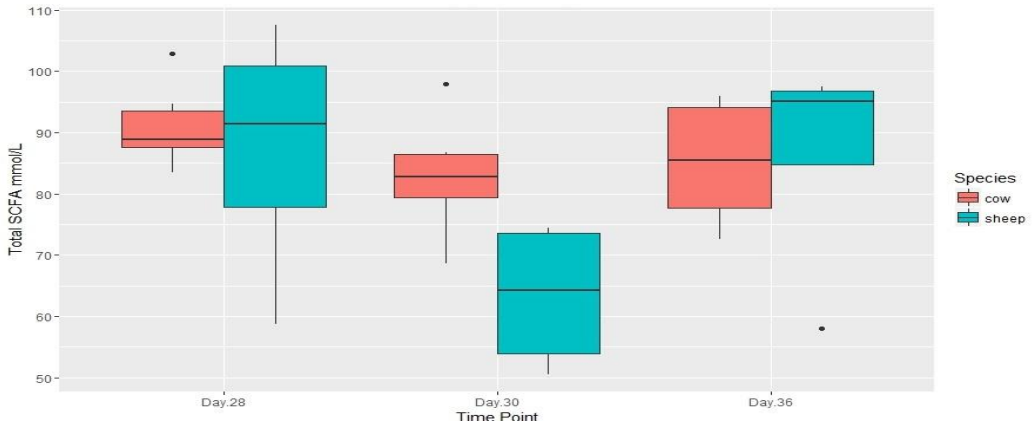

Figure 5-9: Box and whisker plot showing the total SCFA concentration at D-28, D-30 and D-35 for each species. Change was not significant between species or overall time-point but both species showed similar responses, tending to decrease at D-30 and increase at D-35.

5.4.6 **Histamine and lipopolysaccharide (LPS)**

Reticuloruminal fluid (RF) histamine concentration was significantly affected by overall time-point and species (p <0.05) but not their interaction term, as shown in table 5-10. Plasma histamine was not significantly affected by overall time-point in cattle but could not be analysed in sheep using the Abnova kit. The kit uses antibodies (Ab) which are raised in goats and resulted in non-specific binding. The sheep plasma bound to nonspecific sites with the goat Ab and disrupted the ELISA. To attempt to overcome this, a pooled plasma sample was tested alongside mouse IgG or a phosphate buffered saline/ bovine serum albumin mix (PBS/BSA) to try to block the non-specific binding sites. However, this was unsuccessful and there were too many missing and erroneous values to present the data. Ruminal fluid LPS was significantly different between species (p <0.05) but was not affected by time-point or the time-point/species interaction term. As shown in table 5-11, in both species the mean RF histamine increased at D-30 then decreased at D-35 (as did plasma histamine in cattle).

Table 5-10: Table showing the significance of the effect of time-point, species and their interaction on reticuloruminal fluid and plasma histamine concentration and reticuloruminal LPS concentration (one way ANOVA with time-point and species as fixed effects), for both species (plasma histamine only for cattle), across all time-points and the significance of the change between D-28 and D-30 and between D-28 and D-35 (t-test, mu = 0).

	Significance				T-Test significance	
Variable	Time-point	Species	T-P*Species	Species	D-28 and D-30	D-28 and D-35
RF Histamine (ng/ml)	**0.005**	**0.001**	0.438	Cow	**0.004**	0.078
				Sheep	0.217	0.832
Plasma Histamine (ng/ml)	0.775			Cow	0.151	0.451
				Sheep		
RF LPS (ug/ml)	0.204	**0.023**	0.335	Cow	0.969	0.092
				Sheep	0.760	0.240

Table 5-11: Table showing the mean, median and SD for reticuloruminal fluid and plasma histamine and reticuloruminal LPS for cattle and sheep across all time-points and at each individual time-point (D-28, D-30 and D-35). Plasma histamine could not be determined in sheep.

	Cow All T-P	Cow D-28	Cow D-30	Cow D-35	Sheep All T-P	Sheep D-28	Sheep D-30	Sheep D-35
RF Histamine mean	2.03	1.83	2.27	2	2.52	2.33	2.94	2.28
RF Histamine median	2.05	1.82	2.29	2.03	2.38	2.33	2.78	2.23
RF Histamine SD	0.27	0.26	0.16	0.19	0.53	0.19	0.72	0.35
Plasma Hist. mean	0.04	0.04	0.02	0.08				
Plasma Hist. median	0.03	0.04	0.01	0.04				
Plasma Hist. SD	0.68	0.42	0.43	1.08				
RF LPS mean	14018	17639	18227	4622.4	60121	91583	79244	9536
RF LPS median	6833.8	13474	5849	5062.8	14418	52033	78207	11615
RF LPS SD	20265	12874	31964	1325.2	80478	114735	72422	6773.9

5.4.7 **16S ribosomal RNA sequencing**

The results of the phylogenetic whole tree diversity (PD) are shown in figure 5-10. Results are shown for each animal at D-28 and D-30 (where D0 refers to D-28 and D2 to D-30) up to 5,000 reads. The results for the majority of animals were clustered together and did not show a huge range in PD at each time-point, but D-30 (D-2) results in sheep tended to be lower than in cattle. Table 5-12 shows the values for rarefaction measure (PD) for each animal at D-28 and D-30 for 5,000 reads. All animals showed a decrease in PD at D-30, except FC4, which showed a slight increase. The majority of sheep showed a larger decrease in comparison to the cattle, with the exception of sheep FS6 who showed a small change in PD.

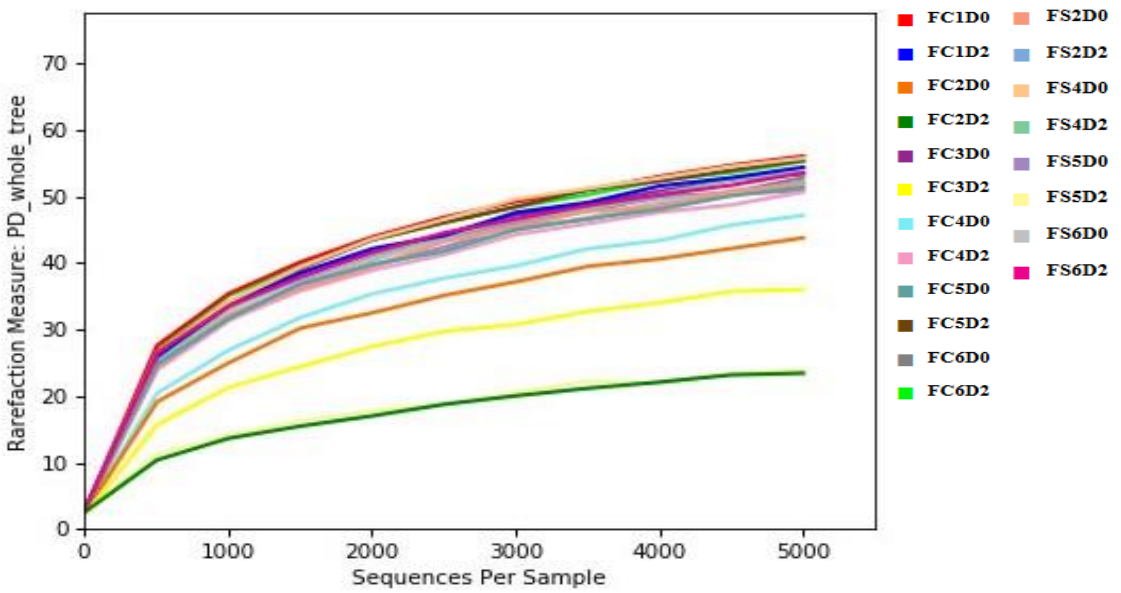

Figure 5-10: PD whole tree diversity at 5,000 reads for each animal at D-28 and D-30 (basal diet and challenge diet). The PD value for the majority of animals at 5,000 reads clustered between 50-60 with some noticeable decreases to below 20 for FS4 and FS5. In the legend, DO refers to D-28 and D2 to D-30.

Table 5-12: Mean rarefaction measure (Phylogenetic diversity (PD)) results for D-28 and D-30 for all animals and the difference in PD between the two time-points. The majority of animals decreased PD from D-28 to D-35. Sheep generally showed larger losses than the cattle, except sheep FS6 showed a small loss.

	Day-28		Day-30		
	Mean Rarefaction Measure	Mean SD	Mean Rarefaction Measure	Mean SD	Difference (D-30 – D-28)
FC1	56.04	1.06	53.55	1.20	-2.49
FC2	52.27	1.01	51.85	0.89	-0.42
FC3	55.26	0.97	54.37	0.62	-0.89
FC4	55.42	1.04	55.74	0.72	0.34
FC5	53.61	1.08	51.83	1.21	-1.78
FC6	53.43	0.80	52.85	1.04	-0.57
FS2	50.94	1.03	36.04	0.85	-14.90
FS4	43.78	0.98	23.88	0.90	-19.90
FS5	47.14	1.28	23.45	1.42	-23.70
FS6	51.37	0.94	50.67	1.16	-0.71

As shown in figure 5-11, the results of the unweighted UniFrac plot for the 16S data showed that at D-28 (red points) and D-30 (blue points), cattle were clustered together (green circle), generally as were sheep (orange circles). However, the sheep that appeared to be most affected by the diet change (FS4 and FS5), showed a wide divergence at D-30, separating completely from the other sheep and the cattle (shown at the far left of the plot). These 2 animals showed a vast change in their community structure following the introduction of the challenge diet.

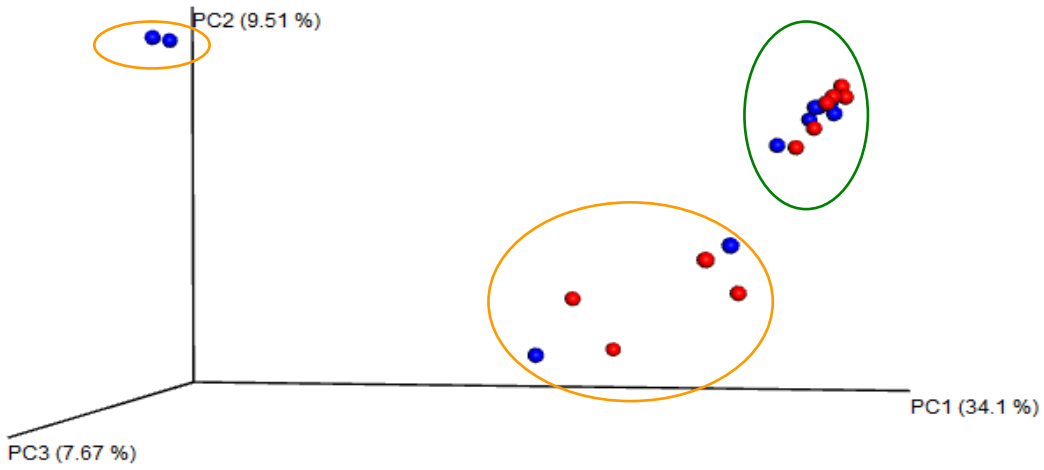

Figure 5-11: Unweighted UniFrac plot for cattle and sheep at D-28 and D-30. Red dots refer to D-28 values and blue points refer to D-30. The group circled in green represent cattle and the groups circled in orange represent sheep. Those at the far left of the plot represent sheep FS4 and FS5, which were most affected by the challenge diet.

Figure 5-12 shows a bar chart of the percentage of the total phyla observed in each individual animal that was contributed by each bacterial or archaeal phylum observed at D-28 and D-30. The graph shows that for cattle, although there were changes in the phyla observed pre and post challenge, the animals tended to show the same phyla before and after the introduction of the challenge diet. However, for those sheep that ate the challenge diet (FS2, FS4 and FS5), there was a substantial change in the phyla observed post challenge, with some phyla disappearing completely and new ones appearing. Sheep FS6, which refused the majority of its feed showed slight changes between D-28 and D-30 but tended to show the same species at both time-points.

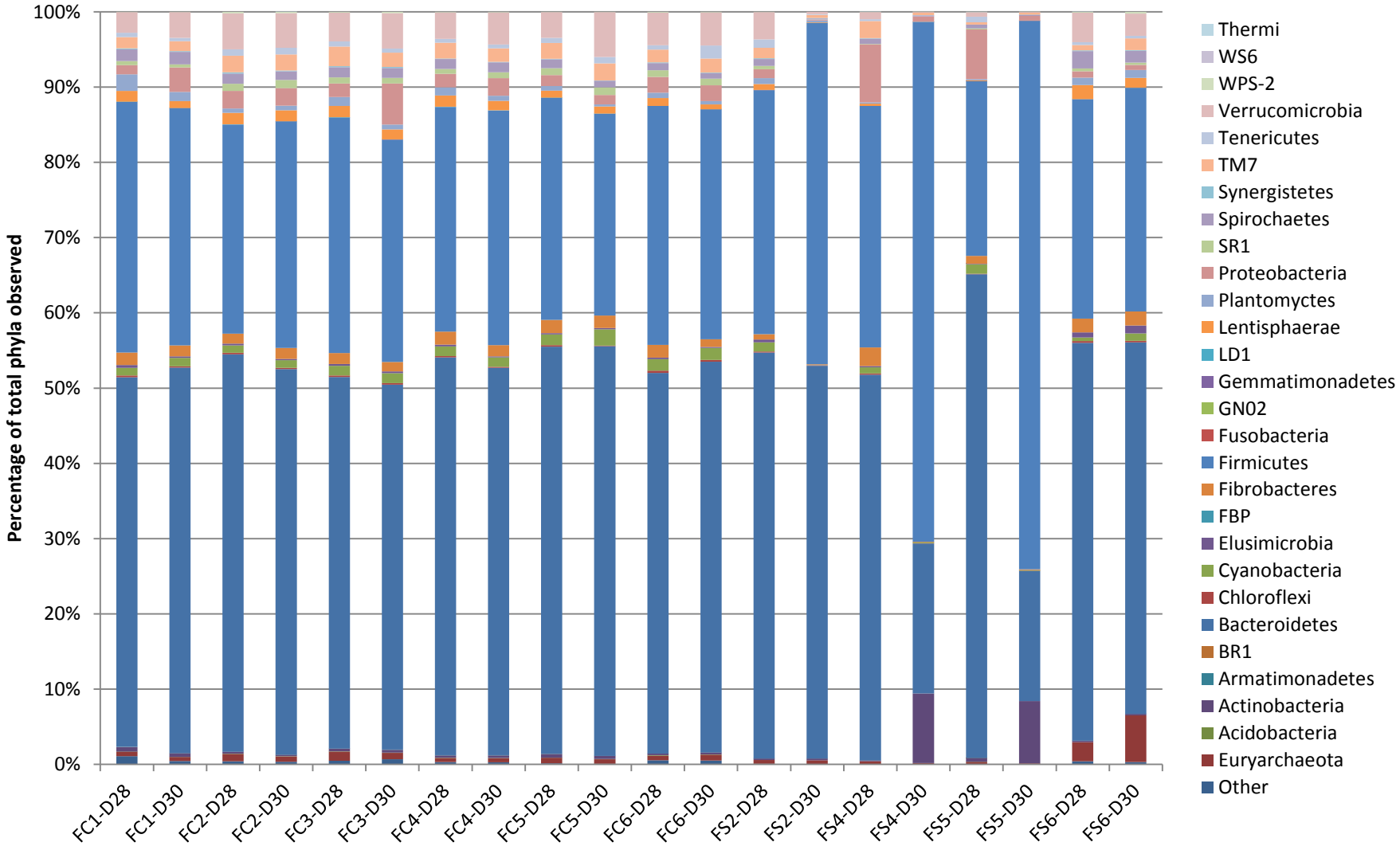

Figure 5-12: Stacked bar plot showing those phyla observed at D-28 and D-30, for each individual animal and the percentage of the total phyla represented by each for each individual bacteria or archaea present as created in Qiime.

Figures 5-13 and 5-14 show LefSe cladograms of the biomarkers (discriminative bacterial/archaea species significantly different in abundance between groups) that were observed at D-28 and D-30 for each species. These cladograms highlight those taxa that were significantly higher in that specific species at that time-point, where red represents cattle and green represents sheep. Overall, 21 biomarkers were identified for cattle and 16 for sheep at D-28, and 31 were identified for cattle and 8 for sheep at D-30. These were considered to be biomarkers for animals on the basal diet and for an animal following introduction of the high carbohydrate diet respectively. Only those biomarkers which were significant from 4 levels of biological classification (family level) or further are detailed here. LefSe classifies according to biological classification/standard taxonomy to the level it can identify to: phylum, class, order, family, genus then finally species. Tables 5-13 and 5-14 list the biomarkers for each species at D-28 and D-30 that were significant from 4 levels (family), their phylum and significance level. These biomarkers show a clear change in the abundance and type of phyla present pre and post-challenge for both cattle and sheep, with sheep showing a much larger loss of biomarkers at D-30.

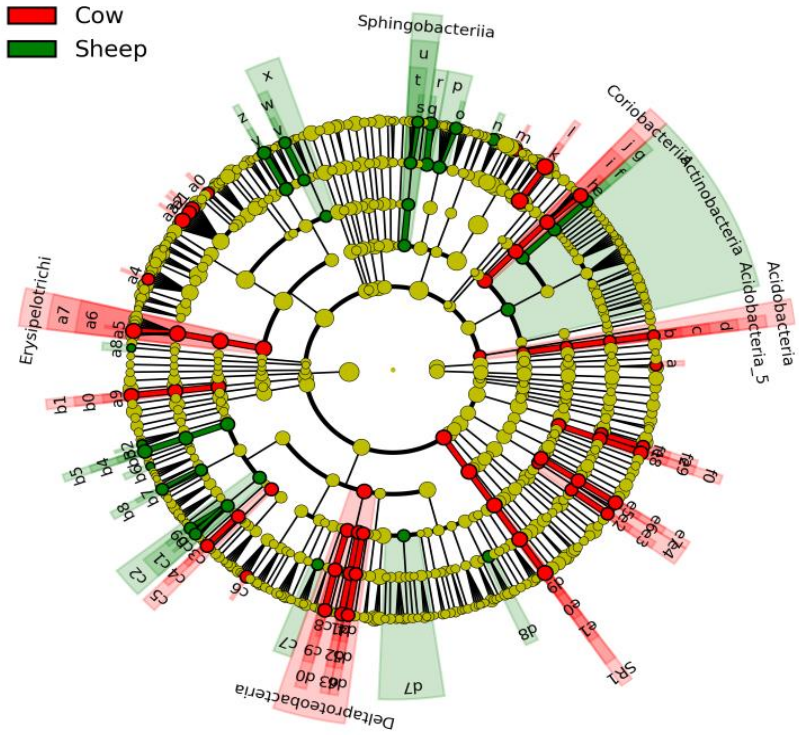

Figure 5-13: Taxa biomarkers for D-28 (basal ration) for both species. Biomarkers associated with cattle are highlighted in red and biomarkers in sheep are highlighted in green. 21 biomarkers were identified for cattle and 16 for sheep.

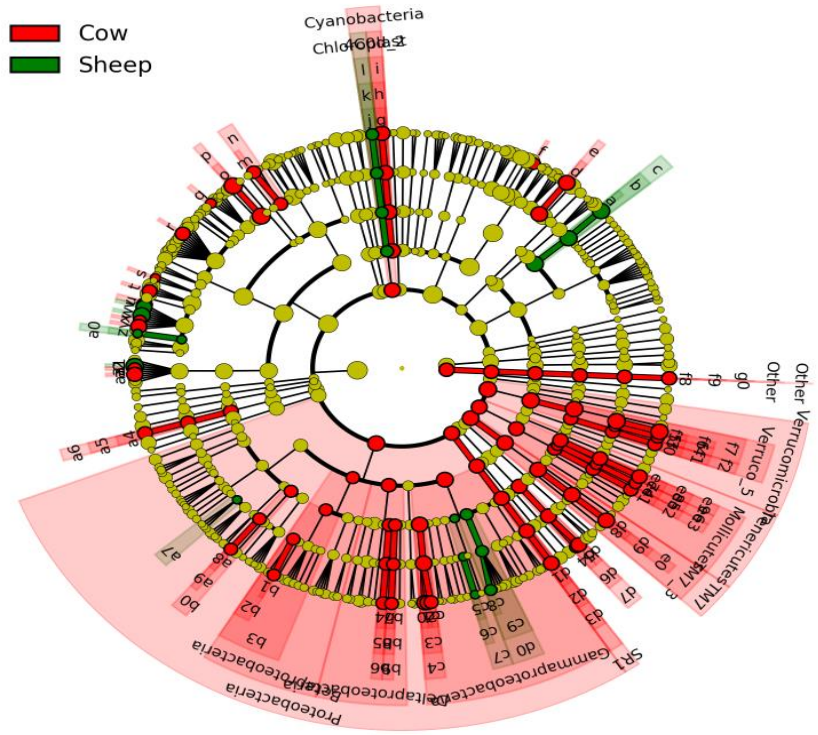

Figure 5-14: Taxa biomarkers for D-30 (challenge diet) for both species. Biomarkers associated with cattle are highlighted in red and biomarkers in sheep are highlighted in green. 31 biomarkers were identified for cattle and 8 for sheep.

Table 5-13: Table showing the biomarkers identified at D-28 for both species. Phylum and significance level[1] are detailed. Those taxa that are listed as "unknown name" were recognised via Greengenes in LefSe but did not have an associated name in the database.

Time-point A		
Species	Phylum	Significance Level
Cow	Actinobacteria	Family – Coriobacteriaceae
Cow	Acidobacteria	Genus - unknown name
Cow	SR1	Genus - unknown name
Cow	Proteobacteria	Genus- Various
Cow	Firmicutes	Genus- RFN20
Sheep	Bacteroidetes	Family – Sphingobacteriaceae
Sheep	Actinobacteria	Family – Bifidobacteriaceae

1. **Significance level refers to the most specific taxonomic biological classification level that the phylum could be identified down to by the Greengenes Database in LefSe.**

Table 5-14: Table showing the biomarkers identified at D-30 for both species. Phylum and significance level are detailed. Those taxa that are listed as "unknown name" were recognised via Greengenes in LefSe but did not have an associated name in the database.

Time-point B		
Species	Phylum	Significance Level
Cow	Cyanobacteria	Genus - YS2, unknown
Cow	Other – Unknown	Species – unknown
Cow	Verrucomicrobia	Family - RFP12
Cow	Tenericutes	Species – Anaeroplasma
Cow	TM7	Family - CW040
Cow	SR1	Genus - Unknown name
Cow	Proteobacteria	Species – Various
Sheep	Cyanobacteria	Family - Chloroplast

5.4.8 **Histology**

Histological score was only significantly different between species for stratum corneum thickness (SCT) and MHC2 positive cell count ($p < 0.05$), and was not affected by overall time-point or the overall time-point/species interaction term as shown in table 5-15. Change in variables between time-point was not significant for any variable, for either species. Table 5-16 shows the mean, median and range for histological parameters for each species over all time-points and for each species at each individual time-point. Change between time-points was statistically similar in both species; the means for SCT, SGT and vascular diameter were consistent with an increase at D-30 in both species. Immunohistochemistry results were statistically similar again between species but mean counts were consistent with a decrease at D-30 in cattle and an increase in sheep.

Table 5-15: Table showing the significance of overall time-point, species and their interaction term on histological parameters (GLM) and the significance of the change in values for histological parameters between D-28 and D-30 and D-28 and D-35 (t-test, mu = 0).

	Significance				T-Test significance	
Variable	Time-point	Species	T-P*Species	Species	TPA and TPB	TPA and TPC
SCT	0.62	**0.01**	0.67	Cow	0.05	0.53
				Sheep	0.19	0.48
SGT	0.80	0.17	0.52	Cow	0.87	0.57
				Sheep	0.34	0.31
VASCD	0.06	0.13	0.23	Cow	0.14	0.05
				Sheep	0.13	0.12
CD3	0.90	0.30	0.66	Cow	0.62	0.83
				Sheep	0.13	0.90
MHCII	0.26	**0.001**	0.26	Cow	0.11	0.11
				Sheep	0.73	0.78

Table 5-16: Table showing the mean, median and SD for histological parameters for cattle and sheep across all time-points, and at each individual time-point (D28, D-30 and D-35).

	Cow All T-P	Cow D-28	Cow D-30	Cow D-35	Sheep All T-P	Sheep D-28	Sheep D-30	Sheep D-35
SCT mean	3.24	3.24	3.45	3.05	8.85	5.06	15.63	5.87
SCT median	3.13	3.13	3.31	2.93	5.96	4.72	14.89	5.47
SCT SD	0.57	0.78	0.56	1.21	7.94	1.24	12.32	2.56
SGT mean	35.32	34.68	35.04	36.25	40.88	38.70	46.90	37.05
SGT median	34.20	32.77	35.38	35.35	36.30	33.45	45.48	36.74
SGT SD	5.71	7.35	6.30	14.35	12.55	13.56	16.65	9.72
VASCD mean	15.48	11.22	18.42	16.79	20.43	13.62	21.24	26.42
VASCD median	13.09	12.23	14.99	16.86	17.96	14.38	20.46	24.01
VASCD SD	7.21	2.30	11.50	7.29	8.59	4.45	7.49	10.43
CD3 mean	57.72	59.00	53.33	60.83	72.75	64.75	85.75	67.75
CD3 median	60.00	58.50	53.50	64.50	74.00	63.00	89.00	70.50
CD3 SD	13.54	16.99	12.56	26.00	19.94	26.61	16.09	16.70
MHCII mean	63.78	77.00	57.33	57.00	28.00	24.25	31.75	28.00
MHCII median	61.50	82.00	60.00	48.50	23.00	19.50	31.00	25.50
MHCII SD	17.37	17.39	12.36	26.98	15.84	21.23	20.82	9.06

Figures 5-15 and 5-16 show the significant difference between species in the changes observed in SCT and MHC2 across time-points. SCT showed a wide range

of values in sheep at D-30 in comparison to cattle.

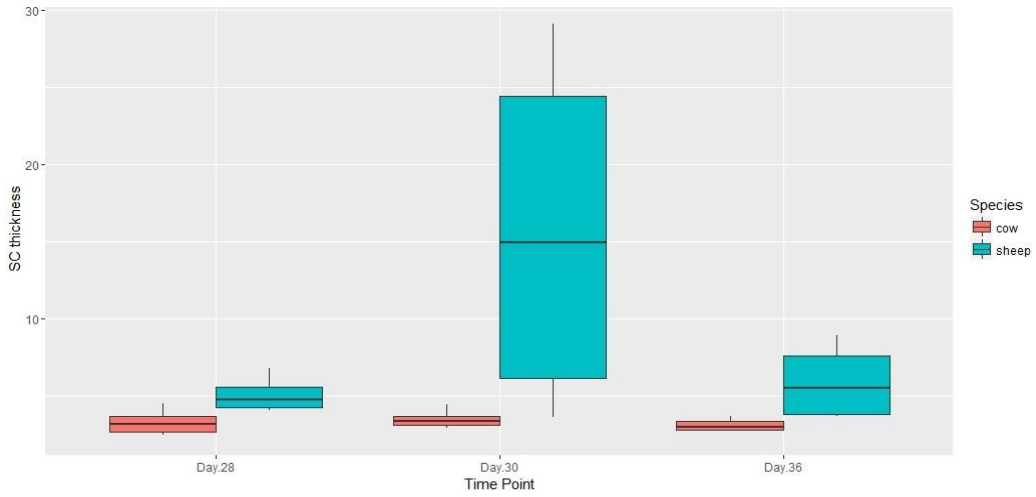

Figure 5-15: Box and whisker plot showing average stratum corneum thickness at D-28, D-30 and D-35 for each species. Difference between species was significant.

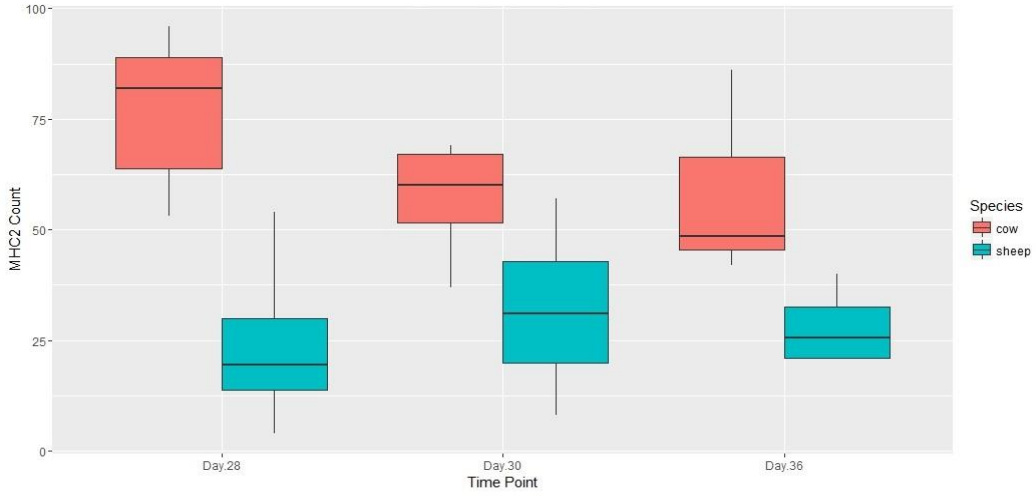

Figure 5-16: Box and whisker plot showing the average MHC2 positive count at D-28, D-30 and D-35 for each species. Difference between species was significant.

For categorical variables, the sample numbers were not compatible with effective Chi-squared testing. Tables 5-17 and 5-18 show the count for each categorical score for each of the categorical variables as a proportion of the total, for all cattle and all sheep across all time-points, and the count for each score for each species at each time-point. Stratum corneum integrity scores (SCINT) appeared to be lower in cattle than sheep and sheep showed no microabscesses in any samples.

Table 5-17: Table showing the proportion (%)of each categorical score[1] for the histological variables for cattle and sheep across all time-points.

SWELLSCORE	**2**	**3**	**4**	**5**	**6**
Cow	5.55	16.66	66.66	0	11.11
Sheep	8.33	33.33	25	8.33	25
VACSCORE	**0**	**1**	**2**		
Cow	33.33	38.88	27.77		
Sheep	58.33	33.33	8.33		
SCINT	**1**	**2**	**3**	**4**	**5**
Cow	44.44	27.77	5.55	16.66	5.55
Sheep	0	0	33.33	16.66	50
SLOUGH	**1**	**2**	**3**		
Cow	61.11	33.33	5.55		
Sheep	33.33	16.66	50		
MICROABSCESS	**0**	**1**			
Cow	61.11	38.88			
Sheep	100	0			

1.Histological score created using the scoring system detailed in section 3.3.7

Table 5-18: Table showing the proportion (%) of each categorical score[1] for the histological variables for each species for each sampling day.

	SWELLSCORE	**2**	**3**	**4**	**5**	**6**
Cow	D-28	0	16.66	66.66	0	16.66
Cow	D-30	0	16.66	66.66	0	16.66
Cow	D-35	16.66	16.66	66.66	0	0
Sheep	D-28	0	50	25	0	25
Sheep	D-30	25	25	25	0	25
Sheep	D-35	0	25	25	25	25
	VACSCORE	**0**	**1**	**2**		
Cow	D-28	33.33	33.33	33.33		
Cow	D-30	50	0	50		
Cow	D-35	16.66	83.33	0		
Sheep	D-28	50	50	0		
Sheep	D-30	75	25	0		
Sheep	D-35	50	25	25		
	SCINT	**1**	**2**	**3**	**4**	**5**
Cow	D-28	33.33	50	0	0	16.66
Cow	D-30	66.66	16.66	16.66	0	0
Cow	D-35	33.33	16.66	0	50	0
Sheep	D-28	0	0	0	25	75
Sheep	D-30	0	0	25	0	75
Sheep	D-35	0	0	75	25	0
	SLOUGH	**1**	**2**	**3**		
Cow	D-28	50	50	0		
Cow	D-30	66.66	16.66	16.66		
Cow	D-35	66.66	33.33	0		
Sheep	D-28	25	25	50		
Sheep	D-30	0	25	75		
Sheep	D-35	75	0	25		
	MICROABSCESS	**0**	**1**			
Cow	D-28	83.33	16.66			
Cow	D-30	50	50			
Cow	D-35	50	50			
Sheep	D-28	100	0			
Sheep	D-30	100	0			
Sheep	D-35	100	0			

1 Histological score as scored using the scoring system detailed in section 3.3.7

5.5 Discussion

The main objectives of this study were to determine the extent to which sheep and cattle varied in their responses to a challenge diet high in rapidly fermentable carbohydrates and thus, to what extent sheep should be used as a model for dietary manipulation in cattle, and to provide baseline data on the comparative 16S microbiome of sheep and cattle.

The challenge diet was formulated using an allometric scaling technique that considered the surface area and rumen capacity of sheep and cattle, resulting in a 5-fold increase in barley proportion for cattle and a 10-fold increase for sheep. Gut capacity is a constant proportion of body weight in mammalian herbivores (Clauss et al., 2007, Weckerly, 2010) and the isometric scalar of gut capacity, in relation to the allometric scalar of metabolic rate, has been shown to explain differences in digestive efficiency among various herbivore species (Han et al., 2015). Larger species have greater gut capacity, a higher feed intake, and should be expected to retain digesta for longer in the reticulorumen for fermentation (Clauss et al., 2007). From the overall reaction to the diet, feed refusals, ruminal content appearance/smell and clinical observations, sheep appeared to be considerably more affected in the challenge period in comparison to the cattle. Immediately post challenge (D-30), sheep FS6 showed noticeable changes in the appearance of her rumen contents, which persisted throughout the challenge week and she refused the majority of her ration from the beginning of the challenge week. As a result of the very high continued refusal of food over the challenge week, changes in parameters in sheep FS6 could not be attributed to a result of the challenge diet. Initially it was proposed that from the acute acidosis-type reaction and immediate refusal observed in sheep FS6 to the first day of the challenge ration, that she was possibly accidentally double fed her grain ration. However, as FS6 did not show decreased pH, it appears another factor was affecting her. There was no significant effect on any results by excluding FS6 from analysis and therefore she was left in for the analyses. Although sheep FS6 was the only sheep who showed such a marked and swift change in her rumen content appearance and an immediate refusal of the diet, all sheep refused a large majority of their challenge ration throughout the challenge week. In comparison, no cattle refused feed at any point throughout the challenge week and there were no obvious clinical symptoms of

any ill effects of the challenge diet in the cattle. . This clear difference in the refusal rate of the sheep and cattle in response to the challenge diet could reflect a species difference, or more likely could reflect an inaccuracy in the scaling calculations of the diets, leading to a greater challenge for the sheep in comparison to the cattle. The feed refusal observed in sheep is an expected possible consequence of voluntary feeding in challenge studies versus involuntary feeding. As the animals in this trial were all fistulated, it would have been possible to carry out an involuntary feeding challenge where grain was introduced directly into the rumen via the fistula or leftover feed was introduced directly, as is common practise in many studies (Keunen et al., 2002, Chiquette, 2009, Dionissopoulos et al., 2012). However, involuntary feeding does not allow direct comparison of results to results observed on farm and eliminates the adaptive mechanisms of saliva production from chewing the ration. To allow a direct comparison between scientific studies and on-farm practice, the trial needs to reflect those practices observed on farm as closely as possible.

Reticuloruminal pH was significantly different between time-points and species and differed significantly among individuals. Sheep showed a much wider range in reticuloruminal pH at D-30 in comparison to cattle and consistently had a lower mean pH across the trial. Only sheep showed a pH below 6 at any point in the trial, with increased hours spent below pH 6 at D-30 and two sheep showing minimum values of pH 5.1 and pH 4.23. Both of these minimum pH observations in the sheep would be considered to be acidotic and have SARA by standard definitions in the literature (< pH5.5 (Garrett et al., 1999), < pH5.6 (Cooper et al., 1999, Gozho et al., 2005), < pH6 (Plaizier, 2004)), but the pH minimum values observed in cattle would not. Although cattle did not show substantial changes in pH across the trial, both species significantly decreased pH between D-28 and D-30 and both increased significantly between D-30 and D-35. This suggests an initial effect of the challenge diet in both species at D-30, though more severe in sheep, and a compensatory effect raising the pH at D-35. This pattern, although more pronounced in sheep, followed the pattern reported in numerous SARA challenge studies, such as Steele et al (2011). This previous study reported an initial reduction, followed by a gradual increase when dairy cattle were suddenly changed to a diet high in rapidly fermentable carbohydrates. As pH is strongly linked with SCFA proportions and absorptions, the severity of this pH decrease and following increase, could be expected to follow the changes observed

in SCFA proportions and changes in microbial communities and diversity (Penner et al., 2009b).

The concentration of SCFA in the reticulorumen is a function of SCFA production from carbohydrate fermentation and the removal of SCFA from the rumen via absorption through the rumen wall or passage through to the omasum in the liquid phase (Peters et al., 1990). As the proportion of rapidly fermentable carbohydrates in the diet increases, so too does the production of SCFA (from the fermentation of these carbohydrates) and the corresponding rate of absorption of SCFA through the rumen wall. Therefore, high SCFA concentrations in the reticulorumen can arise as a result of high production of SCFA, low rate of removal of SCFA or any combination of these which results in accumulation. Only the change in propionate and butyrate was significant in cattle between D-28 and D-30 and D-28 and D-35 respectively, both decreasing across each time-point. The reduction in propionate between D-28 and D-30 was initially unexpected from the literature (Plaizier et al., 2008, Dijkstra et al., 2012) but was consistent with the observations in the study described in chapter 4, as well as being consistent with the findings at post-mortem in animals on high and low concentrate beef finishing diets described in chapter 3. However, it is hypothesised that the decreased propionate noted in this trial in cattle between D-28 and D-30 occurred as a result of rapid adaptation to high SCFA concentration and increased rate of absorption of SCFA via the rumen wall. Any reduction in SCFA after the challenge would be consistent with the significant increase in the relative expression of NHE3 in the rumen epithelium after the introduction of the challenge diet. NHE3 relative expression tended to increase in all animals between D-28 and D-30, with the exception of FS6, which did not follow similar response patterns to any other animal. As discussed in chapter 3, increased absorption of SCFA is thought to occur via increasing the surface area of the rumen epithelium which in turn leads to an increase in the activity and therefore relative expression, of ion exchangers such as NHE3 (O'Shea et al., 2016). Although not a significant change, the mean total SCFA observed for each species showed a decrease between each time-point, as observed in chapters 3 and 4 as concentrate in the diet increased.

LPS is a component of the outer membrane of gram-negative rumen bacteria, released during bacterial lysis occurring as a result of decreased reticuloruminal pH

(Gozho et al., 2007). LPS has been shown to migrate from the rumen into circulation as a result of failure of the rumen epithelial tight junctions, due to decreased reticuloruminal pH, where it is capable of eliciting a host immune response (Plaizier et al., 2012). The release of LPS is associated with grain induced SARA and has been shown not to occur as a result of SARA induced with other carbohydrate sources such as alfalfa hay pellets (Khafipour et al., 2009, Li et al., 2012b). LPS concentrations observed in this trial were significantly higher in sheep than cattle across the whole trial but did not vary significantly between time-points for either species despite the significant pH decreases noted in both species. LPS results have been shown to be extremely varied between individuals in previous studies, such as those in chapter 3 and 4, so this variation could reflect true individual variation in animals. The wide variation between species and individual animals suggests that LPS concentration may not be a suitable variable for quantifying the effect of challenge diets. The LPS concentrations in this study did not reflect the large pH drop noted in sheep at D-30 as expected.

As with reticuloruminal LPS, reticuloruminal fluid histamine is also of ruminal bacterial origin and is produced as a result of decarboxylation of histidine by ruminal bacteria (Wicki and Schatzmann, 1977). Histamine is commonly measured in SARA/acidosis studies due to the proposed relationship between ruminal acidosis and bovine laminitis and the possible causative effect of histamine in the development of bovine laminitis (Nocek, 1997) as a result of decreased reticuloruminal pH (Dain et al., 1955, van der Horst, 1961, Irwin et al., 1979). Additionally, histamine producing bacteria have been shown to be present in high numbers in grain fed animals but were undetectable or present in low concentrations in forage fed animals (Garner et al., 2002). In this study, reticuloruminal fluid histamine level was significantly affected by time-point, but the increase was only significant between D-28 and D-30 for cattle, and was significantly different between species, with sheep showing a higher mean concentration over all time-points. The mean reticuloruminal fluid histamine concentration for both species followed the same pattern, increasing between D-28 and D-30 and decreasing between D-30 and D-35. Plasma histamine was only analysed in cattle and was not significantly different between any time-points.

Overall, it was expected that given the severity of their pH and appetite responses, sheep would show significantly higher concentrations of LPS in comparison to cattle. However, the lack of increase noted in LPS in sheep at D-30 could reflect the fact that LPS concentrations are dependent on the types of bacteria present in the rumen to begin with; it is possible that gram-negative bacteria were lower in sheep in comparison to cattle at this point as a result of the diet change.

The results for both species for reticuloruminal pH, SCFA and histamine were broadly consistent with each other. However, the results from the 16S sequencing showed a larger variation between the 2 species, particularly at D-30, following the dietary challenge. 16S ribosomal RNA (rRNA) sequencing is a commonly used and well-established method for studying phylogeny and taxonomy of samples from complex microbiomes, used to identify and compare bacteria present within a given sample. Unlike PCR-based approaches, next-generation sequencing (NGS) allows for a culture free method, which allows for analysis of the total microbial community in a sample (Illumina, 2016).

The 16S results were consistent with initial observations that sheep were more affected by the introduction of the challenge diet than cattle. The introduction of the challenge diet resulted in a larger loss of diversity in the ruminal microbiome in sheep than in cattle. Previous studies have shown that there is a core microbiome present in ruminants, regardless of host species (Henderson et al., 2015). In this study, the 2 main bacterial phyla present at both time-points, in both species, were *Firmicutes* and *Bacteroidetes*, consistent with the idea of a core microbiome. Beyond this similarity, substantial differences were observed in the phyla present in the two species, shown clearly in phyla taxa summaries. In cattle, there was variation between animals but the variation was not large. In sheep, as well as large pre and post-challenge variation, each animal showed more variation over time than each of the cows. UniFrac plots showed that cattle and sheep, despite changes in their overall pre and post-challenge microbiome, were relatively closely grouped before and after challenge. The 2 sheep which showed the lowest pH also showed a wide divergence between pre and post-challenge UniFrac grouping, again suggesting that these animals were affected very strongly by the dietary change and therefore that their rumen environment was very different to the rest of the animals.

LefSe biomarkers were as expected at D-28. In cattle at D-28, 5 biomarkers were identified from the phyla *Actinobacteria*, *Acidobacteria*, *SR1*, *Proteobacteria* and *Firmicutes*. The biomarkers observed in cattle at D-28 were as expected and many were common phyla previously associated with cattle fed a range of diets including pasture and TMR fed cattle (de Menezes et al., 2011) and high forage and high starch (Thoetkiattikul et al., 2013) and so were not surprising biomarkers at D-28. Other biomarkers observed such as *Proteobacteria* have been shown to be present in cattle from pre-weaning (Malmuthuge et al., 2014), in goats on a high hay diet (Liu et al., 2015) and are commonly found in adult cattle on a range of diets, but tended to be found in higher percentages in high or moderately high forage diets in comparison to high grain (Petri et al., 2014). The only biomarker at D-28 which was unexpected was phylum *Firmicutes,* genus *RFN20*. *RFN20* has been shown to be higher in dairy heifers fed a high grain diet (De Nardi et al., 2016) and in cattle fed a higher proportion of grain in comparison to forage diets (Pitta et al., 2014). A bacterial genus present at D-28, which shows a relationship with high grain, was unexpected, as the other biomarkers for cattle were associated with a higher forage diet. The presence of a genus associated with higher grain levels could perhaps mean that the cattle were fed a higher grain percentage prior to the beginning of the basal diet than expected and their rumen environment was still suitable for high grain associated bacteria.

At D-28 in sheep, 2 phyla were identified as biomarkers, *Bacteroidetes* and *Actinobacteria*. The presence of these biomarkers in sheep at D-28 was expected, as they have been shown in previous studies to be the dominant phyla in sheep fed on a maintenance, basal diet and have been shown to be present in higher proportions in animals fed a diet higher in forage (grass silage) (Dryden et al., 1962), fitting with the D-28 diet.

At D-30 in cattle, 7 phyla were considered as biomarkers. Many of the phyla considered as biomarkers have previously been shown to increase in cattle fed on a mixed straw and concentrate diet (Zhao et al., 2015) and are common in the rumen microbiome (Ametaj et al., 2010, Li et al., 2012a). For example, *Verrucomicrobia* and *Anaeroplasma* have been shown to significantly increase in goats fed diets with increased concentrate proportions (Shen et al., 2016) and have been shown to increase during a period of high grain feeding in cattle, intended to induce SARA (Jami

et al., 2013) fitting with the challenge diet fed. One of the biomarkers observed in cattle at D-30, A*naeroplasma,* has been shown to decrease in cattle fed a high concentrate (70%) diet intended to induce acidosis (De Nardi et al., 2016) and are known to have a poor tolerance for low ruminal pH. Their presence suggests that SARA was not induced in the cattle, or more specifically that their pH did not reach extreme levels – fitting with the pH traces observed (Loor et al., 2016). Other biomarkers noted at D-30 in cattle have been shown to increase in cattle fed a diet of grain and alfalfa hay plus histidine (Golder et al., 2014), have been shown to be present in dairy cattle fed a 70% concentrate diet (Jami et al., 2014) and have been shown to be higher in cows after an initial short SARA challenge diet in comparison to a longer period on a diet intended to induce SARA (Wetzels et al., 2016), fitting with the short period of time cattle were exposed to the challenge diet during this study.

In sheep, at D-30 only 1 phylum was significantly higher in sheep and considered as a biomarker. Phylum *Cyanobacteria* was significant down to the family *Chloroplast* and order *Streptophyta*. Sequences recovered from the family *Chloroplast* originate from plant cells and so *Streptophyta* present in the family *Chloroplast* are present either due to consumption of forage (Kim et al., 2014) or are present due to colonisation of barley prior to ingestion. As the barley and the straw bedding was not sterilised prior to the trial, any unexpected bacteria which appeared in the 16S results may have occurred from the barley or surroundings. Without sequencing both the barley and swabs from the environment this cannot be confirmed. However, the fact that this was the only phylum significantly higher in sheep at D-30 suggests that the loss of diversity seen in sheep as a result of the diet change was so extreme that their microbiome levels were vastly depleted in comparison to cattle. Overall, 16S results for both species showed a negative effect of the challenge diet which was far more severe in sheep but the grouping of the species in the UniFrac plots suggest that overall the core microbiome and responses were similar between species.

An increase in the expression of inflammation-related genes in the ruminal epithelium has previously been shown in cattle fed a diet high in rapidly fermentable carbohydrates, specifically those diets high in grain (Zhang et al., 2016). Grain induced SARA has been shown to increase levels of acute phase proteins, such as haptoglobin and serum amyloid A (SAA), as studied in chapter 4, indicating a systemic

inflammatory response in cattle (Gozho et al., 2007). This systemic inflammatory response is thought to partially occur as a result of the previously mentioned translocation of LPS and histamine, due to disruption of the rumen epithelial barrier function and increased permeability of the rumen epithelium due to decreased reticuloruminal pH, though the mechanisms are not clear (Emmanuel et al., 2007, Plaizier et al., 2008, Klevenhusen et al., 2013). As described by Zhang et al, following disruption of the rumen epithelial barrier, lymphoid tissue associated with the rumen epithelium, including innate lymphoid cells and mast cells (Kurashima et al., 2013) respond by triggering local inflammation of the rumen epithelium and increasing cytokine production (Mani et al., 2012, Kurashima et al., 2013). This previously mentioned study by Zhang et al investigated the effects a high concentrate diet and low concentrate diet on the expression of inflammatory related genes in the rumen epithelium. Those genes studied included interleukin and C-C motif cytokines, as studied in this current trial. Zhang et al found that in cattle fed a high concentrate diet, expression of inflammation related genes was up-regulated, indicating local inflammation in the rumen epithelium and a corresponding immune response (Zhang et al., 2016). Additionally, the expression of interleukins was shown to increase with increased LPS and lowered pH suggesting the combination of these two factors is important in the bovine rumen immune response (Zhang et al., 2016). This increased expression of inflammation related genes has been associated with decreased feed intake and a decrease average daily body weight gain in cattle, particularly in those animals with increased expression of *IL1β* (Hagg et al., 2010).

In this study, *CCL11, IL1β, NHE3* and *TLR4* were studied further. As previously mentioned, levels of expression of C-C motif cytokines and interleukins such as *CCL11* and *IL1β*, have been shown to increase as a result of dietary induced inflammation in the rumen. *CC11* is a member of the C-C class of chemokines expressed predominantly by T cells and monocytes, which are associated with chronic inflammation that occurs over a period of weeks or years (White et al., 2013). *IL1B* is a pro-inflammatory cytokine that plays a role in acute and chronic inflammation, has been associated with pain and autoimmune conditions and aids in antibody production by acting as a growth factor for B cell proliferation (Ren and Torres, 2009). *NHE3*, although not involved in an immune/inflammatory response was investigated due to its previously mentioned positive relationship with increased SCFA absorption.

Increased absorption of SCFA is thought to be achieved through increasing the epithelial surface, increasing the surface area for SCFA absorption that subsequently leads to an increase in the expression of ion exchangers such as *NHE* transporters (Graham and Simmons, 2004;Henderson et al., 2015; O'Shea et al., 2016). It is known that toll-like receptor 4 (*TLR4*) can recognise by-products of bacterial fermentation, such as LPS, and stimulates the host circulatory and rumen epithelial immune response, as well as promoting the proliferation of epithelial cells and barrier function of intestinal epithelium (Abreu, 2010). *TLR4* can recognise LPS due to its high specificity for the lipid-A portion of LPS (Park and Lee, 2013). Dysregulation of this process can cause chronic inflammatory responses and epithelial barrier dysfunction (Abreu, 2010). Therefore, increased *TLR4* expression indicates not only initiation of innate responses via LPS but may aid in protecting the rumen epithelium by increasing epithelial proliferation, which in turn could lead to an increase in *NHE3* expression.

In the present study, between D-28 and D-30, only the increase in *NHE3* was significant for cattle and the change in *IL1β* tended toward a significant increase in both species. Between D-30 and D-35, expression levels were significantly different for *IL1β* in both species, decreasing and *NHE3* decreased in cattle and *NHE3* tended towards a significant decrease in sheep. Sheep FS6, which refused the largest proportion of the challenge ration from the beginning of the trial, showed the smallest changes in gene expression of all animals studied. Overall, the response in gene expression levels for both species were broadly similar and tended to follow the same patterns expected from the literature, showing a pro-inflammatory effect of the challenge diet, increasing initially and then decreasing after a longer period exposed to the diet.

Histological scoring of rumen papillae biopsy, using the scoring system created for chapter 3, showed significant differences between species only for average stratum corneum thickness (SCT) and MHC2 count. No histological parameters were significantly different between time-points and no other parameters were affected by species or the overall time-point/species interaction term. There is no previous work detailing comparative histology of sheep and cattle maintained on the same diet to allow comparison, but this present study found that the mean score for all continuous histological parameters was higher in sheep than in cattle, except for MHC2 count.

However, no change in MHC2 was significant so this was not investigated further. This higher mean in sheep could reflect the more severe response observed in sheep, as increased SCT, SGT and vascular diameter have been associated with a grain fed diet in cattle and lambs (Hinders and Owen, 1965, Steele et al., 2012). Although not significant, it was noted that the mean score for the majority of histological parameters in both species increased at D-30 and decreased at D-35, suggesting a slight, but rapid histological change in the rumen of both species as a result of dietary manipulation. As the samples collected from the ruminal biopsy contained only papillae samples, it was not possible to carry out the full range of scoring on the samples collected in this study. If the full rumen wall had been collected and a wider range of histological parameters scored, there may have been a more significant change in variables between time-points. For the categorical histological variables, the main difference between the 2 species was a higher stratum corneum integrity score in sheep and a lack of microabscess in sheep. A higher SC integrity score reflects poorer SC integrity with the majority of papillae in the sample showing SC vacuolation and interruption to the SC. This higher score in sheep was noted in samples from D-28 and so could not be attributed to the challenge diet alone and may simply be part of the normal appearance of the sheep rumen. Microabscesses, although found only to be present in cattle, were not thought to be of major importance. Little is known about the presence or absence of microabscess and they were suggested in a study into reindeer rumen pathology to arise due to forage scratching the rumen epithelium and allowing bacterial entry (Josefsen et al., 1997). The histological results suggest that overall, as the majority of parameters did not vary significantly between species, the histological response of the rumen epithelium is likely to be comparable between species. However, as sheep had a higher mean score for the majority of parameters, it may not be possible to directly compare scores, rather the pattern observed. Histological scoring of both species using the full rumen wall – to allow full use of the scoring system - and a larger sample set would allow a better understanding of whether the histological responses were comparable between species.

Of the haematological variables studied, the majority were significantly affected by species. In cattle, the majority of variables were significantly different between D-28 and D-30 and D-30 and D-35. In sheep only neutrophil and PDW were significant between D-30 and D-35. Surprisingly, despite sheep showing more clinical symptoms

and a bigger decrease in pH, cattle showed a more pronounced haematological response. However, the mean values for all variables tended to follow the same patterns. The fact that so many variables were significantly different between species was expected, as it is well established that the normal range for parameters varies between cattle and sheep (Jackson and Cockcroft, 2007). Therefore, the significance of the change in each variable between time-points according to the t-test was compared, rather than the value for each variable itself.

RBC count and haemoglobin (Hb) increased significantly in cattle from D-28 to D-30 then increased again from D-30 to D-35. In sheep, the mean for both RBC and Hb followed a similar pattern between D-28 and D-30 but not D-30 and D-35 but change was not significant. An increase in RBC and Hb was previously noted in the trial detailed in chapter 4 after the introduction of a challenge diet. It is hypothesised that the increase in both RBC count and Hb arises due to increased osmotic pressure in the rumen. An increase in osmolarity would cause water to move from the circulation into the rumen, resulting in a higher RBC count and Hb level in the blood. Increased ruminal osmotic pressure as a result of increased concentrate feeding has been noted previously (Bennink et al., 1978). This would also account for the statistically significant increase in haematocrit (Hct) in cattle between D-28 and D-30 and between D-30 and D-35 and the tendency in sheep for the mean Hct to be increased and D-30. Increased RBC, Hb and HCT has been shown previously in heifers induced with SARA (Marchesini et al., 2013), suggesting that the diet was challenging both species. Mean cell volume (MCV) did not change greatly for either species at any time-point, which was expected, as it should not be affected by increased osmotic pressure in the rumen.

The mean overall white blood cell (WBC), neutrophil, lymphocyte, monocyte and eosinophil counts followed the same patterns in both species at both D-30 and D-35. However, change was only significant in cattle between D-28 and D-30 for neutrophil and monocyte count and between D30 and D-35 for WBC and neutrophil count. For sheep, change was significant only between D-30 and D-35 for neutrophil count. Both species showed a decrease in neutrophil count between time-points.

Overall, despite the significant species difference, the majority of haematological results showed similar response patterns in both species at D-30 and D-35, again

suggesting that sheep could be used as a model for cattle if the overall response were compared as opposed to the specific haematological response value.

Of the majority of variables studied in this trial, cattle and sheep showed similar response patterns to the challenge diet, and results were broadly consistent with previous SARA studies in cattle and sheep. A similarity in the response in both species was observed despite the fact that clinical symptoms of SARA were observed only in sheep and only sheep were considered acidotic by standard literature definitions of pH thresholds.

Overall it seems reasonable to conclude from this trial that sheep are a suitable model for further bovine nutrition studies. Both species showed similar responses in the majority of parameters investigated in response to a diet intended to induce acidosis, with the exception of 16S sequencing. If further testing was carried out to investigate the effect of an increased challenge in cattle or a decreased challenge in sheep, the clustering of the majority of animals in the 16S plots suggest that the 16S results could be comparable. The apparent divergence in the severity of the response to the challenge diet observed in this trial between cattle and sheep highlights the need for accurate dosing in sheep. The allometric scaling approach used in this trial to determine the challenge dosage may be inferior to dosing on a simple bodyweight basis. Future studies to determine the correspondence between challenges for cattle and sheep would be useful and allow a greater understanding of the use of sheep as a model for future bovine nutrition studies.

6 General Discussion

SARA is widely reported globally in animals from a range of management systems, fed on rations high in rapidly fermentable carbohydrates (Titgemeyer & Naaraja 2006; Kleen et al., 2009; Kleen & Cannizzo, 2012), and estimates of the financial impact of SARA are consistently high (Enemark, 2008). Occurrence of the condition varies with region, but has been suggested in a review by Kleen and Cannizzo (2012) to be up to 40% in the USA (Garrett et al., 1997). SARA has been shown to be associated with numerous ill effects, leading to increased culling rates and consequential financial

losses (Plaizier et al., 2008), lower production efficiency (Abdela, 2016) and lower welfare (Enemark, 2008).

The main objectives of the work presented in this thesis were to:

- Examine the range of responses in commonly studied traits to diets expected to induce ruminal acidosis. The variables investigated included haematological and biochemical variables, positive acute phase proteins, expression of genes associated with inflammation, reticuloruminal pH, reticuloruminal histamine, reticuloruminal LPS, reticuloruminal SCFA and production parameters.
- Assess the ability of new direct fed microbial agents (DFM) for controlling the effects of ruminal acidosis in cattle
- Identify the extent to which sheep might legitimately be used as a model organism for cattle in nutrition studies
- Define a phenotype that is a strong indicator of resistance to ruminal acidosis for use in future studies

In chapter 3, the on-farm and post-mortem observations associated with ruminal acidosis in beef and dairy cattle were investigated. This enabled the creation of a scoring system for bovine rumen epithelium, shown in this thesis to be capable of differentiating between animals from different management groups from their histological score. The recent literature on the normal histological appearance of the bovine or ovine rumen epithelium, without dietary manipulation, is quite limited (Dobson, 1956, Graham and Simmons, 200). Often, histological investigations into the rumen epithelium use small sample sizes and focus on the acute response to a diet high in rapidly fermentable carbohydrates, not on the normal appearance associated with long-term adaptation to any given type of diet (Steele, 2009; Steele, 2011; Steele, 2012; Liu, 2013). The scoring system created in this chapter allows for future investigations into the long term effects of dietary inputs and the adaptive ability of the rumen with long term exposure to a diet, in comparison to the acute effects of dietary manipulation that are often studied. It is likely that variation in the ability of the rumen epithelium to adapt and increase its absorptive capacity is one of the key factors

behind variation in susceptibility to SARA observed among animals on different farms and on the same farm. The removal of SCFA via absorption and passage through the rumen, together with the buffering capacity of saliva are important for maintaining the reticuloruminal pH and those variables associated with a change in pH. Therefore, variation in these traits is important and an animal in which the rumen epithelium adapts quickly and efficiently is less likely to suffer from reduced pH and the associated negative consequences.

An unexpected result of the study in chapter 3 was the overwhelming effect of farm of origin on all variables studied. Farms that had a similar *a priori* risk classification based on the major recognised criteria nonetheless showed very diverse patterns of response in commonly studied variables. Therefore, as farm of origin has such a strong effect on results, care is needed when assuming that farms which appear similar superficially, would have a similar risk of animals in the herds developing acidosis. With finishing cattle, groups are often formed from animals which originated from many diverse farms and therefore from different neonatal and genetic backgrounds. It is possible that with the importance of the environment in developing the neonatal microbiome (Malmuthuge et al., 2015), microbiome variation could explain in part the strength of the effect of farm of origin on a wide range of variables. An animal's ruminal microbiome has been shown to develop and be influenced by the maternal microbiota, surroundings, diet and diet delivery and antibiotic treatment during early life (Fanaro et al., 2003, Penders et al., 2006) – all factors which would vary greatly among farms. The ruminal microbiome has been shown to be maintained throughout life and microbiome profiles have been shown to revert to original levels in cattle within 14-61 days following complete transfaunation (Weimer et al., 2010). Therefore, with the importance of the ruminal microbiome for the overall function of the rumen, it would be expected that the strong variation among farms and groups of animals on 1 farm in a finishing group could partly be explained by variation in the microbiome.

In addition to variation in the microbiome, another possible explanation for the strong effect of farm of origin is epigenetics; biochemical modifications to DNA and to histones (proteins that provide stability to DNA) induced by environmental stimuli, which alter gene expression and are potentially heritable (Doherty et al., 2014). There

is growing evidence that prenatal nutrition and stress can have a long term effect on progeny (Singh et al., 2010) although the relationship between epigenetic changes and the resulting phenotype is still not clear (Skinner et al., 2010). There is evidence to suggest that nutritional status during early development is associated with disease risk later in life (Heijmans et al., 2008) and dietary intervention in pregnant animals has been shown to affect their offspring (Liu et al., 2011). In pigs, variation in maternal dietary protein intake resulted in epigenetic changes that altered transcription of the myostatin gene (MSTN) (Liu et al., 2011). MSTN is responsible for production of myostatin protein, which plays a key role in determining muscle mass and meat quality (Kocamis and Killefer, 2002, Wiener et al., 2009). Offspring of pigs fed a low protein diet had upregulated MSTN gene expression, which showed specifically at the finishing stage and was not present at weaning (Liu et al., 2011), thus suggesting a potential role of epigenetic regulation in the determination of carcase yield and meat quality – particularly important in finishing beef cattle (Liu et al., 2011).

Butyrate is often added to animal feeds as it has been shown to reduce *Salmonella* infection by inhibiting large intestine colonisation (Lawhon et al., 2002). However, SCFA, including butyrate, have been shown to act as histone inhibitors (Ho and Dashwood, 2010) and have been shown to be involved in inflammatory gene up-regulation (Doherty et al., 2014). Transcriptomic changes have been shown to be induced by butyrate and some are thought to be epigenetically regulated (Doherty et al., 2014). Therefore, variation in the reticuloruminal levels of SCFA, such as butyrate, in dams could be expected to have an effect on their offspring and could reflect part of the strong effect of farm and variation noted among animals. Additionally epigenetics can have an effect on the immune cell development and function in cattle, and epigenetic changes in response to inflammation and infection are thought to have an impact on the level of future immune responses (Doherty et al., 2014). Research has shown that stimulation with LPS creates an increase in the expression of epigenetic enzymes associated with immune response, and histone inhibitors have been shown to inhibit the expression of pro-inflammatory cytokines like *IL2* and *IFN-γ* in response to LPS (Doherty et al., 2013). Therefore, epigenetic regulation of gene expression could help explain variation in response to infection and differences in disease susceptibility or severity in cattle (Doherty et al., 2013). Increased LPS is often associated with acidosis as a result of decreased pH leading to reticuloruminal

bacterial lysing. In this thesis, wide ranges in LPS concentrations were noted among animals. It stands to reason that epigenetic variation could be partly responsible for explaining this wide range noted, reflecting the range of farms of origin noted. This variation as a result of epigenetic factors, coupled with the variation in the ruminal microbiome, could again account for the strong effect of farm of origin noted among farms which superficially appeared similar.

This trial measured particle size between farms and considered it as a parameter when assigning the *a priori* risk category. However, we did not measure time of feeding, style of feeding or management of the feed bunk. All of these variables could account for some of the strong effect of farm of origin on the measured parameters. Time of feed delivery has been shown to affect the feeding and lying patterns of cattle (Devries and von Keyserlingk, 2005), and the type of flooring present at a feed bunk has been shown to affect both the time spent standing near the feed bunk and the feed intake (Tucker et al., 2006), with cattle showing a preference for softer flooring such as sawdust in comparison to concrete. Cleanliness and the surface of the feed bunk also affect intake in cattle. Therefore, despite considering the composition and particle size of the diets when considering the risk of acidosis, actual intake could have varied greatly among farms. Considering all of these factors when looking at the effect of a diet intended or expected to induce acidosis in cattle in future studies is important.

The second study, detailed in chapter 4, investigated the effects of a diet high in soluble carbohydrates on variables associated with SARA in lactating dairy cattle, with and without the use of direct fed microbials (DFM). The challenge diet induced an inflammatory response in cattle that was associated with an increase in milk production and markers of improved metabolic status but only weak tendencies to reticuloruminal acidosis. The inflammatory response pattern was characterised by elevations in monocytes, acute phase proteins and inflammatory gene expression and has previously been observed in other studies (Li, 2006; Hernandez *et al*., 2014). To a lesser extent, a slight anti-inflammatory effect of the DFM introduction was also observed, as has been previously shown in the literature (Jensen et al., 2008; Gao, 2008) but not specifically in cattle. It seems sensible to consider that an inflammatory type response in cattle may be a precursor to SARA, becoming evident before clinical symptoms arise in individuals. It is possible that by early detection of inflammatory

markers, fermentation disorders could be prevented before clinical symptoms arise and prior to a decrease in production. The DFM used in this study had little or no effect on most of the changes in response to the challenge diet. Some increased the number of observations noted below pH 6 and caused a significant increase in mean pH but these changes were very small numerically. The group fed the commercially available DFM showed the lowest number of instances of pH below 6 in comparison to the novel DFM. The proportion of treatment consumed in each group suggested that the palatability and volume of DFM should be considered, as both types of yeast DFM were refused more than the bacterial DFM, which were a smaller volume. The changes observed also tended to suggest a slight anti-inflammatory effect following the introduction of the DFM. However, this was not as marked as the inflammatory response that followed the introduction of the challenge diet. As the rumen was not challenged to the extent that was intended in this trial and no animals showed symptoms of SARA, it was not possible to fully investigate the ability of the DFM to control the effects of reticuloruminal acidosis or SARA. It appears that all the DFM were beneficial some way, increasing mean pH or showing a slight anti-inflammatory effect but they did not alter pH or other studied variables vastly and the established commercially available DFM was the most successful at altering the rumen environment. While the cost of the initial inclusion of DFM in the diet may be prohibitive for some farms, the possible returns via increased health and production are thought to outweigh this initial cost (Kalebich and Cardoso, 2018). Despite the results in this trial not showing a large effect of the DFM, the literature has shown a positive relationship with health and production and the feeding of DFM over the last few decades. For example, addition of DFM to the ration has shown increased growth rate (Dawson, 1990 ; Lehloenya et al., 2008), improved milk production (Dawson, 1990 ; Lehloenya et al., 2008), greater production efficiency (Dawson, 1990; Isik et al., 2004; Jouany, 2006; Oetzel et al., 2007 ; Lehloenya et al., 2008), increased average daily gain and efficiency in feedlot cattle (Krehbiel et al., 2003 ; Guillen, 2009), improved health (Krehbiel et al., 2003 ; Guillen, 2009), improved immunity (Krehbiel et al., 2003 ; Guillen, 2009), performance in young calves (Krehbiel et al., 2003; Guillen, 2009 ; Novak et al., 2012), decreased potential for ruminal acidosis (Krehbiel et al., 2003 ; Guillen, 2009), and the potential to decrease the effect of endotoxins (Eckel and Ametaj, 2006). Variation in the results of DFM may include proper dosage, lactation stage, variation in strains of DFM, and animal stress conditions (Seo et al., 2010). In

this trial as all animals were dosed in the same manner, lactation stage was equally split between groups and animals were maintained in the same conditions, it is unclear why the DFM did not have a larger impact. It may be possible that with a larger dietary challenge, a more significant effect of the introduction of DFM would have become apparent between the control group and those fed DFM.

The final study in this thesis, detailed in chapter 5, aimed to compare the responses of cattle and sheep to the rapid introduction of a diet high in soluble carbohydrates. This study suggested that sheep are likely to be suitable for use as a model for cattle in future studies. The patterns of response were similar between species, although the greater effect size suggested that sheep were more strongly challenged than the cattle. To the best of our knowledge, there are no published reports of a direct comparative response in sheep and cattle to the same diet intended to induce ruminal acidosis or SARA in both species, although there have been independent studies into SARA/acidosis in both species. There have been 3 reports of side by side comparisons in cattle and sheep in dietary studies, but none in which there was an attempt to induce SARA. The first study looked at the use of sheep as a model to predict the relative intakes of silages by dairy cattle and found the mean intakes of silages by cows were 2.25 times those of the sheep, and it was concluded that sheep were not a useful model system to predict the intakes of a range of grass silages of similar chop length by dairy cows (Cushahan, 1994). The second study looked at the comparative digestion in sheep and cattle fed different forage to concentrate ratios and found that at high intake, digestion values in the cows were less than those in the sheep for all diets (Colucci et al, 1989) – as has previously been shown in larger ruminants where digesta is expected to be retained in the rumen for longer in larger ruminants in comparison to smaller ones (Clauss et al., 2007). From this variation in the digestive physiology, the group concluded that sheep could not be used as a model. However, if this variation of digestion rate was taken into consideration via allometric scaling of diets, it is possible that the model would work – as noted in this present trial. The final study in the literature followed on from the previously discussed trial, investigating the digesta kinetics of the previous trial. Colluci et al (1990) found that passage values of particulate and liquid markers were faster at higher intakes in both species and no substantial differences were found in particulate passage values between sheep and cattle. They found that liquid passage rates were higher in cows

than in sheep for all diets and suggested that although care should be undertaken when comparing results, the variation does not preclude the use of sheep as a model for future cattle studies (Colucci, 1990). The baseline comparative 16S data was the trait that varied most between species, suggesting that the challenge diet was too harsh for sheep, with sheep showing vast losses of biomarker species following the introduction of the challenge diet. Despite variation in the 16S results post challenge, the majority of animals of both species showed similar results and were consistent with previous work that suggested a core microbiome was present in all ruminant species (Henderson, 2015). As described, 16S results were also consistent with the species observed in previous studies in forage and grain fed animals.

In all studies, results for the majority of commonly investigated traits such as reticuloruminal pH, reticuloruminal LPS concentrations and histamine concentrations followed patterns previously described in the literature – tending to increase with increased levels of rapidly fermentable carbohydrates. In contrast, one of the consistently observed results across all trials in this thesis – significantly decreased reticuloruminal SCFA concentrations with the introduction of a challenge diet – conflicted with the commonly reported finding that increased levels of SCFA occur with increased levels of concentrate feeding (Nordlund and Garret, 1994). It is considered likely that the reason for decreased levels of SCFA noted in all 3 trials in this thesis is as a result of increased levels of absorption through the ruminal wall and increased passage of SCFA through from the rumen. A possible reason for decreased SCFA in the trials observed in this thesis could be increased water intake. As this was not measured, it is not possible to confirm. However, it is unlikely and as the finishing cattle in chapter 3 would have water limited prior to slaughter, it would be expected that if water intake were causing the decreased SCFA concentrations observed, it would not have been noted in this group. Further studies looking at absorption and movement of SCFA through the rumen using ^{14}C labelled acetate, propionate and butyrate would allow a greater understanding of whether increased absorption or another mechanism could be responsible for the lower SCFA levels noted in these trials. ^{14}C labelling of SCFA has been used previously in *in vitro* studies to investigate movement of SCFA using Ussing chambers (Sehested et al, 1999) – if it had been possible to extract ruminal wall from the fistulated animals and test this hypothesis *in vitro* or to use labelled SCFA in live animals it may have been possible to confirm increased

absorption as the mechanism responsible for the decreased SCFA levels observed in this thesis.

In conclusion, the feeding of a diet high in rapidly fermentable carbohydrates suggests that there is a systemic inflammatory response prior to any clinical symptoms commonly associated with SARA in cattle. This inflammatory response is characterised by elevations in monocytes, acute phase proteins and inflammatory gene expression and has previously been observed in other studies (Li, 2006; Hernandez *et al*., 2014). The effect of farm of origin was far stronger than initially expected in this study and suggests that when considering the effect of a diet on the ruminal function and overall animal health, it is necessary to consider the presentation of feed, time of feed and possible genetic and microbiome factors, as well as commonly studied variables such as percentage of concentrate and particle size. Finally, the work suggests that it should be possible to use sheep as a model for future cattle nutrition and acidosis studies if the variation in the rate of digesta and liquid passage through the rumen is considered through allometric scaling.

This work looked to identify a possible phenotype for indication of resistance to acidosis. It is possible, with further investigation, that the level of inflammatory response observed in individual animals prior to clinical symptoms could be used as a phenotype for indication of acidosis resistance. It is likely that animals with lower inflammatory responses to a diet high in rapidly fermentable carbohydrates are less likely to develop negative consequences associated with such a diet in comparison to

animals with strong inflammatory responses .

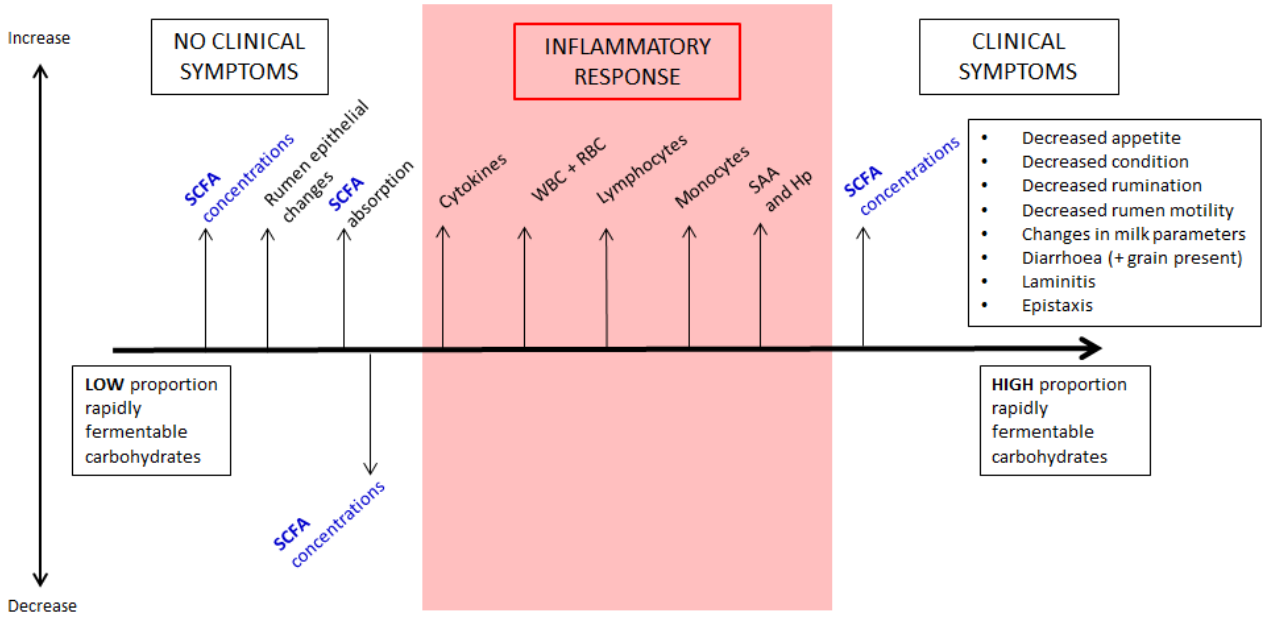

Figure 6-1: Schematic of the hypothetical sequence of events which explain the observed responses in this thesis to a diet high in rapidly fermentable carbohydrates.

Figure 6-1 details the hypothetical sequence of events that explain some of the observed responses in this thesis. Increased rapidly fermentable carbohydrates in the diet are proposed to lead to an initial increase in SCFA as commonly observed in literature. This initial increase in SCFA is thought to lead to increased SCFA absorption and rapid consequential decrease in reticuloruminal SCFA, reflecting the decreased concentrations observed in every trial in this thesis. The changes observed in the histological mean scores for all variables in chapter 5 after the initial introduction of the challenge diet, though not significant, suggest the ability for rapid change in the rumen epithelium in response to the diet and could therefore support change associated with increased absorption. It is hypothesised that this initial increase in SCFA, coupled with the increased movement of SCFA across the rumen epithelium results in the inflammatory response noted systemically and locally through various parameters, including circulating and ruminal wall cytokine gene expression levels, blood biochemistry and haematology, ruminal and plasma histamine and ruminal LPS – as suggested in the current work. It is hypothesised that this decrease in the rumen SCFA levels cannot be maintained long term, eventually leading to the build-up of SCFA in the rumen, as commonly reported in the literature, which eventually leads to the associated clinical symptoms of SARA as the pH declines and the disorder progresses.

Animals which show a lower inflammatory response during this critical period (red section) or which can maintain decreased SCFA levels in the rumen for longer are more likely to be resistant to the ill effects of SARA and thus less likely to suffer from any negative consequences or show any clinical symptoms. With further investigations, it may be possible to confirm this hypothesis and allow for the use of this low inflammatory phenotype to be actively selected for through breeding. It is also possible that the novel scoring system created could be used to further investigate cattle with rapid changes in their rumen histology in response to high carbohydrate diets, in conjunction with the inflammatory parameters, to attempt to find the level of inflammatory response noted in animals showing a rapid/large histological response. If the combination of these mechanisms led to the creation of this low inflammatory phenotype that could be selected for, animals could be bred to reduce the incidence/ negative effects of SARA – increasing welfare and decreasing financial losses to farmers globally.

7 References

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Appendix 1

Analysis of feedstuff (Sciantec Ltd.)

Determination of neutral detergent fibre (NDF) in feedstuff was carried out by Sciantec using enzymatic gravimetry. Starch in the sample was converted to soluble sugars by use of the enzyme α-amylase after de-fatting of the sample with petroleum spirit. The residue was boiled with a neutral solution and the soluble nutrients separated from those that required microbial fermentation. The insoluble matter remaining was designated NDF. The uncertainty for the determination of NDF by Sciantec is +/- 8.6%.

Determination of Nitrogen (N2) and Crude Protein in feedstuff was carried out by Sciantec using the DUMAS method with a LECO FP528 nitrogen analyser. The sample was weighed into a nitrogen free foil parcel and placed into a furnace. It was flushed with pure oxygen to produce rapid combustion. Products of this combustion were passed through filters with a thermoelectric cooler to remove water and collected in a ballast tank to equilibrate. An aliquot of this gaseous mixture was swept through hot copper, removing oxygen and reducing NOx to N2. Next, CO2 and H2O were removed by chemical absorption and the remaining N2 was measured via thermal conductivity cell. N2 content was used to estimate protein content.

Determination of Crude Fibre in feedstuff was carried out by Sciantec using an Ankom 220 analyser. Crude fibre was defined as the organic material that remains insoluble after successive treatments with 0.1275M Sulphuric acid and 0.313M Sodium hydroxide solution. Samples were de-fatted and digested successively with the acid and alkali under controlled conditions and the remaining organic matter was recorded as crude fibre. Uncertainty for the determination of crude fibre by Sciantec is +/- 12%.

Determination of Moisture in feedstuff was carried out by Sciantec via gravimetry. The moisture content of a sample was calculated by measuring the loss of weight after heating at 103 - 105°C for 2 hours 45 minutes ± 15 minutes. Uncertainty for the determination of moisture by Sciantec is +/- 5%.

Determination of Ash (total mineral matter) in feedstuff was carried out by Sciantec using gravimetry. Ash in a sample was considered the residue remaining after a sample had been incinerated at 510°C for at least 4 hours. Uncertainty for the determination of ash by Sciantec is +/- 5.2%.

Determination of Oil/Fat in feedstuff was carried out by Sciantec using a modified Weibull acid hydrolysis method. Samples were analysed for oil A using extraction by ether and then boiling the residual meal or liquid in hydrochloric acid to release the bound fat. The resulting “digest” was filtered and washed until neutral. Fat was retained in the filter and after drying, residue was extracted with light petroleum ether via extraction tube and measured. Uncertainty for the determination of oil by Sciantec is +/- 7.6%.

Determination of Starch in feedstuff was carried out by Sciantec using polarimetry. The method contains two determinations. In the first determination, the sample was

treated whilst warm with dilute hydrochloric acid, clarified and filtered and the optical rotation of the solution measured by polarimetry. In the second determination, the sample was extracted with 40% denatured ethanol. After acidifying the filtrate with hydrochloric acid, clarifying and filtering, the optical rotation of the solution was measured under the same conditions as the first determination. The difference between the two readings, multiplied by a known factor, gave the starch content of the sample. Uncertainty for the determination of starch by Sciantec is +/- 7.7%.

Determination of Cellulose and Hemicellulose from NDF in feedstuff samples was carried out by Sciantec via calculation from results obtained from determinations of acid detergent fibre (ADF), acid detergent lignin (ADL) and NDF fractions.

Determination of Total Sugars in feedstuff was carried out by Sciantec using the Luff-Schoorl method. Sugars were extracted from the sample by shaking with water. The solution was clarified and sugars determined by the Luff-Schoorl method either before or after inversion. Uncertainty for the determination of sugars by Sciantec is +/- 21 %.

Determination of Water-Soluble Carbohydrates in herbage was carried out by Sciantec. The sample was shaken in water and then filtered. An aliquot of the sample was reacted with an anthrone solution while boiling. The resulting solution was measured at 625 nm on a visible spectrophotometer and the absorbance compared to a set of glucose standards treated in the same way.

Determination of Acid Detergent Lignin (ADL) was carried out by Sciantec using an Ankon 220 Analyser. Acid detergent digestion removes the hemicellulose leaving cellulose and lignin. The acid detergent removes the cell contents and other acid soluble material that would otherwise interfere with the lignin determination. Residue from the ADF determination is primarily lignin and cellulose. Cellulose was dissolved using 72% sulphuric acid. The remaining residue consists of lignin and acid-insoluble ash.

Determination of Biogenic Amines (histamine) in feedstuff was carried out by Sciantec using ion exchange chromatography. The sample was homogenised for one hour at 40˚C with 10% thicholoacetic acid solution. The pH of the resulting extract was adjusted to pH 2.2, centrifuged, and filtered. The biogenic amine content of the resulting sample solution was determined via ion exchange chromatography and post column reaction with ninhydrin reagent and photometric detection on the visible region at 750nm.

Appendix 2

Variables investigated in chapter 4, abbreviation, units and whether the distribution of the variable and the change in the variable between time-points A and B and B and C was considered normal.

			Normal Distribution		
Variable	**Abbreviation**	**Units**	**Values**	**B-A**	**C-B**
Liveweight	LWT	kg	Y	Y	N
Milk yield	MILK	l/d	Y	Y	N
Milk butterfat	BF	%,	Y	Y	Y
Milk protein	PROT	%	Y	N	N
Milk lactose	LACT	%	Y	Y	Y
Milk somatic cell count	SCC	10^3 cells/ml	N	N	N
Red blood cell count	RBC	10^12 cells/l	Y	Y	Y
Blood haemoglobin conc.	Hb	g/dl	Y	Y	Y
Packed cell volume (haematocrit)	PCV	%	Y	N	Y
White blood cell count (leukocytes)	WBC	10^9 cells/l	Y	Y	N
Neutrophil count	NEUT	10^9 cells/l	N	Y	N
Lymphocyte count	LYMPH	10^9cells/l	N	Y	Y
Monocyte count	MONO	10^9 cells/l	Y	Y	Y
Eosinophil count	EOS	10^9 cells/l	N	N	N
Basophil count	BASO	10^9 cells/l	N	Y	Y
Platelet count	PLT	10^9 cells/l	Y	Y	N
Plasma cholesterol conc.	CHO	mmol/l	Y	Y	Y
Plasma triglyceride conc.	TG	mmol/l	Y	Y	Y
Plasma β-hydroxybutyrate conc.	BHB	mmol/l	N	Y	Y
Plasma potassium conc.	K	mmol/l	Y	Y	N
Plasma calcium conc.	Ca	mmol/l	Y	Y	N
Plasma phosphate conc.	PO4	mmol/l	Y	Y	Y

Plasma magnesium conc.	MG	mmol/l	Y	Y	Y
Plasma urea conc.	UREA	mmol/l	N	Y	Y
Plasma creatinine conc.	CREAT	µmol/l	N	Y	N
Plasma bilirubin conc.	BILI	µmol/l	N	Y	Y
Plasma alkaline phosphatase activity	ALP	U/l	N	N	N
Plasma aspartate aminotransferase activity	AST	U/l	N	N	N
Plasma γ-glutamyl transferase activity	GGT	U/l	N	N	N
Plasma glutamate dehydrogenase activity	GLDH	U/l	N	N	N
Plasma total protein conc.	PROT	g/l	Y	Y	Y
Plasma albumin conc.	ALB	g/l	Y	Y	Y
Plasma globulin conc.	GLOB	g/l	N	Y	N
Plasma haptoglobin conc.	HP	g/l	N	N	N
Rumen fluid histamine conc.	RFHIST		N	N	N
Plasma histamine conc.	PLASMAHIST		N	N	N
Rumen fluid lipopolysaccharide conc.	RFLPS		N	N	N
Rumen acetate conc.	ACETATE	mmol/l	Y	N	N
Rumen butyrate conc.	BUTYRATE	mmol/l	Y	Y	Y
Rumen propionate conc.	PROPIONATE	mmol/l	Y	Y	Y
Rumen valerate conc.	VALERATE	mmol/l	Y	Y	Y
Rumen isobutyrate conc.	ISOBUTYRATE	mmol/l	Y	Y	Y
Rumen isovalerate conc.	ISOVALERATE	mmol/l	Y	Y	Y

Appendix 3

Sheep Fistulation Protocol

1. Selection of animal
    a. Candidates for cannulation should be healthy adults, preferably with no history of chronic conditions such as mastitis or lameness. The para-lumbar fossa must be checked for size sufficient to take the cannula. Occasionally, the last rib may project across the fossa, making cannula placement difficult, and these animals should be rejected. A template made from the cannula was used to determine this. Sheep had bloods taken for haematology and biochemistry, had their mouths checked, and were body condition scored to ensure they were physically fit enough to be chosen for surgery. This left only 4 animals from the 6 that were initially chosen.
2. Surgical preparation
    a. 24 -48 hours before the operation, the left flank was clipped.
    b. Solid food was withheld for 2 hours before the operation. If the rumen is full, it will obviously increase the risk of spillage of contents once the rumen is incised. However, a full rumen does more closely mimic the normal anatomy of the flank, and will assist the surgeon to position the rumen sutures more accurately. Water was not withheld.
    c. The sheep were brought into the theatre individually and pre-meds given just before surgery: Diazepam (0.2-0.3mg/kg) and IV Torbugesic (0.2mg/kg). Local anaesthesia was then induced by the injection of Alfaxan (1-2mg/kg). The sheep were then intubated and maintained on Sevofluorane and oxygen. Throughout the surgery, temperature and heart rate were monitored every 15 minutes manually.
    d. Once under anaesthetic, the template was placed on the site, such that the edges of the flange would just touch on the last rib and the transverse processes of the lumbar vertebrae. Permanent marker was used to mark a guide for scalpel.
    e. Left side prepared for surgery using Hibi-Scrub.
    f. Animal draped with a sterile cloth
    g. Surgeon gowned and gloved
3. Surgical technique
    a. Using a scalpel, the skin was incised along the marker line. The circle of skin was then lifted and excised with blunt scissors.

b. The subcutaneous fat was then cut through until the muscle layers were visible.

c. An incision was made using blunt scissors in the external oblique abdominal muscles, in the plane of the fibres, and then blunt dissection by hand was used to part the muscle to give a hole of roughly 3 inches diameter.

d. The internal oblique abdominal muscles were incised in the plane of the fibres, and blunt dissection used again to part the muscle to give a potential hole of 3 inches as above. This exposed the peritoneum.

e. In the centre of the muscle wound, the peritoneum was incised. This creates a small rush of air into the abdomen at this stage, and it is important to immediately grasp the exposed rumen wall. This was done with a pair of Allis tissue forceps. The forceps act as a landmark to identify the correct position of the rumen, before it falls away from the wound, back into the abdomen.

f. Viewing the skin wound as a clock, a 5 metric Polysorb suture (CL929 - Vetoquinol) was placed, from the skin surface to the sub-cutis at 3-o-clock. Bypassing all of the muscle layers, a deep bite was taken through the serosal surface of the rumen, picking up the rumen musculature, but not penetrating the mucosa. The suture was then taken back through the skin from sub-cutis to surface and tied on itself, taking care to leave a long end.

g. Using a continuous Lambert pattern, the rumen wall was sutured to the skin around the circumference of the skin wound, working in a clockwise direction. The object of this continuous suture was to make a water tight junction between the rumen wall and the skin, before the rumen is opened.

h. Once the continuous suture is completed, it was tied off and the rumen wall incised. Using scissors, a circle approximately matching the size of the skin wound was trimmed, being careful not to cut the continuous suture pattern.

i. Using a monofilament suture material of high strength and long half-life (Maxon 4 metric – 6769-71 - Vetoquinol), simple interrupted sutures were used to fix the rumen to the skin, each suture going through skin and full thickness of the rumen wall.

j. Once the skin was firmly sutured to the rumen wall, blue spray was used and the edge of the wound lubricated with vet lube and the cannula introduced and the inner flange everted, making sure that it lies flat against the mucosa of the rumen. Prior to this, the cannula was warmed in a bucket of very hot water for several minutes, to allow it to be flexible enough to invert and fold the inner flange for introduction. The bung was then inserted.

4. Post-Operative care

Fed approximately 50% rations for the first 24 hours, thereafter fed as normal.

Pain relief – Metacam (2.5ml per 100kg)

Antibiosis – Marbocyl (1ml per 50kg)

5. Recovery
   Recovery took place in a bedded pen in the theatre to allow observations. The sheep were allowed to come round from anaesthetic and then taken in a bike and trailer back to their pen.