A batch culture study of the rumen bacterial community and the fermentative digestion of forage in cattle

Katie McDermott

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Abstract

Environmental pressures of ruminant livestock production could be lessened by improving feed digestion efficiency. As most feed is digested by the rumen microbial community there is interest in its manipulation. Attempts at doing so *in vivo* have largely been unsuccessful. The aim of this thesis was to determine if, by uncoupling the rumen bacterial community from its host, manipulation would be possible.

Experiments were conducted using an *in vitro* batch culture fermentation model using cattle rumen fluid as inoculum. Parameters of fermentative digestion were measured, and the bacterial community studied using next generation sequencing methodology, the pipeline for which was tested. The role of epiphytic bacteria and concentration of rumen fluid within the model were also explored.

Rumen fluids differing in their ability to digest dry matter *in vitro* (IVDMD; Good, Bad) were cross inoculated (1:1 Mix). After 24 hours of fermentation the IVDMD of the Mix (0.29) was intermediate ($P<0.001$) of the Good (0.34) and Bad (0.20), a result supported by the measured fermentation parameters. However, by the end of the sixth consecutive batch culture (CBC6) there was no difference in IVDMD between rumen fluid treatments, but the overall IVDMD had significantly ($P<0.001$) improved; compared to the average 24 hr IVDMD of CBC1 that of CBC9 was 69% higher. When this experiment was repeated there was no effect of cross inoculation on IVDMD, but again overall IVDMD significantly improved with each consecutive batch culture. Surprisingly there were no differences in bacterial community composition between the rumen fluids, however, the diversity of the community decreased significantly ($P<0.001$) with time.

Differences in IVDMD performance in the absence of differences in bacterial community composition would suggest either differences in community function or differences in communities not studied here. The improved performance with time, associated with reduced bacterial diversity, may indicate bacterial activity within the rumen is restrained.

Table of contents

List of Figures

List of Tables

List of abbreviations

ADF	Acid detergent fibre
ADL	Acid detergent lignin
ARISA	Automated ribosomal intergenic spacer analysis
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CBC	Consecutive batch culture
CC	Continuous culture
CDI	*Clostridium difficile* infection
CH_4	Methane
CHX	Charolais cross
CV	Coefficient of variation
DEFRA	Department of environment, food and rural affairs
DM	Dry matter
EU	European union
DAI	Daisy II ANKOM incubator
DHP	Dihydroxy-pyridine
DLWG	Daily live weight gain
DNA	Deoxyribonucleic acid
FAO	Food and agriculture organisation of the united nations
FISH	Fluorescence *in situ* hybridisation
FCR	Feed conversion ratio
FCE	Feed conversion efficiency
FID	Flame ionising detector
FOM	Fermentable organic matter
FRSGB	Filtered rumen fluid, sterilised grass and buffer
FR	Filtered rumen fluid
FRB	Filtered rumen fluid and buffer

FT	Faecal transplant
GB	Grass and buffer
GC	Gas chromatography
GIT	Gastrointestinal tract
GV	Gas volume
HC	High clover
HFX	Holstein Friesian cross
HSG	High sugar grass
IFN	Interferon
IL	Interleukin
IS	Internal standard
IVDMD	*In vitro* dry matter digestibility
KW	Kruskall-Wallis
MCP	Microbial crude protein
NDF	Neutral detergent fibre
NH_3-N	Ammonia nitrogen
NGS	Next generation sequencing
NMDS	Non-metric multidimensional scaling
NWFP	North Wyke farm platform
RB	Rumen fluid and buffer
RF	Rumen fluid
RGB	Rumen fluid, grass and buffer
RFI	Residual feed intake
RSGB	Rumen fluid, sterilised grass and buffer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-DGGE	Polymerase chain reaction – denaturing gradient gel electrophoresis
PCW	Plant cell wall
PERMANOVA	Permutational analysis of variation

PP	Permanent pasture
PUFA	Polyunsaturated fatty acid
QK	QIAamp DNA stool mini kit
qPCR	Real time (quantitative) PCR
OTU	Operational taxonomic unit
RUSITEC	Rumen simulation technique
SE	Standard error
SEM	Standard error of the mean
SCFA	Short chain fatty acid
SGB	Sterilised grass and buffer
SOP	Standard operating procedures
SSA	Sulphosalicylic acid
TAE	Tris-acetate EDTA
TLR	Toll-like receptor
T-RFLP	Terminal restriction fragment length polymorphism
VFA	Volatile fatty acid
WB	Wheaton bottle
WSC	Water soluble carbohydrate
ZK	ZymoBIOMICS DNA Mini Kit

Chapter 1 General Introduction

1.1 Background - The challenges facing global food production

It is projected that an additional 50% of today's food production will be needed to support 10 billion people towards 2050 (FAO, 2017) (Figure 1-1). Along with the growing human population, there comes competition for land space. Urbanisation is predicted to increase by 20% towards 2050 (FAO, 2009), reducing rural areas, land available for crop production and grazing animals. Therefore, there is cause to increase the output and efficiency of our current systems. Intensification of food production, however, needs to be achieved sustainably, reducing the current levels of environmental pollution associated with food production and maintaining economic stability.

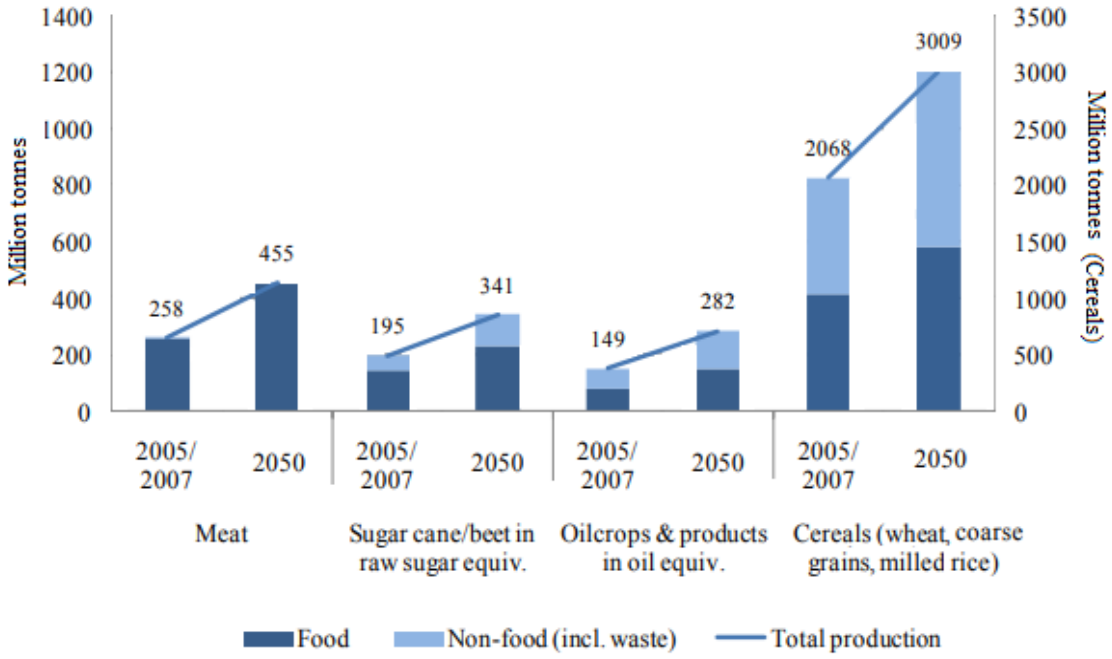

Figure 1-1 World production and use of major products and their predicted increase from 2005/7 to 2050. Modified from Alexandratos and Bruinsma (2012).

Ruminants can alleviate some of the competition between humans and livestock, as they are able to utilise land that is not suitable for human crop production (Varga and Kolver, 1997) and ruminants do not have to compete for human edible foodstuff unlike monogastric livestock such as pigs and poultry (Gill et al., 2009). This is because ruminants are capable of utilising lignified, cellulose rich, and fibrous plant material as their sole source of energy due to the mutualistic relationship with the microbial population that resides within the reticulorumen. There are over 3.6 billion individual ruminants worldwide, comprising 150 species and domesticated ruminants graze an estimated 26% of the planet's terrestrial land. Many species of ruminant are valuable to livestock worldwide as producers of milk, meat, fibres and draft power. In the UK alone, there are 9.8 million cattle and 23.3 million sheep (DEFRA, 2017), indicative of a substantial agricultural economy.

However, ruminants are also associated with negative environmental impacts especially due to their contribution to greenhouse gas emissions through the eructation of methane. Methane (CH_4) is a potent greenhouse gas that has 25 times greater global warming potential than carbon dioxide (Yvon-Durocher et al., 2014). CH_4 is produced in the rumen by strictly anaerobic archaea and, by so doing, remove excess hydrogen from the rumen helping to maintain a low partial pressure of hydrogen. This allows hydrogen-sensitive hydrogenase enzymes to function, which are important for fibre digestion (Hegarty and Gerdes, 1999). As well as its environmental impact, CH_4 also represents a 2-12% loss of dietary energy for the animal (Johnson and Johnson, 1995).

Ruminants are less efficient than non-ruminants at utilising dietary protein due, in particular, to the rapid and extensive degradation of forage protein in the rumen (Calsamiglia et al., 2010). Consequently, another important environmental pollutant associated with ruminant livestock is nitrogen excretion in manure and urine (Kebreab et al., 2001). Nitrogen (N) can leech from agricultural fields into waterways, resulting in eutrophication of lakes and rivers (Adrian et al., 2015). Another form of nitrogen pollution is volatilisation in the form of ammonia, which returns to the land or water via rainfall or direct absorption (Bussink and Oenema, 1998). Dispersal of manure is also an issue.

Therefore, there is much interest in improving efficiency of forage digestion in the ruminant forestomach. As well as reducing the amount of feed required to produce the same amount of meat or milk, efficient cattle emit approximately 25% less CH_4 than

inefficient ones and excrete less N. Through utilising the evolutionary pathways that have enabled ruminants to be so successful, there is scope to maximise their use in the food production system.

1.2 Digestion in the rumen

The rumen is the first chamber of the compartmentalised ruminant fore-stomach. Ruminant animals, such as cattle, have evolved a four-chambered stomach capable of digesting fibrous plant material. The ability to digest plant fibre is due to the mutualistic relationship with the microbial community that reside within the first two compartments – the rumen and reticulum. Together the rumen and reticulum make up 20% of the animal's body weight and are considered a large fermentation vat (Huhtanen et al., 2006). When food enters the rumen, it is colonised by the microorganisms that initiate digestion. Through microbial fermentation and rumination of the feedstuff, the substrate is broken down.

Microorganisms, in a cooperative effort, produce enzymes that hydrolyse the bonds between the sugar monomers. This hydrolysis is usually the rate limiting step, as a complex cocktail of enzymes are required for the effective release of monosaccharides (Chesson and Forsberg, 1997). Microbial fermentation of these monosaccharides results in the production of volatile fatty acids (VFAs) and these provide around 70% of the animal's energy requirements when absorbed across the rumen epithelium (Bergman, 1990).

However, these VFAs are toxic to the rumen microorganisms in high concentration and their build up can limit further degradation. ATP is produced during the fermentation process and this is used as a substrate for microbial growth (Hackmann and Firkins, 2015). It is the production of acetate, a C2 volatile fatty acid, which is implicated in the production of methane. Diets high in fibre are associated with higher acetate production than diets rich in concentrates, therefore, there is much interest in manipulating fibre digestion to increase its efficiency and reduce methane production.

1.2.1 Fibre digestion

Ruminant animals are unique in their ability to utilise cellulose rich, highly lignified, plant material as a source of energy, and referred to herein as lignocellulose (cellulose, hemicellulose and lignin). When feed enters the rumen, it is rapidly colonised by

microorganisms (*ca* 15 minutes) (Huws et al., 2012). With fresh perennial rye grass, primary colonising bacteria were shown to be replaced by a secondary bacterial community following 2 - 4 hours of incubation, likely due to a change from digestion of the soluble fraction to that of the plant cell wall (Huws et al., 2014; Huws et al., 2016). For grazing livestock, fibrous substrate such as grasses can make up 100% of the animal's diet. In the rumen, hydrolysis of lignocellulose occurs up to 30 times faster than is observed for industrial anaerobic digesters used to produce biogas (Mason and Stuckey, 2016). However, despite the rumen's impressive ability to digest plant cell walls, when low quality forage is fed (such as straw) less than 50% of the structural carbohydrates are digested (Horton, 1978; Ribeiro et al., 2017).

The constituents of the plant cell wall (PCW) comprise lignocellulose (cellulose, hemicellulose and lignin) as well as pectin, each of which require a different enzymatic cocktail to hydrolyse. The structure of the PCW is complex, and the components are interlinked (Figure 1-2), thereby reducing physical access of microbial enzymes (Chesson, 1988).

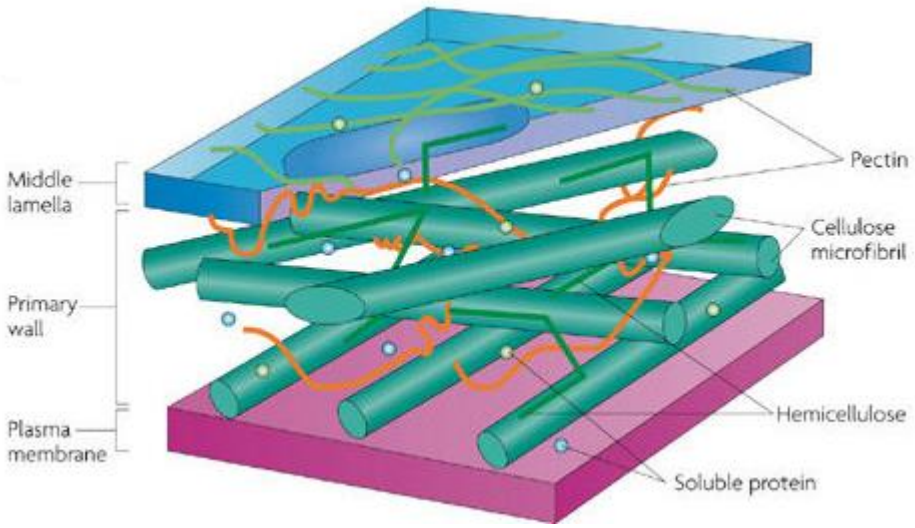

Figure 1-2 The structure of a primary plant cell wall Taken from Sticklen (2008)

1.2.1.1 Cellulose

Cellulose is the most abundant polymer on Earth and is the major constituent of the plant cell wall constituting 20-40% of plant dry matter (Ünay et al., 2008). Cellulose is composed of glucose units (up to 3,000) linked by ß1-4 glycosidic linkages (in contrast to the (α1-4) linkages observed in starch and glycogen) with no branching (O'Sullivan,

1997). The straight chains form strong hydrogen bonds between neighbours, resulting in high tensile strength which gives the plant cell wall its structure.

Mammalian enzymes are unable to hydrolyse the ß1-4 linkages found in cellulose links, however, enzymes derived from microorganisms can (Flint and Bayer, 2008). Degradation of cellulose, through cellulase enzymes secreted by micro-organisms releases glucose molecules which are then utilised by the microorganisms as an energy source. The major cellulolytic bacteria in the rumen (*Fibrobacter succinogenes, Ruminococcus flavefaciens* and *R. albus)* can utilise cellulose as their sole source of energy and are considered the most active mesophillic cellulolytic microorganisms known to date, however, their ability to digest cellulose is limited by accessibility to the susbtrate (Weimer, 1996). The crystallinity of cellulose can also affect the rate of cellulose enzymatic degradation (Hall et al., 2010). It has been estimated that the digestibility of cellulose (from forage) is 62% *in vivo* (Jung and Deetz, 1993).

1.2.1.2 Hemicellulose

Hemicellulose is a polysaccharide that differs from cellulose in that it is a heteropolymer, made of multiple β-linked sugars, including glucose, xylose, mannose, galactose, rhamnose and arabinose (Ren and Sun, 2010). Hemicellulose is also branched with shorter chain lengths and its primary role is to strengthen the plant cell wall through linkages with cellulose and, to some extent, lignin (Scheller and Ulvskov, 2010). Hemicellulose is amorphous and represents around 15-30% of lignocellulosic mass by weight (Sella and Trajano, 2014). The diversity of the hemicellulose structure can limit its decomposition rate to simple monomers (López-Mondéjar et al., 2016), as many enzymes are required to act synergistically to cleave the different pentose and hexose sugars present (Dutta and Chakraborty, 2018).

1.2.1.3 Lignin

Lignin is a highly indigestible constituent of the plant cell wall and consists of many cross-linked phenolic polymers which surround cellulose and hemicellulose (Sanderson, 2011). After cellulose, lignin is the second most abundant natural polymer (Norgren and Edlund, 2014). It is the most recalcitrant of the three components of lignocellulose (Rahimi et al., 2014). Lignin plays a protective role in the plant cell wall, and is a major limiting factor in microbial PCW degradation (Jung and Lamb, 2003; Vanholme et al., 2010). In grasses lignin can account for 10-15% of the total plant mass (Li et al., 2015a).

Lignin is primarily located in the secondary cell wall, hence it is not shown in Figure 1-2 above (a diagram of the primary cell wall).

1.2.1.4 Pectin

Pectin is considered the most structurally complex polysaccharide family in nature, accounting for 2-10% of the primary cell wall of grasses (Mohnen, 2008). Pectin is formed of α-1,4 linked galacturonic acid-rich polysaccharides including homogalacturonan, ryamnogalacturonan-I and rhamnogalacturonan-II (Willats et al., 2001) and is thought to play a large role in the structure and function of both primary and secondary cell walls (Mohnen, 2008).

1.2.1.5 Fructans

Although not associated with the plant cell wall, fructans are important water-soluble carbohydrates found within plant tissue that can also be produced by both bacteria and fungi (Anadón et al., 2016). In the plant, fructans act as an energy storage system, especially in temperate grasses, and usually consist of one glucose unit attached to one or more fructose chains via ß-2,1 or ß-2,6 fructosyl bonds (Jensen et al., 2016). Along with starch, fructans are rapidly and completely digested in the rumen to produce VFAs (Ramirez-Lozano, 2015).

1.2.1.6 Factors affecting fibre digestion

There are many factors that affect the rate and/or extent of fibre digestion within the rumen. The first of these to consider is the physiology of the animal itself. Studies have shown that the efficiency of fibre digestion increases with larger ruminant species (Van Soest, 1994). The pH of the rumen is also an important factor, as cellulolytic bacteria require a pH between 6-7 to efficiently digest fibre (Sung et al., 2007). Also, the animal's dry matter intake can affect rumen retention time. The more the animal eats, the quicker the rate of passage, decreasing the amount of time spent in the presence of the microorganisms which can break down cellulose (Oba and Allen, 1999). The animal also plays a role in physically digesting lignocellulose through chewing and frequent rumination, reducing particle size (Allen and Mertens, 1988).

A second factor to consider is the structure of the substrate and the components of the diet provided to the animal. As mentioned above, lignin is a major determinant of the rate of PCW degradation (Section 1.2.1.3) and the concentration of lignin has been shown

to increase with maturity of the plant (Aurangzaib et al., 2016). The crystallinity of cellulose was also mentioned as a reason by which degradation of the PCW can be decreased (Hall et al., 2010) with amorphous cellulose hydrolysed a magnitude of order quicker than microcrystalline cellulose (Zhang et al., 2006). A further factor that can affect fibre digestion is the amount of water soluble carbohydrates present in the diet. Due to the relative ease of water soluble carbohydrate digestion, high concentrations in the diet can lead to rapid fermentation and therefore a reduced pH, limiting fibre digestion (Hiltner and Dehority, 1983). The supplementation of dietary lipid over 5% of the diet has also been shown to reduce fibre digestion due to toxic effects on some members of the rumen community such as fibryolytic bacteria, methanogens and protozoa (Henderson, 2009). In addition, the functional specific gravity of the feedstuff can affect its retention time within the rumen. Smaller particles with higher functional specific gravity will sink and exit the rumen at the reticulo-omasal orrifice, whereas larger particles with lower functional specific gravity will remain within the rumen (Welch, 1986). The longer the feed particles are present in the rumen, the greater the extent of digestion that occurs.

The presence of secondary plant compounds can also affect fibre digestion. Polyphenolics (such as tannins) and saponins have been shown to reduce fibre digestion. In pure culture experiments, a steroidal saponin extract from *Yucca schidigera* was shown to reduce the cellulolytic ability of both bacteria (*F.succinogenes, R. flavefaciens, R. albus*) and rumen fungi (*Neocallimastix frontalis* and *Piromyces rhizinflata*) (Wang et al., 2000). It is well known that tannins reduce the digestibility of protein within the rumen, but there is also evidence that the presence of tannins can reduce fibre digestibility. It is thought that this is achieved by interfering with microbial attachment and inhibiting enzymatic activity (Frutos et al., 2004). The amount of silica present can also affect fibre digestion. Silica can represent up to 10% of the dry matter in grasses (Epstein, 1999), and has been shown to reduce fibre digestion in herbivorous species (Hartley and DeGabriel, 2016) and the feeding behaviour of sheep (Massey et al., 2009).

The concentration of minerals such as sulphur, magnesium and phosphorous within the rumen can also affect fibre digestion. Sulphur is required at a minimum concentration of 1µg/ml (Bray and Till, 1975) for bacteria to synthesise sulphur containing amino acids (such as methionine and cysteine) and it is also an essential mineral for fungal growth

(Gordon and Phillips, 1989). A deficiency of either phosphorous or magnesium results in a decline of microbial growth, therefore reducing fibre degradation (Griffith, 2017).

Finally, digestion of fibre within the rumen is made possible due to the relationship between the host animal and the microbial community that resides within the reticulo-rumen. It therefore follows that the composition and activity of that community is associated with the efficiency with which ruminants digest fibre. This is something that is explored further in Section 1.4. The synergism and antagonism of the different microorganisms within the rumen likely contribute to the rate and extent of fibre digestion (Wang and McAllister, 2002). Furthermore, the cellulolytic bacteria (such as *Fibrobacter succinogenes, Ruminococcus albus* and *R.flavefaciens*) require attachment to the substrate to initiate fibre digestion. Bacteria that are not able to attach to the substrate show little cellulolytic activity (Morris and Cole, 1987). This is probably due to proteolysis of the secreted enzymes before they contact the substrate, or alternatively their wash out from the rumen (Wang and McAllister, 2002).

1.2.2 Protein

There is an important balance required in the rumen between energy and the availability of nitrogen to produce microbial protein and therefore microbial growth. Microbial protein produced in the rumen is responsible for 60-85% of the amino acids that reach the small intestine of the animal (Storm et al., 2007). Fibre digestion in the rumen is supressed when there is insufficient protein in the diet (or unsynchronised delivery of nitrogen) to support microbial growth (Sampaio et al., 2010).

When protein enters the rumen it is largely broken down by the microbial community to ammonia, which is then used by the bacterial community to synthesise its own amino acids and protein. This means ruminant animals can be fed a diet containing a source of low quality protein with no negative effect on performance, as microbes improve the quality of low quality dietary protein. In fact, as long as there is a source of dietary nitrogen, ruminants can be fed a diet containing no protein. Urea is a common source of non-protein dietary nitrogen included in ruminant diets (Hunter and Vercoe, 1984; Muralidharan et al., 2015). Microorganisms break down urea to ammonia through the use of urease enzyme and utilise ammonia as a source of nitrogen for the anabolism of amino acids. Feeding a ruminant unprotected high-quality protein is therefore not

necessary. For dietary protein to be efficiently converted to microbial protein, the timing of protein degradation and energy release must be synchronised (Sinclair et al., 2009).

Up to 50 % of the microbial protein present in the rumen can be degraded back to non-protein nitrogen and recycled through a number of causes such as predation by protozoa, autolysis and bacteriophages (Wells and Russell, 1996; Oldick et al., 2000; Hackmann and Firkins, 2015). As well as its use in microbial protein synthesis, ammonia is also absorbed across the rumen wall, converted to urea in the liver and either recycled back to the rumen or excreted in urine. High levels of urea (and therefore nitrogen) in the urine are responsible for some of the environmental pollution associated with livestock production (Kebreab et al., 2001).

In the rumen, microbial crude protein (MCP) and ammonia-nitrogen (NH_3-N) can be quantified as an indicator of protein use efficiency (nitrogen metabolism). Ideally, the MCP should be high and free NH_3-N low (dependent upon time after feeding). A large pool of NH_3-N would indicate a large amount of digested proteins in the rumen pool that had not been used by the microorganisms, which may be an indicator of imbalance between protein and energy release.

1.3 Feed use efficiency

Animals differ in their ability to digest and utilise their feed. As feed is a major variable cost in animal production, feed use efficiency has become an important factor in breeding programs (Pryce et al., 2013). Many factors are associated with an animal's ability to utilise feed, such as the breed of the animal, the size of the rumen, retention rate within the reticulo-rumen and the passage rate of feed. More recently, the microbial community that resides within the rumen has also been implicated in efficiency of feed digestion (Guan et al., 2008; Hernandez-Sanabria et al., 2012; McCann et al., 2014; Jewell et al., 2015; Myer et al., 2015; Paz et al., 2018). Dietary energy can be lost as heat, methane or excreted in the urine and faeces of the animal and many of these are causes of environmental damage e.g. CH_4.

There is scope to improve the feed efficiency of cattle. By increasing efficiency, not only does the animal produce fewer waste products and greenhouse gasses directly, but less feed is required for the same amount of growth signifying economic savings.

1.3.1 Residual feed intake

Residual feed intake (RFI) was first proposed by Koch et al. (1963) as a method by which to compare the feed utilisation efficiency of individual animals. RFI is independent of production measures such as size and growth making it a better measure of efficiency than both feed conversion ratio (FCR; feed intake per unit of weight gain) and feed conversion efficiency (FCE; amount of product produced (such as meat or milk) divided by feed intake) (Meyer et al., 2008). RFI is defined as the predicted amount of feed required for a certain level of growth, maintenance of body weight, or output (e.g. milk volume, meat) divided by the actual intake of the animal. RFI defines the variation in feed intake that remains after maintenance and growth requirements have been met. If an animal has a low RFI (LRFI) it is deemed a more efficient animal than one that has a high RFI (HRFI) and will eat less feed than predicted for its current performance. However, for any of these measures to be used as a measure of an animal's feed use efficiency, accurate recording of an animal's feed intake must be made. This makes feed efficiency a difficult measure in the grazing animal.

Feed efficiency has been shown to be a moderately heritable trait with heritability estimates of 0.28 - 0.58 for RFI (Moore et al., 2009) and 0.06 – 0.46 for FCR (Arthur and Herd, 2008). This highlights the importance of an animal's genetics in determining its feed utilisation efficiency.

Variation in RFI between animals has been associated with metabolic rate, feed intake, activity and thermoregulation (Herd et al., 2004). The greatest source of variation was shown to be caused by protein turnover, tissue metabolism and stress susceptibility (Herd and Arthur, 2009). Cattle divergent in RFI have been found to host different microbial communities within the rumen despite the same management suggesting a role of the microbial community in animal's feed efficiency (see Section 1.4.7).

1.4 The rumen microbial community

The rumen microbial community is a complex ecosystem comprising of bacteria, archaea, fungi, protozoa and viruses. Studies looking at the microbiota and their associated fermentation date back to the 1800s (Hungate, 1966). There are three identifiable communities of microorganisms in the rumen, the particle associated, free floating and epimural. Around 70-80% of the bacterial community is associated with feed particles, 10-20% float freely in the rumen fluid and 1-2% are associated with the

mucosal epithelium (Craig et al., 1987). A core microbiome has been established (Jami et al., 2014; Petri et al., 2014; Henderson et al., 2015). The global census study of Henderson et al. (2015) identified a group of microorganisms present across a wide geographic range as well as across a range of ruminant species highlighting their integral role in rumen function.

Evidence of a potentially heritable microbial population has been established largely through the effect of sire breed on the microbial composition of the offspring (Hernandez-Sanabria et al., 2013; Roehe et al., 2016). Although dam breed is also likely to have an equal effect, sire breed has received more attention due to the number of offspring that can be included in studies. Some bacterial species are thought to be more heritable than others (Sasson et al., 2017). Sasson et al. (2017) identified 22 heritable operational taxonomic units (OTUs), with the order *Bacteriodales* especially represented. These 22 OTUs were phylogenetically related and shown to have moderate heritability (heritability estimate > 0.7). Heritable species were identified in 50 to 100% of the study animals (n =146).

The rumen microbial community is established shortly after birth and is sourced from random acquisition of the surrounding environment including the birth canal during delivery, the skin of the mother and other animals during suckling and grooming, and colostrum and milk (Curtis and Sloan, 2004; Rey et al., 2013). Microbial activity is thought to have been observed in the rumen as early as two days of age in the pre-ruminant animal (Rey et al., 2012) and previous studies, using classical culture techniques, showed that colonisation of the lamb rumen by bacteria could be seen from two to seven days of age (Fonty et al., 1987). The phylum Proteobacteria represented more than 70% of the total bacteria present at two days of age and large variation was seen between individual calves in bacterial genera despite the same raising environment (Rey et al., 2013). Functional maturity of the rumen was shown to be achieved at a month of age, however, at 83 days of age the bacterial community still showed large difference to that of the adult animal (Rey et al., 2012; Rey et al., 2013). The mature rumen bacterial community is thought to establish as the animal reaches adulthood and once established, has proved difficult to manipulate (see Section 1.4.8). There is emerging evidence to suggest that early life manipulation may be more successful (see General Discussion).

1.4.1 Bacteria

Bacteria are the highest concentration of microorganism in the rumen ($10^{10} – 10^{11}$ cells per ml; (Hungate, 1966)) depending upon the diet they represent 95% of the total rumen microbial community (Zhou et al., 2015). The majority of the bacteria within the rumen are obligate anaerobes, although some facultative bacteria are also found, mostly in the epimural community attached to the rumen wall and it is thought that a role of these facultative bacteria is to scavenge oxygen and maintain anaerobis (Nagaraja, 2016).

Firmicutes, Bacteroidetes and Proteobacteria represent the most populous phyla of bacteria in the rumen (Kim et al., 2011) with *Prevotella*, a Bacteroidetes, identified as the most abundant genus across a range of diets and ruminant species. The global rumen census project identified seven dominant bacterial genera that were identified across a range of geographic locations and ruminant species, highlighting their roles as members of the 'core' ruminant bacterial species. These seven genera were *Prevotella*, *Butyrivibrio*, and *Ruminococcus*, as well as unclassified *Lachnospiraceae*, *Ruminococcaceae, Bacteroidales* and *Clostridiales* (Henderson et al., 2015).

The majority of studies that explore the composition of the microorganisms within the rumen have focused on the bacterial population due to their abundance. Bacteria will be the focus of this thesis.

1.4.2 Archaea

The archaea that are found within the rumen are methanogenic and strictly anaerobic at a concentration of $10^{8} – 10^{9}$ cells per ml (Wang et al., 2017). Archaea, which belong to the order Methanobacteriales, are most commonly found in the rumen (Jarvis et al., 2000). Methanogenic archaea use mostly hydrogen, and in some cases formate, methyl groups and rarely acetate, to reduce carbon dioxide to methane (Janssen and Kirs, 2008). Archaea are known to be associated with protozoa, both extra- and intra-cellularly (Sharp et al., 2006), and anaerobic fungi (Bauchop and Mountfort, 1981). It has been shown that efficient cattle in terms of RFI, host a less diverse methanogenic community than inefficient cattle thus highlighting the potential role of archaea in feed use efficiency (Zhou et al., 2009).

1.4.3 Protozoa

Protozoa are ubiquitous yet non-essential members of the rumen community (Morgavi et al., 2012) and represent *ca* 50% of the biomass within the rumen due to their large size (Puniya et al., 2015). The majority of rumen protozoa (63 - 90 %) are found attached to feed particles or the rumen wall (Hook et al., 2012). Protozoa in the rumen were first described in 1843 (Gruby, 1843) and there are two major types of protozoa within the rumen which differ in both structure and activity: the Holotrichs and the Entodiniomorphid protozoa (Belanche et al., 2015; Williams and Coleman, 2012). Protozoa are involved in the intracellular degradation of feedstuff entering the rumen, producing hydrogen as a by-product of fermentation and VFAs (Saminathan et al., 2017).

Protozoa predate upon bacteria and *in vitro* studies have shown that a typical protozoal population are capable of breaking down *ca* 17% of the available rumen bacterial population per hour of incubation (Belanche et al., 2012a). Protozoa are also thought to play a role in stabilising fermentation through the consumption of sugars and starches, converting these to reserve carbohydrates, thus preventing their rapid fermentation by bacteria, resulting in a more stable rumen pH (Williams and Coleman, 2012; Denton et al., 2015).

1.4.4 Anaerobic fungi

Anaerobic fungi (phylum Neocallimastigomycota) are eukaryotic species that are thought to play a dominant role in fibre digestion (Puniya et al., 2015) and represent around 20% of the biomass in the rumen at a concentration of $10^2 – 10^3$ per ml (Rezaeian et al., 2004). Fungi have a syntrophic relationship with archaea and there are currently nine described genera of anaerobic fungi (Edwards et al., 2017). Through enzymatic degradation, fungi create access to the substrate for bacteria and they are the only rumen microorganisms able to penetrate the plant cuticle (Akin and Borneman, 1990).

1.4.5 Viruses (bacteriophages)

Virus particles are emerging as the most abundant microorganism on Earth (Koonin, 2010) and are found in most environments including sediments (Yoshida et al., 2018), the ocean (Suttle, 2007) and the gastrointestinal tract (Egert et al., 2006). The major viral particles found in the rumen are bacteriophages (Berg Miller et al., 2012; Ross et al., 2013). Phages have co-evolved alongside bacteria resulting in an arms race of invasive and defensive mechanisms respectively (Stern and Sorek, 2011). Different phages infect

specific bacterial cells, resulting in either death of the cell (lytic phage) or incorporation of phage DNA into the bacterial cell chromosome (prophage). Phages can encode proteins which enhance the fitness of the host bacterium (Hartley et al., 2012) and have been found to regulate bacterial genes through active lysogeny, acting like a molecular switch in genes that have been disrupted during the integration of phage DNA (Feiner et al., 2015).

1.4.6 Factors affecting the microbial community

A global study of the rumen microbial community of a range of ruminant species revealed that the largest effect on microbial structure was due to what the animal was fed with a core community observed across a range of ruminant species, diets and global locations (Henderson et al., 2015). More recently, redox potential has been highlighted as a possible mechanism by which diet affects the microbial community composition (Friedman et al., 2017). Both the age (Li et al., 2012; Jami et al., 2013) and breed (Guan et al., 2008; Bainbridge et al., 2016; Paz et al., 2016; De Mulder et al., 2018) of the animal have also been shown to affect the microbial composition within the rumen.

The individual animal itself is also a factor to consider when examining the microbial population. Jami et al. (2014) found that animals within the same herd, fed the same diet and occupying the same environment shared only *ca* 50% of bacterial OTUs. When taxon phylogeny was taken into account, this did increase to 82%, suggesting that although the OTUs were different, many of them were phylogenetically related. Several host physiological features have also been shown to correlate to the microbial community in the rumen such as glucocorticoid levels as indicators of stress (Deng et al., 2017) and response to acidotic challenge (Plaizier et al., 2017). Inter-animal variation is not limited to just the bacterial community; it has also been observed for both the protozoal and archaeal communities (Zhou et al., 2012a).

The use of antibiotics can also alter the rumen community. Monensin, for example, is an ionophore which was commonly fed to cattle as a growth promoter up until 2006 when it was banned under EU law due to antibiotic resistance concerns (Franz et al., 2010). Monensin has been shown to selectively act upon Gram positive bacteria (Ishlak et al., 2015) and has been shown to improve the feed efficiency of the animal (Russell and Houlihan, 2003) and reduce methane emissions through selection of propionate producing bacteria and the inhibition of hydrogen, formate and acetate producing

bacteria (Chen and Wolin, 1979; Spirito et al., 2018). Specifically, monensin has been shown to reduce the abundance of *Ruminococcus*, *Erysipelotrichaceae* and *Lachnospiraceae* in the rumen of feedlot steers (Thomas et al., 2017).

A variety of feed additives, such as essential oils, have been explored as alternatives to monensin with the hope of manipulating the microbial community in such a way that promotes growth and improves efficiency (Khorrami et al., 2015; Gholipour et al., 2015). Vitamin E (α-tocopheryl acetate) has also been examined as a ruminal supplement *in vitro*. Addition of Vitamin E led to an increase feed digestibility (8 %), which in turn led to higher numbers of both bacteria and protozoa (Belanche et al., 2016). Additionally, some methanogen species were also affected.

1.4.7 Link between the microbial community and the host phenotype

There is a growing interest in understanding the link between the microbial community that resides within the gastrointestinal tract and the phenotype of the host animal. Beginning in the human literature, there has been a growing number of papers published highlighting the correlation between the microbial community and a range of human diseases including type 2 diabetes (Qin et al., 2012), obesity (Ridaura et al., 2013) and non-alcoholic fatty liver disease (Zhu et al., 2012). There is also evidence that the gut microbial community can modulate neuro-behaviour (Soto et al., 2018). As well as correlative studies, causation studies, mostly in mice, have shown a direct link between the microbiome and disease phenotypes (Upadhyay et al., 2012; Ridaura et al., 2013; Cox et al., 2014; Surana and Kasper, 2017) and the concept of the pathobiome has been introduced (Vayssier-Taussat et al., 2014).

In the rumen, many correlations between the microbiota and a range of production measures have been observed. An animal's feed efficiency has been shown to correlate with their microbial population (Guan et al., 2008; Hernandez-Sanabria et al., 2012; Myer et al., 2015; Jewell et al., 2015). Shabat et al. (2016) measured the feed efficiency of 146 lactating Holstein Friesian dairy cows and analysed the microbial community, gene content and metabolomics composition of the 78 animals at the extremes of feed efficiency (40 most efficient, 38 least efficient). They showed that the most efficient phenotype was correlated with the lowest richness of both microbial taxa and gene content. In the feed efficient group, both microbial taxa and metabolic pathways were associated with improved energy harvest and lower methane emissions. The authors

suggested that the efficient animal microbiome was less complex, but more specialised to support the host animal's requirements for energy harvest from a particular feed.

As well as feed efficiency, there is also evidence for a relationship between the presence of certain microbial taxa and milk fat content (Jami et al., 2014), an animal's susceptibility to acidosis (Chen et al., 2012; Khafipour et al., 2009) and methane production (Shi et al., 2014; Roehe et al., 2016). A causal relationship between the microbiota and production measures has yet to be elucidated partly due to the effect of the individual host animal on its microbial composition (discussed below in Section 1.4.8) and the varying factors between studies (e.g. breed, genetics, feed, age and diet).

The link between the microbiome and the host's production highlights a key area where manipulation of the microbial community could be used to improve an animal's efficiency, therefore reducing the amount of feed needed for the same amount of production and at the same time reducing the environmental pollution associated with livestock production.

1.4.8 Manipulations of the microbial community

The microbial community, as described above, has shown a clear correlation with performance traits in the host animal. Therefore, there is much interest in manipulating the microbial community of the rumen with the idea of improving fermentative digestion of feeds and/or improving animal productivity and products whilst decreasing environmental pollution (Díaz et al., 2017).

As described briefly above (1.4.6), both the diet and additives can alter the microbial population within the rumen. There have been a multitude of studies that explore the effects of additives, such as plant extracts and essential oils (Busquet et al., 2006; Kamel et al., 2008; Adesogan, 2009; Kolling et al., 2018), enzymes (reviewed by Beauchemin et al. (2003)) and active dry yeasts (reviewed by Chaucheyras-Durand et al. (2008)) on rumen fermentation. More recently, the effect of these additives on the microbial composition have also been explored (Kišidayová et al., 2018; Mannelli et al., 2018). When treatment with additives is stopped, the effect on both fermentation and the microbial community is lost with parameters reverting back to their pre-treatment levels. In some cases, the microbial community has been shown to adapt to the presence of essential oils within a continuous fermenter, rendering the treatment ineffective (Cardozo et al., 2004; Busquet et al., 2005; Benchaar et al., 2008).

As the additive needs to be continually fed in order to exert an effect on rumen fermentation and the rumen microbial population, there has been interest in more direct methods to alter the microbial composition through inoculation of 'beneficial' microbial species into the rumen. In these studies, the authors aimed to increase the number of a certain species of bacteria (or fungi) to improve performance traits (described below).

1.4.8.1 Previous attempts to inoculate species into the rumen have had mixed results

Previous attempts to manipulate rumen fermentation through introduction of one or more bacterial species have had mixed results. Multiple studies whereby species of bacteria or fungi were introduced into the rumen reported that the inoculated species did not persist, and in many cases had declined by 24 hours (Table 1-1). Experiments using *Megasphaera elsdenii* are described in the text after the table and for bacteria that have been introduced into a new niche see sections 1.4.8.2

Table 1-1 A summary of studies in which bacteria or fungi were dosed into the rumen of cattle, sheep, goats, reindeer or buffaloes.
Modified from Weimer (2015)

Dosed strain	Source	Recipient animals	Result	Notes	Reference
Selenomonas ruminantium SS2	Non lactating cow	Two cannulated adult sheep	Dosed strain failed to establish	Numbers increased only slightly when substrate was continuously supplied compared to twice daily doses	Wallace and Walker (1993)
Lactobacillus plantarum (1193 pM25)	Lab strain	Two cannulated adult Suffolk x Mule wether sheep	Does strain rapidly lost from the rumen	No detection of dosed strain after 24 hours	Sharp et al. (1994)
Ruminococcus albus A2	Mutated lab strain from *R.albus 7*	Cannulated two year old Tokara goat	Reduced from 10^8 to 10^4 within 4 hours of inoculation. Reduced to 1/100 of this within a day	Persisted at low level for 14 days in multiple trials	Miyagi et al. (1995)
Clostidium longisporum B6405 and *C.herbivorans* 54408	Bison (B6405), Pig (54408)	Three cannulated 6 year old cows	Dosed strain not detected (<10^3 cells/ml) within 24-48 hours of dosing	Rumen nearly emptied prior to dosing, and feeding resumed immediately after dosing	Varel et al. (1995)
Ruminococcus albus (Y1, LP9155 or AR72), or *R. flavefaciens* (SY3 or AR67)	Lab strains	Total of 16 cannulated adult Merino sheep	Stains dosed daily for 9 days at 5 x 10^{12} cells/dose reached abundances of up to 6.5% of bacterial community but did not persist	No improvement observed in dry matter digestibility of Rhodes grass incubated *in situ* during dosing period	Krause et al. (2001a)
Recombinant *Butyrivibrio fibrisolvens* (xynA)	Lab strain	Four cannulated Brahman x Friesian cattle and four mature cannulated Merino sheep	Dosed strain did not persist	Concentration of dosed strain (10^10 - 10^12) declined over time and was not detectable in ether species after 22 days	Krause et al. (2001b)
Recombinant *B. fibrisolvens* (NO4)	Lab strain	Sheep	Strain did not persist and was no longer detectable after 144 hours	*In vitro* disappearance was also tested (48 hours) and was affected by protozoal number	Kobayashi et al. (2001)

Table continued…

Dosed strain	Source	Recipient animals	Result	Notes	Reference
R. flavefaciens NJ + probiotic	Wild moose	Six cannulated non-lactating dairy cows	Dosed strain (6.8×10^{11} cells) did not persist	Dosed strain declined by ~10^3 fold within 24 hours and was undetectable 50h after dosing	Chiquette et al. (2007)
		Calves (21-35 days old)	Dosed strain showed weak persistence	Dosed strain detected at low levels (~10^2 cells/ml) 7 days after cessation of dosing	
R. flavefaciens 8/94-32	Norwegian reindeer	Three starved male reindeer	Dosed strain did not persist	Population size of the abundant *Ruminococcaceae* family did not change. Some change in overall bacterial community composition observed	Præsteng et al. (2013)
R.flavefaciens FD-1	Lab strain	Six lactating Murrah buffaloes	Equivocal results: populations of *R.flavefaciens* increased from 1.46×10^7/ml prior to dosing to 2.52×10^7/ml during the week after dosing concluded but also increasing in control buffaloes fed autoclaved cultures.	Very heavy oral supplementation of dosed strain (9×10^{14} cells on alternate days for 1 month)	Kumar and Sirohi (2013)
*Propionibacterium acidipropionici*strain P169, *P. acidipropionici* strain P5 or *P. jensenii* strain P54	Lab strains	Twenty cannulated beef heifers	Dosed strains failed to persist and returned to pre-treatment levels within 9h	Although strains didn't persist, CH4 emission intensity was reduced	Vyas et al. (2014)
Bacillus foraminis (KP245773), *B. firmus* (KP245774), *B. licheniformis* (KP245781), *B. licheniformis* (KP245789), and *Staphylococcus saprophyticus bovis* (KP245800)	Wild moose	Twenty Dorset-cross lambs (4-7 days old)	Experimental animals had higher diversity initially, but decreased when probiotic was stopped	Resolution of sequencing technology not high enough to determine if dosed strains persisted	Ishaq et al. (2015)

Table continued…

Dosed strain	Source	Recipient animals	Result	Notes	Reference
L. acidophilus and Enterococcus faecium (LAB) or L. acidophilus and Propionibacterium (LAB/LU)	Lab strains	Seventy two beef steers	Average daily gain and efficiency greater for LAB/LU than LAB	Persistence of dosed strain was not recorded	Kenney et al. (2015)
L. acidophilus and *E. faecium*		Twelve cannulated steers	Did not impact growth but modulated rumen fermentation	Persistence of dosed strain was not recorded	
P. freudenreichii 53-W, *L. pentosus D31*, or *L. bulgaricus D1*	Lab strains	Twelve cannulated Texel wethers	Dosed daily for 4 weeks. Dosed strains were unable to persist but *L. pentosus* had greater 24 hour survival than others	Changes to ruminal parameters were minor, some evidence of modification to CH4 emissions	Jeyanathan et al. (2016)
L. plantarum (GF103) or *L. plantarum* (GF103) and *Bacillus subtilis* (B27)	Obtained from farm soil	Twelve Holstein calves	Dosed strains failed to persist	Affected the rumen bacterial community. Number of cellulolytic bacteria decreased	Zhang et al. (2017)
Ruminal fungi *Orpinomyces sp*. C-14 or *Piromyces sp*. WNG-12	Cattle	15 lactating Murrah buffaloes	Increased feed digestibility and up to 5.6% improvement in milk production	Zoospore density higher in dosed animals, but level of dosage not reported	Saxena et al. (2010)
Ruminal fungi *Saccharomyces cerevisiae*	Commercial product	Eighty Holstein cows	Amount of fungi increased with level of supplementation, increased some cellulolytic bacteria. Persistence after trial not measured	Feed efficiency effect was more apparent as the length of supplementation was extended	Zhu et al. (2017)

Mixed results for the use of inoculations have been observed in grain fed dairy cattle. For example, to explore their role in milk fat depression, Weimer (2015) dosed *Megasphaera elsdenii* directly into the rumen of Holstein cows. In most cases, the levels of *M. elsdenii* had returned to low baseline levels within 24 hours. This effect was observed even when the *M. elsdenii* was sourced from cattle within the same herd ensuring that the cows had shared common environmental factors and diet prior to inoculation. The same effect was observed when four dosings were executed over a five day period. Similarly, in milk fat depressed cows, rumen inoculation from cows that were non-milk fat depressed was not able to improve the yield of milk fat, but was seen to slightly improve both the speed of recovery for fatty acid synthesis and biohydrogenation (Rico et al., 2014)

More promising results were observed for five steers that were inoculated with both *M. elsdenii* YE34 and *Butyrivibrio fibrisolvens* YE44 at the time of adaptation to a high grain-based diet (Klieve et al., 2003). *M. elsdenii* established quickly and increased 100-fold over the first four days following introduction to the rumen. *B. fibrisolvens* on the other hand declined rapidly and was not detectable after eight days of adaptation to the high grain diet. The *B. fibrisolvens* strain used was selected for its ability to degrade wheat starch *in vitro.* Although the initial concentration of *B. fibrisolvens* was high when the animals were brought in from pasture, the dosed cells alongside the native *B. fibrisolvens* were unable to survive in the rumen when the animals were switched to a 75% barley grain diet.

Studies in lambs have found that the inclusion of fresh rumen fluid (FRF) drenched directly into the rumen decreased feed conversion ratio over a 56-day study (3.74 *vs* 3.24) and improved average daily gain (0.163 *vs* 0.191 kg/d), but significant differences in average daily gain were seen only for days 0-8 after drenching with FRF (Zhong et al., 2014). However, De Barbieri et al. (2015) found that dosing of lambs prior to weaning did not result in improved performance at weaning or at five months of age despite some modulation of rumen fermentation parameters.

1.4.8.2 Establishing populations within the rumen is possible, but only when they fill an unoccupied niche

Successful introduction of a bacterial species into the rumen has been achieved, but only when the bacterial species fill an unoccupied niche. Two examples of this are introductions of *Synergistes jonesii* and a recombinant *Butyrivibrio* strain into the rumen.

S. jonesii was isolated from the rumen of Hawaiian goats that showed no toxicity when fed *Leucaena leucocephala* (Jones and Megarrity, 1986). An amino acid in *Leucaena*, mimosine, is broken down into 3, 4-dihydroxy-pyridine (3, 4-DHP) which is a goitrogen causing low appetite, alopecia, enlarged thyroid, low thyroxin and death in ruminants. *S. jonesii* is able to rapidly degrade 3, 4-DHP in the rumen and persist when *Leucaena* was fed, thus preventing toxicity.

The other successful integration of a bacterial species into the rumen was a recombinant strain of *Butyrivibrio*. Monofluoroacetate has a 50% lethal dose in ruminants of 0.3 mg per kg of live weight (Annison et al., 1960) and is found in plants across Australia, Brazil and Africa. Monofluoroacetate was first identified as a toxic compound in the leaves of the Gifblaar plant (*Dichapetalum cymosum)*(Marais, 1944). Fluoroacetate poisoning is caused by disruption of the Krebs cycle by irreversible binding of fluorocitrate to acontinase (Proudfoot et al., 2006). In a study by Gregg et al. (1998), four strains of *Butyrivibrio fibrisolvens* bacteria were transformed to include fluoroacetate dehalogenase activity and established it in the rumen of sheep prior to fluoroacetate challenge. The fluoroacetate challenge had markedly reduced toxicity in the test animals compared with control animals and the test animals maintained a concentration of modified bacteria of 10^6 to 10^7 cells per ml of rumen fluid over the 5 week trial period.

More recently, similar results have been shown in the mouse model (Shepherd et al., 2018). Dosage of a strain of Bacteroides (*Bacteroides ovatus* NB001) was able to establish in the colon due to the strain harboring an uncommon gene cluster for porphyran utilisation (a marine polysaccharide) when porphyran was fed to the animal. Through the development of a unique metabolic niche, the authors were able to overcome the priority effect (an early colonising species is able to limit the resources available and therefore reduce colonisation of a late arrival (Fukami, 2015)) and largely replace a native strain of bacteria in the colon as long as porphyran was provided to the animal. Feeding of porphyran had no effect on the underlying gut microbial community. Introduction of the gene family responsible for porphyran utilisation into two different Bacteroides species (*B. stercoris* and *B. thetaiotaomicron*) which did not previously have the capacity to utilise this fructan rich substrate showed an increased growth when porphyran was supplemented into drinking water. This highlights a possible technique for the selection of a specific microbial strain through establishment of a metabolic niche.

1.4.8.3 Exchange of rumen content

Due to the mixed results observed when introducing an individual species or strain into the rumen, studies were designed whereby whole rumen content was exchanged between animals that differed in digestive performance. By exchanging the whole rumen community, it was hypothesised that the resilience of the microbial community to perturbation would be overcome. Early experiments tested for nutritional/physiological outcomes rather than effects on the microbial composition (Satter and Bringe, 1969; Cockrem et al., 1987; Cole, 1991) and the first study to explore the effect on the microbial population was performed by Weimer et al. (2010). In this study, near total rumen content (> 95 %) was exchanged between two pairs of dairy cows that showed the largest difference in their rumen bacterial community composition as analysed by ARISA. Both animals differed in their pre-transfer pH and VFA concentration. After 24 hours post-transfer, both pH and VFA concentration had reverted back to that of the host animal prior to the exchange of rumen fluid. Similarly, the microbial population was shown to revert back to that of the original host animal, but with varying levels of success in terms of the time taken to do so ranging from 14-61 days in their first experiment. In the second experiment, neither cow showed a return to the pre-inoculation bacterial community after 62 days, but the composition was more like that of the host than the donor.

Following the Weimer study, further cross inoculation studies have been performed (Ribeiro et al., 2017; Zhou et al., 2018) and these are described in more detail in Chapter 4.1. As for the Weimer et al. (2010) study, both studies showed that even when repeated inoculation (Ribeiro et al., 2017) and multiple washing steps (Zhou et al., 2018) were included, the microbial community was most similar to that of the host animal pre-transfer, highlighting the importance of the host animal in the maintenance of its microbial population.

The RuminOmics project (www.ruminomics.eu) has also attempted rumen content transfer between reindeer and cattle and again saw a re-establishment of the bacterial community toward that of the host animal over time. This highlighted the stabilising effect the host animal exerts with time to shape the bacterial community. Both cows and reindeer had been adapted to the same diet prior to the experimental period. In a second study, rumen community composition was explored in identical twins and non-related animals (Yáñez-Ruiz, 2018). Rumen exchange was then performed. It was hypothesised that the twins would have a more similar microbial community prior to rumen exchange

and that both animals would re-establish their microbial population in a similar way if the host animal was controlling the population through genetics. However, neither hypothesis was supported by the results: the twins did not have more similar microbial populations, and they showed large inter-individual variation in response to rumen exchange. However, members of the Firmicutes phylum did recover their original status in the host animal and this supported findings from human studies that the families *Ruminococcaceae* and *Lachnospiraceae*, both Firmicutes, show the strongest heritability (Wallace, 2016).

The lack of maintained improvement in cross inoculation studies has highlighted the apparent resilience of the rumen microbiome to perturbation. The native microbiota is robust and appears to be able to withstand inoculation, unless perhaps this is associated with an additional shock to the community such as the rapid dietary change from pasture to barley grain in the study by Klieve et al. (2003) described above (Section 1.4.8.1). The *in vivo* studies performed by Weimer et al. (2010), Ribeiro et al. (2017), Zhou et al. (2018) and the RuminOmics project show a clear reestablishment towards the original host bacterial population, highlighting the influence of the host animal in determining its rumen bacterial population.

1.4.9 Host effect on microbial community

Ecological theory would suggest that a host is under a strong selective pressure to harbour a beneficial microbial population (Foster et al., 2017). Due to the close interaction between the host and its gut microbial community, it follows that the host may influence which microbial species can establish and persist within a region of the GIT. The potential for a host-specific microbiota within the rumen was first identified for protozoa (MacLennan and Kofoid, 1933; Eadie, 1962) and much later for the fibrolytic bacterial community (Weimer et al., 1999) prior to the establishment of molecular techniques to characterise the organisms present. Despite much interest in the interaction between the host and its microbes, the mechanisms by which the host exerts control is still largely unclear, especially in the ruminant animal.

In the large intestine, which is home to another large microbial community capable of digesting fibrous substrate, the microbial community has been shown to interact with the host immune system at the mucosal barrier. In a paradoxical way, the immune system has been shown to determine the 'safety' of a species using the same environmental

sensors by which it identifies 'danger' through pattern recognition receptors (PRRs), such as toll-like receptors (TLRs) (Swiatczak and Cohen, 2015). Swiatczak and Cohen (2015) suggested that a microflora can be maintained in the intestines due to the PRRs favouring ligands that have long been established within the gut. Indeed, PRRs have been shown to produce an inflammatory response when a new ligand-interaction is experienced whereas this response is not induced by the native microflora therefore allowing them to persist within the environment (Pradeu et al., 2013). Recently, epithelial cells have been shown to play a major role in controlling the microbial ecosystem through the release of antimicrobial peptides by Paneth cells in the small intestine and maintenance of an anaerobic environment in the colon through epithelial hypoxia (Byndloss et al., 2018).

In many studies, to understand the relationship between the host and the microbiota, the mouse gut has been 'humanised' by faecal transplant into gnotobiotic animals. There has been shown to be a connection between the immune system and the microbiota in the hind gut, as development of the immune system is impaired in animals that are raised in germ free conditions. Animals that are raised in germ-free conditions were found to have smaller Peyer's patches, a reduced number of CD4+ T cells and IgA producing cells (Mazmanian et al., 2005; Belkaid and Hand, 2014).

In the ruminant, there is less information available regarding the mechanisms by which the host controls its microbiota. The rumen epithelium (stratified squamous) contains far fewer immune capabilities than that of the hind gut (simple columnar) and is said to be more similar to the skin epidermis in terms of its immunological profile than a gastrointestinal tract membrane (i.e. intestinal mucosa) (Xiang et al., 2016). However, there is some evidence to suggest a relationship between the rumen and the microbial population present through interactions with immune cells in both the epithelium (TLR4, IL-1B, IL-10 and caspase-1) and rumen fluid (T-lymphocytes, B-lymphocytes, IFN-γ and myeloid lineage cells) (Trevisi et al., 2014). TLR expression has further been shown to correlate with bacterial diversity and be dependent upon the diet fed to the animal (Chen et al., 2012; Malmuthuge et al., 2012; Liu et al., 2015). The expression of TLR-5 has been shown to positively correlate to *Roseburia* abundance (Malmuthuge et al., 2012).

Trevisi et al. (2014 and 2018) suggested that the rumen may be able to participate in cross-talk with the lymphoid tissue in the oral cavity through rumination. Indeed, Fouhse et al. (2017) suggested that secretory immunoglobulin A (SIgA) produced in the saliva

of cattle can coat both commensal and pathogenic microbes within the gut and may therefore may be a mechanism by which the host exerts specificity over its residing microbial community. The authors showed that the composition of the SIgA tagged microbiota in the saliva was very similar to that of the SIgA tagged microbiota in the rumen and suggested that this indicated that SIgA was able to act as a selection tool for commensal bacteria.

In addition to the immune system, the specific environment within the rumen (e.g. pH, osmolality, and redox potential) as well as water intake, feed intake and rumen content (solid and liquid phase) turnover rate likely controls the microorganisms present.

Due to the effect that the host animal appears to exert over the microbial populations within the gastrointestinal tract, there is scope to explore microbial population dynamics in the absence of host control. Using an *in vitro* model, it is possible to remove the direct influencing effect of the host animal and therefore it is possible to study the effect of microbial manipulation without the confounding effect of the animal itself.

1.5 Studying the rumen community

Robert Hungate is considered the father of ruminant and anaerobic microbiology, developing the roll tube technique to culture anaerobic bacteria (Chung and Bryant, 1997). Since then, the rumen microbial community has received much attention due to the importance of the microbes in digestion of feed to produce human desired products such as meat and milk. Due to the anaerobic nature of the rumen inhabitants, and the complexity of the ecosystem, attempts to enumerate the rumen microbial community has underestimated the true diversity within. It is estimated that early culturing efforts represented only 8% of the bacterial community within the rumen (Weimer, 2015). Of the culturable microorganisms within the rumen, not all were represented by genomes in public databases. The Hungate1000 project (http://genome.jgi.doe.gov/TheHunmicrobiome/TheHunmicrobiome.info.html) therefore aimed to produce a reference set of microbial genomes for culturable bacteria, methanogenic archaea, ciliate protozoa and anaerobic fungi that reside within the rumen.

The rumen microbial community has been studied by taking samples directly from the rumen through the use of a stomach tube or through surgical modification of the rumen wall to allow for fitting of a cannula (referred to as a rumen fistula). Samples can also be collected indirectly through buccal swabs (Kittelmann et al., 2015) or, alternatively,

samples can be collected from the animal at time of slaughter (Chaudhry and Mohamed, 2012; Lutakome et al., 2017). Fermentation parameters such as the concentration of VFAs, pH, ammonia nitrogen and microbial protein concentration have been measured in these samples alongside analysis of the microbial community. As well as examining the fermentation parameters and microbial community directly from the animal, samples collected from the rumen have also been used to inoculate *in vitro* models of the rumen.

1.5.1 *In vitro* model of rumen fermentation

Rumen fermentation is studied by measuring fermentation parameters of rumen fluid taken directly from animals, and frequently by simulating rumen fermentation via *in vitro* models. The *in vitro* model traditionally provides a platform on which to screen a large number of potential feeds and treatments in a controlled laboratory setting, reducing the cost associated with animal trials (Lengowski et al., 2016). *In vitro* models of rumen fermentation have been regularly used to document the kinetics of feed digestion and the effectiveness of feed additives and are discussed further in Chapter 6. It is accepted that *in vitro* models are generally representative of digestibility of different feed and feed components in the rumen (Minson and McLeod, 1972; Terry et al., 1978; Prins et al., 1981; Weimer et al., 2011). Although some difference in VFA profiles between the rumen of a sheep and *in vitro* models have been reported (Brown et al., 2002), which may be due to the nature of some *in vitro* models as described below (Section 1.5.1.1)

Rumen fluid used as an inoculum can be sourced as described above (Section 1.5). For maximal microbial activity and diversity it has been observed that rumen samples should be collected three hours post feeding where possible (Belanche et al., 2018). Different types of *in vitro* model exist and these can be either batch, semi-continuous or continuous in nature. The batch culture model will be the focus of this thesis.

1.5.1.1 Batch *in vitro* model of rumen fermentation

The history of the batch culture model has been described previously (Muetzel et al., 2014; Yáñez-Ruiz et al., 2016). Briefly, Tilley and Terry (1963) described the use of an *in vitro* model to measure end-point products such as volatile fatty acid concentration and the extent of substrate degradation. Czerkawski and Breckenridge (1975) used a glass syringe to measure the displacement of a piston by fermentation gasses, which acted as a basis for the 'Hohenheim gas test' developed by Menke et al. (1979). Blümmel and Ørskov (1993) introduced more frequent gas production sampling allowing

determination of fermentation kinetics. The use of a pressure transducer with a sealed fermentation bottle, containing rumen fluid, buffer and substrate was described by Wilkins (1974). A pressure transducer was used to manually measure gas accumulation in the headspace as described by Theodorou et al. (1994).

Increased pressure within the *in vitro* system has been shown to potentially affect fermentation end products, and the rate and extent of fermentation (Tagliapietra et al., 2010) when pressure increased beyond 48 kPa (Theodorou et al., 1994) highlighting the need to vent (manual or automatic, depending upon the system) the headspace gas so as not to compromise fermentation.

Batch *in vitro* models use an incubation medium (often referred to as a salivary buffer or artificial saliva) to provide the nutrients and buffering capacity to maintain the pH, to allow degradation to continue uncompromised (Mould et al., 2005). Substrates are generally ground to pass through a 1 mm sieve prior to their addition to the model (Yáñez-Ruiz et al., 2016). Batch *in vitro* models are used to ferment substrates over a short period of time, usually 24 (Cattani et al., 2014; Wencelova et al., 2014; Muetzel et al., 2014) or 48 hours (Li et al., 2013; Brooks et al., 2014; Gemeda and Hassen, 2015) with some studies reporting fermentations lasting up to 72 hours (Varadyova et al., 2013; Gemeda et al., 2014; Pang et al., 2014). It is uncommon for the model to be used longer than this as products of fermentation are not removed, as they would be in the animal (e.g. VFAs are absorbed across the rumen wall), therefore it is possible the buffer may lose its capacity as more acidic VFAs build up (Yáñez-Ruiz et al., 2016). Also, the feed substrate becomes limiting with time, especially ones considered easy to digest. These limitations on batch culture fermentation time can be overcome by using the batch culture model in a consecutive fashion with fermentation liquor from an initial fermentation transferred into a new bottle containing fresh feed and new buffer to allow fermentation over an extended period of time (Theodorou et al., 1984; Gascoyne and Theodorou, 1988; Castro-Montoya et al., 2015).

Most of the literature using batch culture *in vitro* models have examined the fermentation and/or digestibility of a feed stuff or an additive using a pooled rumen inoculum from multiple donor animals. However, there has been little consideration for whether the *in vitro* model can be used to identify and explain possible differences in fermentation, digestibility and effects on microbial populations between individual donor animals.

1.5.2 The use of molecular biology

Many different molecular biology techniques have been applied to the rumen to acquire a deeper knowledge of the microbial population that resides there. Molecular biology has revealed a much greater diversity than could be achieved through culturing alone. A range of molecular biology techniques have been used to study the rumen community such as PCR based fingerprinting methods (e.g. PCR-denaturing gradient gel electrophoresis (PCR-DGGE), terminal restriction fragment length polymorphism (T-RFLP) and fluorescence *in situ* hybridisation (FISH), quantitative PCR (qPCR) and construction of clone libraries, which are described elsewhere (Deng et al., 2008; Zhou et al., 2011). Many of these techniques are made possible due to the evolutionary conservation of 16S/18S and internal transcribed spacer 1 (ITS1) genes (Deng et al., 2008).

1.5.2.1 Next generation sequencing

In the last decade, there has been a huge increase in the use of sequencing technologies to catalogue microbial communities associated with a large variety of environments such as the ocean (Louca et al., 2016), built environment (Kembel et al., 2012), soil (Zarraonaindia et al., 2015) and the gastrointestinal tract (Eckburg et al., 2005). Next generation sequencing (NGS) has also been applied to the rumen resulting in, for example, the discovery of a core rumen microbiome (Henderson et al., 2015). The use of NGS technology in the rumen is discussed in more detail in Chapter 3.

1.6 Thesis aims, objectives and hypotheses

With the growing human population there is need to sustainably increase the production efficiency of livestock, due to the growing demand for meat and milk, the limited availability of land for expansion of livestock farms, and the environmental impacts of livestock production. Improving the efficiency of fibre digestion in the rumen is one possible avenue, as ruminants are capable of utilising lignified, cellulose rich fibrous plant material as their sole source of energy due to their mutualistic relationship with the microbial population that resides within their reticulorumen. Ruminant animals can therefore utilise feedstuff unsuitable for mono-gastric livestock and human food production.

However, individuals within a herd, i.e. same breed, feed and management, differ in their ability to digest fibre (Jami and Mizrahi, 2012; Shabat et al., 2016) indicating there is

scope to improve fibre digesting ability of animals. One potential method of doing so, for reasons described in Section 1.4.8, is to manipulate the composition of the rumen microbial community. However, previous attempts to manipulate the rumen community through rumen exchange have proven unsuccessful and it is thought this is due to the 'host-effect' on the residing microbial community

Project aim:

- To understand the role of the rumen bacteria in fibre digestion

The digestion of feed within the reticulorumen is achieved by a combination of physical and chemical means; physically by means of rumination, and chemically by means of enzymes produced by the resident microbiota. The microbiota are host specific, being regulated by the rumen environment (e.g. nutrient supply, temperature, pH, osmolality, redox potential, and rumen outflow rate) and the host's immune system. These regulatory mechanisms are thought to be a reason why attempts at long-term manipulation of the rumen microbiota have largely been unsuccessful. *In vitro* models of the rumen potentially provide a means to study the rumen microbiota and their fermentative digestion of feed in the absence of host regulatory mechanisms.

The objectives of this thesis were:

1. To determine if an *in vitro* batch culture model of the rumen can be used to study:
    a. The fermentative digestion of high fibre feeds by rumen fluids with different fibre digesting abilities
    b. The microbiota (specifically the rumen bacterial population) of rumen fluids sourced from different animals and with different fibre digesting abilities
2. To determine if it is possible to manipulate the rumen microbiota in favour of fibre digestion *in vitro* where attempts to do so *in vivo* have failed, due to the absence of control by host regulatory mechanisms

1.6.1 Thesis outline

Chapter 2 outlines the methods used throughout this thesis. Here, the batch culture *in vitro* model of rumen fermentation is defined as well as the methodology and bioinformatics associated with 16S amplicon sequencing.

Improvements in sequencing technologies have meant there is a growing interest in their use to document the microbial population in the rumen. As this was the first time that next generation sequencing of the 16S gene had been performed in this laboratory, Chapter 3 aimed to test the sequencing methodology to establish whether the microbial profiles achieved were accurate and repeatable. The pipeline was tested on pig faecal samples obtained from animals fed different concentrations of ZnO, a known antimicrobial compound, to establish whether the methodology could pick up differences in bacterial community composition.

As described above, previous attempts to exchange rumen content between animals has proven unsuccessful and is thought to be due to the effect of the host animal (Section 1.4.8.3). Chapters 4 and 5 used the batch culture *in vitro* model of rumen fermentation to first identify whether the batch culture model was capable of identifying difference between different rumen fluids in terms of dry matter degradation and secondly to identify whether cross inoculation of rumen fluid was possible in the absence of host control. For Chapter 5, rumen fluid sourced from genetically similar cattle (sired by the same bull) raised from birth on a forage-based diet within the same herd was used. In both chapters, the effect of cross inoculation on the rumen bacterial community was examined.

Alongside the microbial ecosystem contained within the rumen inoculum, there is a large, complex microbial community associated with the plant phyllosphere (Lindow and Brandl, 2003; Berlec, 2012). The aim of Chapter 6 was to identify the contribution of this grass associated (or "epiphytic") bacterial community to *in vitro* fermentation across both short and long term fermentations (24 and 144 hours respectively) to establish what role the community plays during *in vitro* fermentation within a batch culture model.

The *in vitro* model is an invaluable tool to allow studies on a wide range of feeds and additives on their effects on rumen fermentation in a cost-effective manner, limiting the need to use animals and reducing the cost associated with animal trials. To date, there is little research examining the behaviour of the microbial community over time within a

batch *in vitro* model of rumen fermentation and the effects of rumen inoculum concentration on this (Yáñez-Ruiz et al., 2016). To be able to study the effect of diet, additives or manipulations on the microbial community using an *in vitro* model, it is imperative to know what effect the model itself is having on the community present. Chapter 7 therefore explored the effect of inoculum concentration on fermentation parameters and the stability of the bacterial community within the batch culture model.

In Chapter 8, the overall findings of the thesis are discussed and future work is identified.

Chapter 2 General Methods:

2.1 Rumen fluid & the *in vitro* model:

2.1.1 Rumen fluid collection

Rumen fluid, a mixture of liquid and solids, was collected from beef cattle immediately after slaughter from four UK abattoirs; John Penny and Sons (Rawdon, UK), ABP (York, UK) and Dawn Meats (Treburley and Hatherleigh, UK). Samples were taken from approximately the middle of the rumen as soon as the rumen was opened and placed into suitable containers, ensuring they were filled to the brim to prevent the presence of an oxygen pocket, for transport back to the laboratory for processing.

From ABP (York, UK) rumen fluid from 57 cattle (13 breeds, 6 farms) was collected. Rumen fluid was collected from 11, live weight recorded, Charolais-cross steers raised from birth at the North Wyke Farm Platform (Okehampton, Devon, UK) on a forage based diet from both Dawn Meats Treburley and Hatherleigh.

2.1.2 Processing of rumen fluid

Rumen fluid was filtered through a double layer of muslin under a constant stream of oxygen-free CO_2. Once filtered, *ca* 45 ml aliquots of rumen fluid was transferred to 50 ml Falcon tubes and frozen at -80°C until use.

2.1.3 Feed source

Throughout all *in vitro* experiments, dried grass (GRAZE-ON, Northern Crop Driers Limited, York, UK) was provided as a high-fibre feed source. This was a mixture of varieties of Tall Fescue (*Lolium arundinaceum*) with some Ryegrass (*L. multiflorum*) and Timothy grasses (*Phleum pratense*) also present (personal communication). The feed was milled to pass through a 1 mm sieve prior to use in incubations and stored in air tight bags. The dry matter content of the feed was determined by weighing approximately 0.75 g of dried grass into three pre-weighed crucibles. The crucibles were placed into an oven at 95°C for *ca* 16 hours, then transferred to a desiccator, cooled to room temperature and reweighed to calculate dry matter (DM) content. The ash content of the feed was then

determined by transferring the crucibles containing the feed DM into an ashing oven (500°C) for *ca* 16 hours, cooling in a desiccator and re-weighing to calculate ash content. A subsample of the grass was sent for chemical composition analysis (Sciantec Analytical, Selby, UK; Table 2-1).

Table 2-1 Chemical analysis of GRAZE-on dried grass Chemical analysis was performed by Scientec Analytical (Selby, UK) with the exception of dry matter and ash which was determined in house. Values are given as g per 100 g DM unless otherwise stated

	g/100g DM
Dry matter (DM)	93.61 g DM/100 g dried grass
Ash	8.08
Neutral Detergent Fibre (NDF)	54.30
Acid Detergent Fibre (ADF)	25.57
Acid Detergent Lignin (ADL)	19.01
Water Soluble Carbohydrates (WSC)	14.73
Crude Protein (Dumas)	13.70
Total Oil	3.14
Calcium	0.44
Magnesium	0.25
Phosphorous	0.22
Potassium	2.16
Sodium	0.15
Sulphur	0.29
Cobalt	0.022 mg

2.1.4 Mould's buffer

Mould's simplified incubation buffer (Mould et al., 2005) was used throughout all *in vitro* fermentations as a buffering system to maintain an optimum pH for fermentation. Stock buffers (Buffer 1 & Buffer 2; Table 2-2), once prepared were stored at 4°C. Buffer 3 (Table 2-2) was always prepared fresh at the beginning of each experiment. Buffer 1, 2 and 3, and distilled water were mixed in the proportions 5:5:1:9 respectively. Resazurin (100 µl/L of a 1.0 g resazurin/L water solution) was added as a redox indicator. The buffer was gassed with CO_2 until the colour of the redox indicator changed from blue to pink. After colour change was complete, the pH of the buffer was recorded (Hannah instruments, USA) and the buffer was transferred to 2.5 L bottle(s) and incubated at 39°C.

Table 2-2 Composition of Mould's simplified incubation buffer

Component	Final Composition (g/ L distilled water)
Buffer 1	
$Na_2HPO_4.12H_2O$	1.985
KH_2PO_4	1.302
$MgCl_2.6H_2O$	0.105
Buffer 2	
NH_4HCO_3	1.407
$NaHCO_3$	5.418
Buffer 3	
Cysteine HCl	0.390
NaOH	0.100

2.1.5 *In vitro* batch model of rumen fermentation

Twenty-four hours prior to starting each fermentation, Mould's buffer was prepared and pre-warmed to 39°C overnight in an incubator and *ca* 0.5 g of dried grass was accurately weighed into each 125 ml serum bottle (Wheaton, USA). Rumen fluid (-80°C) was defrosted for *ca* 2 hours in a water bath at 39°C. Once defrosted, rumen fluid was transferred to a conical flask in which it was maintained at a temperature of *ca* 39°C under a constant stream of O_2 free CO_2, and continuously stirred. Before use, the buffer pH was checked and adjusted where necessary to 6.80 through the use of hydrochloric acid (5 M) or sodium hydroxide (10 M).

To one fermentation bottle at a time, 45 ml of Mould's buffer and 5 ml of rumen fluid were added and the bottle was placed onto a hot plate (*ca* 39°C) under a constant stream of O_2 free CO_2. After 5 bottles were prepared in this way, the first was removed from the hot plate and sealed with a rubber stopper secured in position with an aluminium crimp seal. This was repeated for all bottles. Fermentation bottles containing no feed, only rumen fluid and buffer (blank bottles) were included to allow for correction of gas produced by fermentation of residual organic matter within the rumen fluid. Bottles were gently swirled to mix bottle content and then transferred to an incubator (39°C) for the duration of the incubation.

To reduce any variation caused by local temperature differences within the incubator, trays on which the bottles were placed were rotated from front to back and from top to bottom of the incubator, when gas pressure was recorded (see Section 2.2.1). At the end of the experimental period, bottles were swirled in iced water to stop fermentation. They were then uncapped and samples of the fermentation fluid taken for subsequent analysis. The remaining content (42 ml) was analysed for *in vitro* dry matter digestibility.

2.1.6 Consecutive batch culture

In the case of fermentations longer than 48 hours, a consecutive batch culture technique was used. As well as the experimental bottles, an additional 2-4 bottles were included for each batch culture which were prepared in the same way (feed, buffer and rumen fluid). At the end of the 48 hour fermentation, these bottles were uncapped and 5 ml of content was used to inoculate a new set of fermentation bottles containing fresh feed and buffer following the same procedure as described above (Section 2.1.5).

2.2 Sample Analysis

2.2.1 Gas volume

Each bottle was removed one at a time from the incubator and a digital manometer (Digitron 2023P, Sifam Instruments Ltd, Torquay, UK) was used to record the accumulated gas pressure (kPa) within each fermentation bottle. After recording the pressure the bottle was returned back to atmospheric pressure. Each bottle was swirled gently before placing back into the incubator. Gas pressure was converted to volume using the following equation (López et al., 2007):

$$V = \frac{V_h}{P_a} \, x \, P_m$$

Where V = volume of gas produced (ml), V_h = total headspace volume in fermentation bottle (110.4 ml), P_a = atmospheric pressure (100.52 kPa; altitude of laboratory 71 m) and P_m = pressure recorded on manometer (kPa).

Gas volume was corrected for the volume of gas produced in the blank bottles and standardised to per gram of DM added to each bottle. A minimum of two gas pressure readings were recorded over any 24 hour period.

2.2.2 pH

At the end of each fermentation period, after final gas pressure had been recorded, the bottles were de-capped and pH of the fermentation fluid immediately recorded (Hannah instruments, USA).

2.2.3 *In vitro* dry matter digestibility (IVDMD)

To the contents of each fermentation bottle 5 ml of 20% sulphosalicylic acid (SSA) was added to precipitate solubilised, undigested protein (Boisen, 1991). Bottles were then left to stand at room temperature for 30 minutes. The contents of each bottle were transferred to a pre-weighed centrifuge tube using *ca* 20 ml of 1% SSA to facilitate the transfer of content. Undigested residue was isolated by centrifugation (3,000 x g for 5 minutes; (Udén, 2006)). The supernatant was removed by aspiration. The undigested residue pellet was washed three times with 50 ml of very hot distilled water (80 – 90°C), with centrifugation, as described above, between each wash. After the final wash the tubes containing the undigested residue were placed in an oven at 95°C for *ca* 16 hours, whereupon they were cooled to room temperature in a desiccator and then weighed. IVDMD was calculated as follows:

$$D\ value\ (g/100g) = \frac{(weight\ of\ feed\ in\ (g\ DM) - weight\ of\ feed\ out\ (g))}{weight\ of\ feed\ in\ (g\ DM)} * 100$$

2.2.4 Volatile fatty acid analysis (VFA)

A 1.5 ml aliquot of fermentation fluid was collected from each fermentation bottle into a screw-topped tube and frozen at -20°C until analysis. Samples were thawed at room temperature and analysed via gas chromatography (GC) following the methods of Jouany (1982). Briefly, to 1 ml of sample in a Nalgene™ Oak Ridge high speed centrifuge tube (Thermo Fisher Scientific, MA, USA) was added 250 µl of a solution containing, per litre, 2g of mercuric chloride, 20 ml of concentrated orthophosphoric acid (85% aqueous solution) and 2g of 4- methylvaleric acid. The 4- methylvaleric acid was included as an internal standard (IS). The centrifuge tube was immediately capped and its contents mixed. Samples were then centrifuged at 20,000 x g for 20 minutes at 10°C (Beckman L8-70M Ultracentrifuge, 70.1 Ti rotor). A volume of the resulting supernatant was then transferred into a GC vial ready for analysis. Calibration standards, containing 5 mM

acetate, 5 mM propionate and 5 mM butyrate) were prepared in exactly the same way as the fermentation fluid samples.

A GC fitted with a polyethylene glycol nitroterephthalic acid-treated capillary column (15 m x 0.53 mm, 0.5 µm film thickness; BP21, SGE, Europe Ltd., Bucks, UK) was used to analyse the samples. Samples were injected directly onto the column (0.5 µl; 240°C) with helium as the carrier gas (flow rate *ca* 5 ml/min). The detector was a flame ionisation detector (FID, 280°C).

Concentration of acetate, propionate and butyrate (mM) were calculated relative to the calibration standards using the ratio of their peak areas to that of the IS. Where appropriate the VFA concentrations were blank corrected to remove the VFAs produced from residual organic matter in the rumen fluid.

2.2.5 Ammonia-nitrogen analysis (NH_3-N)

A 1.5 ml aliquot of fermentation fluid was removed from each fermentation bottle into a screw-capped tube for NH_3-N analysis and immediately acidified with an equal volume of 0.2 M hydrochloric acid. The contents of each tube were vortex mixed and then frozen at -20°C until analysis. Following the methods of Cardozo et al. (2004), samples were thawed at room temperature, and 1.5 ml transferred to Nalgene™ Oak Ridge high speed centrifuge tubes (Thermo Fisher Scientific, MA, USA) and centrifuged at 10,000 x g for 20 minutes at 15°C (Beckman L8-70M Ultracentrifuge, 70.1 Ti rotor). Supernatant was diluted 1 in 10 with distilled water. Calibration standards (0 – 10 µg NH_3/ml) were prepared from a stock solution of ammonium sulphate ($(NH_4)_2SO_4$; 5.87 mM) diluted with Mould's buffer (prepared separately without ammonium bicarbonate). Diluted sample supernatant (20 µl) and calibration standards (20µl) were added to wells in a 96 well plate (each sample was assayed in triplicate) in which the NH_3-N assay was then performed using the Berthelot (1859) reaction as described by Chaney and Marbach (1962). Briefly, to each well was added 80 µl sodium phenate (2.5% phenol in a 1.25% NaOH solution), 80 µl sodium nitroprusside (0.01%) and 80 µl sodium hypochlorite (3%). The wells were sealed with acetate foil, gently mixed and then incubated at 40°C for 10 minutes. After removal from the incubator the plates were allowed to cool to room temperature and then the absorbance of the samples was measured at 630 nm using a plate reader spectrophotometer (SpectraMax 340PC, Molecular Devices). The NH_3-N

concentration of the diluted samples were calculated from the calibration line and corrected for dilution.

2.2.6 Microbial crude protein

Microbial crude protein was measured by the Lowry protein assay (1951) with modifications described by Makkar et al. (1982). From each fermentation bottle, a 2 ml aliquot was collected into a screw-topped tube and immediately frozen at -20°C until analysis.

Samples were defrosted at room temperature and centrifuged at 1,000 x g for 5 minutes to remove feed particles and protozoa. The supernatant was centrifuged at 25,000 x g for 20 minutes at 10°C and the subsequent pellet was washed with phosphate buffered saline (PBS) and re-centrifuged. The cells in the remaining pellet were hydrolysed in 0.25 M NaOH (100°C, 10 minutes) and centrifuged again to pellet the cell debris (25,000 x g, 15 minutes). Bovine serum albumen (BSA; 1 mg/ml) was used to prepare a standard curve. Supernatant, a NaOH blank sample and the standards were transferred to a 96 well plate (40 µl) in triplicate and the Lowry protein assay was performed (Lowry et al., 1951). Briefly 200 µl of complex forming solution was added to each well (2% w/v Na_2CO_3 in 0.1 M NaOH, 1% w/v $CuSO_4.5H_20$ and 2% w/v sodium potassium tartrate) and allowed to stand at room temperature for a minimum of 10 minutes. Finally, 1N Folins solution (20 µl) was added to each well, mixed and incubated at room temperature in the dark, for 60 minutes. Absorbance was read at 550 nm. A control sample was run on every plate to allow correction for inter-plate variation.

2.3 Microbial analysis

2.3.1 Sample collection

A 1.5 ml sample of the mixed content, both liquid and solid fractions, of each fermentation bottle was collected into Eppendorf tubes. Collection of both liquid and solid fractions in the 1.5 ml sample aliquot was facilitated by the removal of the ends (*ca* 5 mm) of the pipette tips. The samples were centrifuged at 16,000 x g for 10 minutes at room temperature. The supernatant was removed and the pellets were stored at -80°C until required.

2.3.2 DNA extraction

DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with some minor modifications. Briefly, 0.2 g of 0.1 mm zirconia/silica beads (Thistle Scientific, Glasgow, UK) were added to the microbial pellet of each collected ruminal sample immediately upon removal from the freezer, prior to the addition of Buffer ASL. Buffer ASL was added and tubes were placed into a bead-beater (Tissue Lyser LT– 5 minutes, max speed (50 rps), Qiagen). Samples were then incubated in a water bath for 5 minutes at an increased lysis temperature from 70°C to 95°C to improve lysis of Gram positive bacteria. The remaining protocol was followed according to the manufacturer's instruction. Quantity and quality of DNA was checked spectrophotometrically (NanoDrop ND-1000). Three biological replicates for each sample were pooled in equal ratio to a final concentration of 10 ng/µl and stored at -20°C until PCR amplification.

2.3.3 PCR

Amplification of the V1-V3 region of the 16S rRNA gene was performed using the universal bacterial primers Bact8F (AGAGTTTGATCCTGGCTCAG) and 534R (ATTACCGCGGCTGCTGGC) (Pitta et al., 2014), GoTaq Green Master Mix 2x (12.5 µl; 400µM dNTPs, 3 mM $MgCl_2$; Promega, Madison, Wisconsin, USA), 0.4 µM of each primer (1 µl of each) and 1 µl of extracted DNA was added to each 0.2 mL PCR tube. Volumes were made up to 25 µL with nuclease free water (Promega). All samples were prepared on ice. If DNA concentration was < 10 ng/µl, additional volume of DNA was added up to a total of 5 µl to provide 10 ng of DNA. Amplification conditions were 95°C for 2 minutes followed by 25 cycles of 95°C for 15s, 56°C for 15s, 72°C for 15s and a final extension step at 72°C for 5 minutes. PCR products were checked on a 1% agarose gel (100 V, 20 minutes) in 1 x TAE buffer (Protocols, 2013) for presence of the correct sized band at *ca* 520 bp compared with 1 kb plus DNA ladder (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Each sample was amplified in triplicate and PCR products were pooled prior to purification to give a total volume of *ca* 60 µl.

2.3.4 Amplicon purification

Samples were purified using the QIAquick PCR Purification Kit (Qiagen) following the manufacturer's instruction. To increase yield, after the first spin step (Step 5), the eluent was reloaded onto the column and centrifuged again. Also, at the final step (Step 9),

elution buffer was pre-warmed to 37°C, 30 µl was added to the centre of the spin column, allowed to incubate at room temperature for 2 minutes and eluted via centrifugation. The purified PCR product was reloaded onto the column, incubated for 2 minutes as above and centrifuged a final time. Presence of purified amplicons was confirmed via Nanodrop.

2.3.5 Next Generation Sequencing

Purified PCR products were sent to the University of Leeds Next Generation Sequencing facility at St. James' Hospital (DNA@Leeds, Leeds, UK) for library preparation using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, Massachusetts, USA) without fragmentation. Size selection was performed using Agencourt AMPure XP beads (Beckman coulter, High Wycombe, UK). Amplicon sequencing was performed with 300 base pair, paired end reads using MiSeq V3 chemistry (Illumina, San Diego, California, USA).

In order to validate the sequencing methodology, the same PCR product was sequenced three times to check that the library preparation, sequencing and downstream analysis was consistent. Also, three biological replicates (i.e. PCR amplicons from three bottles of the same treatment) were sequenced independently as well as in their pooled form to confirm that the *in vitro* model produced similar microbial populations in each experimental bottle.

2.3.6 Bioinformatics

2.3.6.1 Mothur

Sequencing reads were processed using Mothur v.1.39.3 (Schloss et al., 2009) following the MiSeq standard operation procedure (SOP) developed by the Schloss group. (Kozich et al., 2013). The SOP was accessed online from March 2017. Briefly, the forward and reverse reads were combined to form contigs. Contigs with ambiguous bases were removed and only those between 500 – 600 base pairs long were included for further processing. Unique sequences were identified and aligned to the SILVA reference database (release version 123). Only contigs that aligned between position 46 and 12,862 were selected with a maximum homopolymer length of 8. Sequences were pre-clustered allowing for 1 difference per 100 base pairs. Chimeras were identified and removed along with any sequences that may have been identified from the 16S rRNA of archaea,

chloroplasts and mitochondria. Sequences were then clustered into operational taxonomic units (OTUs) with 97% similarity. The number of OTUs in each group and their taxonomy were identified. A BIOM file was generated and this was used to transfer the OTU table, associated taxonomy and metadata into a format suitable for use in R (v 3.4.0) where remaining analysis and production of graphics were performed.

2.3.6.2 R

The following packages were installed and used for microbiome analysis: Phyloseq v1.20.0 (McMurdie and Holmes, 2013), Vegan v2.4-3 (Oksanen et al., 2017) , ggplot2 v2.2.1 (Wickham, 2009) and DESeq2 v.1.16.0 (Love et al., 2014). Alpha diversity was estimated based on the Chao1 index (Chao, 1984), an index that is particularly useful for microbiome data as it based upon the number of rare classes (OTUs) in a sample. The Shannon-Wiener index (Shannon, 1948) was also performed as a measure of evenness, i.e. both species richness and abundance. A community with low evenness is dominated by a few abundant OTUs, whereas one with high evenness will have equally distributed abundance across all OTUs (Gotelli, 2008). For both Chao1 and Shannon indices, a general linear model (lme4) was used to identify the effects of the factors (Fluid*Time) upon alpha diversity of the samples. Models were reduced using Analysis of Deviance (AOD; lmerTest).

Beta diversity was plotted using non-metric multidimensional scaling (NMDS) using the Bray-Curtis distance with the number of axes set to 2. PERMANOVA (adonis) was used to identify significant ($p < 0.05$) factor effects and interactions. Finally, DeSeq2 was used on un-rarefied data to identify OTUs, the fold-change of which differed significantly between two groups. P-values were adjusted for multiple comparisons (Benjamin-Hochberg correction). To allow comparisons between single groups (where there was no replication), only the OTUs with the highest fold-change difference were considered.

Chapter 3 Establishing a pipeline for bacterial community composition analysis using an *in vitro* model of rumen fermentation

3.1 Introduction

In 2005, the development of next generation (or "high-throughput") DNA sequencing resulted in reduced cost and an increased ease of use (Loman et al., 2012) compared with previous Sanger sequencing technologies (Metzker, 2005). Although uptake was initially slow (Schuster, 2007), there has been rapid improvement in technology and increased innovation over the last five years (D'Amore et al., 2016), resulting in an explosion of studies documenting microbial communities in a variety of locations for example the human body (Lukens et al., 2014), oceans (Moran, 2015), sediments (Sun et al., 2013) and animal gastrointestinal tracts including the rumen (Petri et al., 2013).

Prior to the advent of sequencing techniques, for many years the rumen was described as a 'black box' and, to some extent still is (Malmuthuge and Guan, 2017), depicting the lack of knowledge regarding the microorganisms that reside within it. The majority of the microorganisms found in the rumen are strictly anaerobic and require a complex medium to grow *in vitro*, which rendered classical culturing techniques difficult, if not impossible, for some species, with early culturing efforts representing only an estimated 8% of the bacterial community (Weimer, 2015). For this reason, the complexity of the rumen was grossly underestimated for a long time. With the development of PCR and molecular biology techniques in the 1980's, new techniques enabled a deeper insight into the microbial community. The use of next generation sequencing (NGS) to study the rumen community emerged in the early 2000s (Brulc et al., 2009; Pitta et al., 2010) and since then has become very much common place with studies examining the bacterial, archaeal, fungal and viral communities (Ross et al., 2013; Lin et al., 2015; Cunha et al., 2017).

The development of NGS techniques has allowed a deeper understanding of the complex microbial ecosystem and has revealed a level of complexity much greater than was possible by culturing alone (Kim and Yu, 2012). However, the use of culturing methods to grow rumen isolates are by no means outdated and can be used alongside sequencing

to predict function and act as a basis for reference databases on which sequencing analysis depends. The Hungate1000 project was designed to produce a reference set of genomes from cultured bacteria, methanogenic archaea, ciliate protozoa and anaerobic fungi. At the beginning of the project, only 12.5% of the 88 bacterial genera in the rumen had a representative genome from a strain of bacteria. As of March 2018, 73 of those 88 genera now have a representative strain belonging to them along with a further 73 strains that can only be classified at the family or order taxonomic level which will lead to improved databases and accuracy during sequence alignment (Seshadri et al., 2018), thereby complementing NGS techniques. The importance of correct database selection and alignment procedures has been discussed previously (Golob et al., 2017).

With advances in sequencing technology NGS techniques are being applied to unravel the complex interactions between microorganisms and their environment as well as between microorganisms and their host. For this to be achieved the methodology needs to be robust and reliable. Amplicon sequencing is a popular method to determine the microbial community of environmental samples using marker genes that are conserved across all members of a domain, e.g. 16S rRNA for bacteria and archaea (Olsen et al., 1986), 18S rDNA for protozoa (Embley et al., 1995; Wright et al., 1997), and ITS1 for fungi (Gardes and Bruns, 1993; Nilsson et al., 2009). The 16S rRNA gene contains nine variable regions that allow determination of bacteria at the genus level (Wang et al., 2007; Chakravorty et al., 2007). The choice of variable region can change the outcome of studies and limits comparison between studies that have used different regions (Yu and Morrison, 2004a; Rintala et al., 2017). The method of DNA extraction has also been shown to play a large role in the composition of the resulting microbial community (Henderson et al., 2013; Yu and Morrison, 2004b) and a bead beating step is recommended to improve cell lysis and maximize diversity (Lazarevic et al., 2013).

As this was the first time 16S rRNA gene amplicon sequencing has been performed in this laboratory, the aim of this chapter was to test the methodology and the sequencing pipeline to determine its ability to reliably and accurately document the bacterial community of samples using primers to amplify the V1-V3 region of the 16s rRNA gene. The pipeline was examined to explore whether it could detect differences between treatments and whether the outcome was repeatable.

3.2 Methods

3.2.1 DNA extraction accuracy and reproducibility

By using a community standard of known DNA content and concentration (ZymoBIOMICS Microbial Community Standard), two commercially available DNA extraction kits were used to identify the ability of each to extract DNA accurately and reproducibly. The two kits were the QIAamp DNA Stool Mini Kit (QK) and the ZymoBIOMICS DNA Mini Kit (ZK). The kits were used as described in the manufacturer's protocol except for the addition of a bead beating step and increased lysis temperature (95°C) for QK as described in the General Methods (2.3.2). The microbial community standard consisted of both Gram-positive (*Lactobacillus fermentum, Enterococcus faecalis, Staphylococcus aureus, Listeria monocytogenes* and *Bacillus subtilis*) and Gram-negative bacteria (*Pseudomonas aeruginosa, Escherichia coli* and *Salmonella enterica*), which were considered a mixture of species both easy and difficult to lyse. Each kit was used to extract DNA three times from the community standard. PCR, purification and sequence analysis were performed as described in the General Methods (2.3.3 – 2.3.6). For the QK samples, 5 µl of DNA was added to each PCR reaction to provide *ca* 10 ng/µl to the reaction. Both kits were also used to extract DNA from the same neat rumen fluid sample to compare the kits on a more complex substrate. Sequences were aligned against the latest SILVA database (v 132).

A Chi-squared goodness of fit test was performed (IBM SPSS Statistics 21) to compare the microbial composition of the microbial standard extracted by each kit with the theoretical values published by the manufacturer at the Genus level.

3.2.2 Testing the sequencing pipeline

To confirm the repeatability of the pipeline from PCR through to sequence analysis, PCR was performed on the same DNA extract independently three times. These three samples underwent the remaining steps in the pipeline as individual samples and were examined to identify any differences in relative abundance. Libraries were prepared by the same person on the same day at the sequencing unit at St James' Hospital (Leeds, UK) and run on the same MiSeq lane. Sequences were processed by the Mothur software (v 1.39.1) and R as described in the General Methods (Chapter 2.3.6). The coefficient of variation (CV) was calculated for each phyla and genera.

3.2.3 Reproducibility of the fermentation bottles

As described in the General Methods (Chapter 2.3.2), DNA was extracted from three experimental fermentation bottles from each treatment and was then pooled to a final concentration of 10 ng/µl prior to PCR and sequencing. To ensure that variation between fermentation bottles was not masked by pooling samples and to ensure that the pooled sample was an accurate representation of the three individual DNA extracts, a set of three bottles were processed as individual samples. The bacterial profile from the three individual bottles was then compared to the bacterial composition of the same three bottles that had been pooled following DNA extraction. Differences between the pooled values and the theoretical values were calculated via Chi-squared analysis in IBM SPSS Statistics 21 (IBM).

3.2.4 Ability to identify a difference between treatments

It was important to establish whether the pipeline was capable of detecting differences between treatments. Therapeutic levels of dietary zinc oxide (ZnO) have been shown previously to modulate the intestinal microbiota of piglets (Yu et al., 2017), therefore it should be possible to detect a difference between treatments using this pipeline. It was also of interest to determine the suitability of the pipeline for different environmental samples.

DNA was extracted from faecal samples obtained from piglets fed either a high (therapeutic) concentration of ZnO (2,500 ppm) for twenty days after weaning, or control concentration of ZnO as in the standard weaner pig diet (100 ppm) with 12 piglets per group. Faecal grab samples were collected at time of defecation from each piglet on Day 20 and stored at -20°C at Spen Farm (Tadcaster, UK). To reduce any variation in microbial profile due to host genetics, animals were matched for litter across a treatment and pens were alternated to remove any variation caused by the environment.

Samples were transferred back to the University and whilst mostly frozen, a *ca* 0.2 g aliquot was removed from multiple, internal locations within each stool sample, transferred to a 2 ml Eppendorf and thoroughly mixed. All samples were frozen at -80°C until DNA extraction. The remaining pipeline was performed as for rumen samples as described previously (Chapter 2.3.2 – 2.3.6). Differences in alpha and beta diversity were compared between animals fed either therapeutic ZnO or control levels of ZnO, as well as between animals of different gender. Results were analysed as described in Chapter

2.3.6. To identify which Phyla differed between control and therapeutic levels of dietary ZnO a t-test (or Mann-Whitney U test when non-normally distributed) was performed in IBM SPSS Statistics 21.

3.3 Results

3.3.1 DNA extraction accuracy and reproducibility

A microbial standard was used for DNA extraction to test the reproducibility and accuracy of two commercial DNA extraction kits. After extraction, DNA concentration was found to be greater for the Zymobiomics kit (ZK) when compared to QIAamp DNA stool mini kit (QK; 22.7 ± 6.21 ng/µl vs 1.23 ± 0.40 ng/µl respectively; t = -5.977, df = 2.02, p = 0.026). However, the microbial community produced by the two kits was found to be very similar (Figure 3-1). PERMANOVA analysis of the community composition revealed no significant difference between the samples from the two kits ($F_{1, 5}$ = 2.0466, p = 0.300). There was also found to be no significant difference between the kits in alpha diversity when measured with both Shannon (2.26 ± 0.034 *vs* 2.26 ± 0.019; $F_{1, 5}$ = 0.0409, p = 0.8496) and Simpson (0.86 ± 0.004 *vs* 0.86 ± 0.005; $F_{1, 5}$ = 0.1217, p = 0.7448) diversity indices for QK and ZK respectively. Chao1 did reveal a significant difference between the two extraction kits with greater species richness for QK (4867.4 ± 87.84 *vs* 3553.6 ± 623.07; $F_{1, 5}$ = 13.079, P = 0.022).

The relative abundance of the eight known species within the microbial community standard were compared to the experimental samples. The eight species in the microbial community standard were detected using both kits (at the Genus level; Appendix A-1). However, both kits showed a significantly different community composition when compared to the theoretical relative abundances as given by the manufacturer (QK X^2 = 39.34, df = 6, p < 0.001; ZK X^2 = 34.29, df = 7, p < 0.001). It can be seen from Figure 3-1 that there is an overestimation of the Gram-negative bacteria especially the genera *Pseudomonas* and *Escherichia-Shigella* and an underestimation of both *Enterococcus* and *Listeria*. Despite this, both QK and ZK showed generally good reproducibility of the same microbial community. Relative abundance of all phyla and genera can be seen in Appendix A-1.

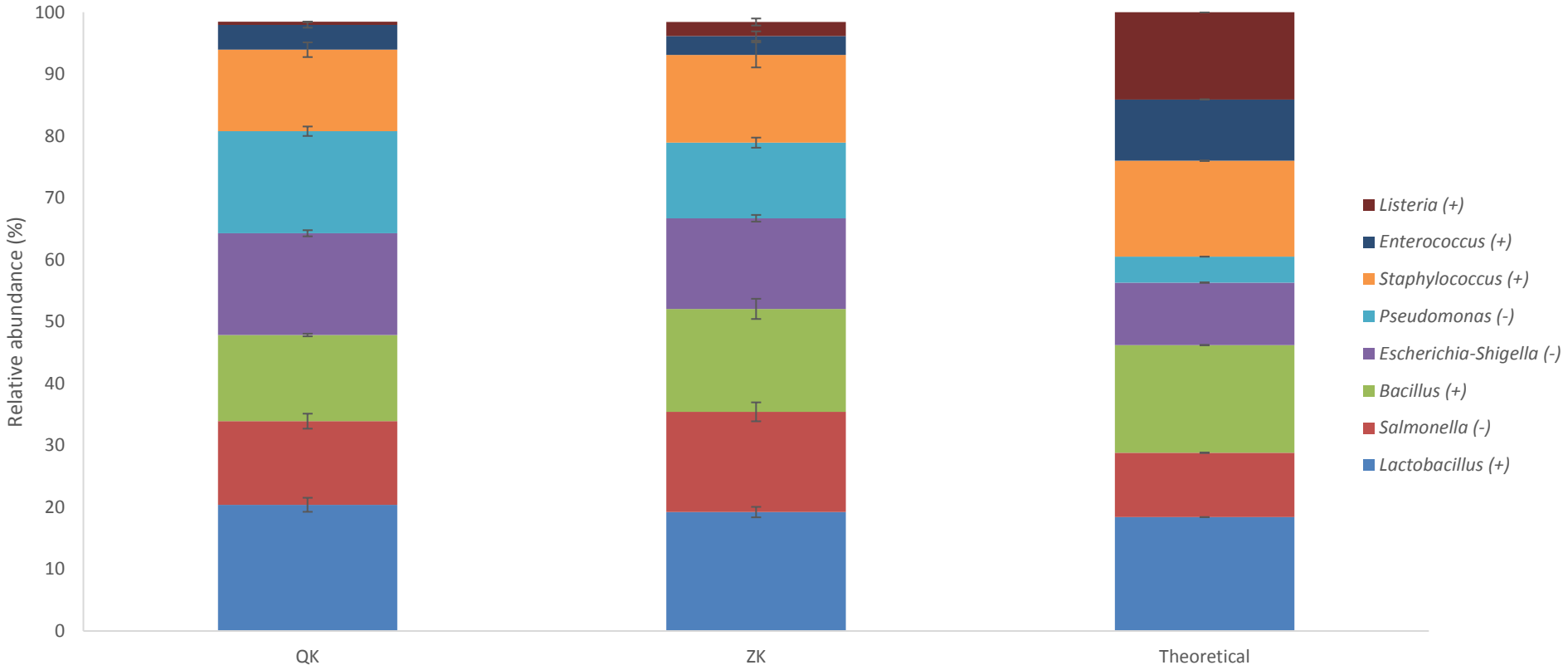

Figure 3-1 The relative abundance of 8 bacterial species isolated from a microbial community standard (ZymoBIOMICS) extracted using two commercial DNA extraction kits alongside the theoretical values Where QK = QIAamp DNA Stool Mini Kit (Qiagen) and ZK = ZymoBIOMICS DNA Mini Kit. Each colour represents a genus with an average value for three replicates. Error bars represent SE. (+/-) represents Gram positive or Gram negative respectively.

Both kits were also used to extract DNA from rumen fluid collected at time of processing to determine whether the extraction process would result in a different profile when a compositionally more complicated starting material was used as compared to the microbial community standard above. Here, the QK was shown to have a higher DNA concentration following extraction (285.9 ng/µl *vs* 225.2 ng/µl for QK and ZK respectively). The relative abundance of the Phyla and Genera differed slightly between the kits with a higher relative abundance of the less abundant genera with ZK (Appendix A-2). However, the most abundant Genera were the same for both kits (31.55, 7.65 and 3.62 % for QK and 27.09, 9.38 and 4.72 % for ZK for *Prevotella 1, F082 ge* and *Rikenellaceae RC9 gut group* respectively) highlighting that despite using a different kit the overall outcome was the same. The abundance of Bacteroidetes and Firmicutes, which were the two most abundant Phyla, were also similar (52.13 and 34.21 % for QK and 48.94 and 31.64 % for ZK) with a Firmicutes to Bacteroidetes ratio of 0.66 and 0.65 for QK and ZK respectively.

3.3.2 Reproducibility of library preparation and the sequencing pipeline

To ensure that the pipeline produced the same bacterial profile each time, PCR was performed on the same rumen fluid DNA extract three times. Figure 3-2 shows the relative abundance of the top 100 OTUs. The profile of the three bottles appeared very similar with no immediately obvious differences between the three profiles. The relative abundance of all phyla and genera present in at least one sample at > 1% can be seen in Appendix A-3 along with the coefficient of variation.

3.3.3 Reproducibility of fermentation bottles

Throughout this thesis, three replicate fermentation bottles from each sample, at each time point underwent DNA extraction before they were pooled in equal ratio to a final concentration of 10 ng/µl prior to PCR and downstream analysis. To ensure that the pooled bottle was a true representative of the three individual bottles, DNA extracted by QK from a set of three bottles was amplified and sequenced alongside the pooled equivalent. A theoretical average of the three replicate bottles at the Phyla and Genera level was also calculated (Table 3-1). At the phylum level, there was no significant difference between observed (pooled) and the theoretical average relative abundances ($X^2 = 4.51$, df =5, p = 0.479) and the same was seen at the genus level ($X^2 = 7.077$, df = 22, p = 0.999)

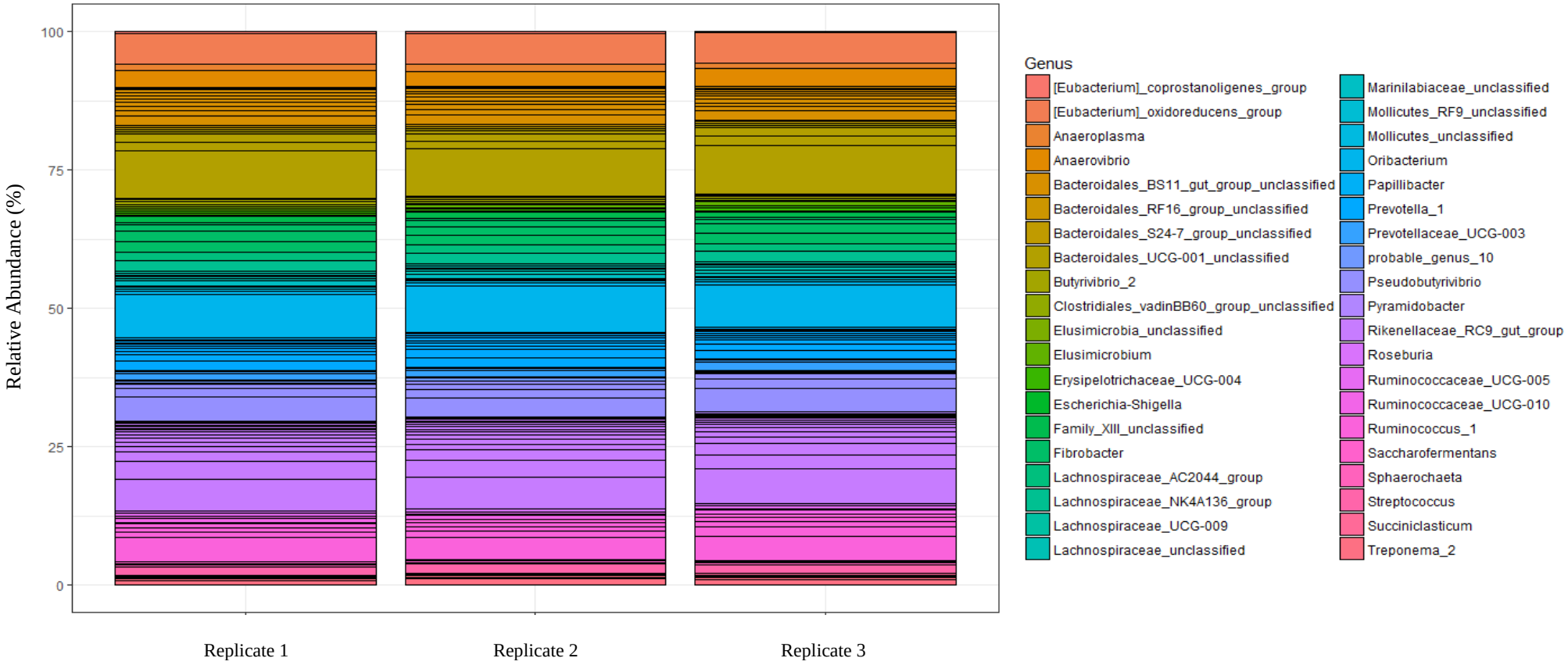

Figure 3-2 The top 100 operational taxonomic units (OTUs) for three library preparations of the same rumen fluid DNA extract. The relative abundance of the top 100 OTUs are represented by each horizontal black line. Each colour represents a different Genus.

Table 3-1 The relative abundance (> 1%) at the Phyla and Genera level from three replicate fermentation bottles and their mean value, and from a sample in which the DNA extracted from three replicate bottles was pooled before PCR and sequencing (pooled)

	Relative abundance (%)				
	Replicate 1	**Replicate 2**	**Replicate 3**	**Average**	**Pooled**
Phyla[a]					
Bacteroidetes	38.05	35.71	32.83	35.53	35.61
Firmicutes	32.13	26.12	27.72	28.66	26.35
Fibrobacteres	12.52	23.18	21.42	19.04	21.04
Tenericutes	8.47	8.88	10.46	9.27	9.60
Spirochaetae	5.62	3.12	3.82	4.19	4.07
Proteobacteria	1.35	0.93	1.68	1.32	1.57
Synergistetes	0.42	0.43	1.41	0.75	0.48
Saccharibacteria	0.20	1.05	0.01	0.42	0.37
Genera[a]					
Prevotella 1	14.47	15.70	14.92	15.03	14.95
Fibrobacter	12.52	23.18	21.42	19.04	21.04
Bacteroidales BS11 gut group unclassified	5.79	6.33	2.24	4.79	4.66
Mollicutes unclassified	5.69	7.05	8.67	7.13	7.25
Lachnospiraceae AC2044 group	5.58	1.27	1.84	2.90	2.73
Prevotellaceae UCG-001	5.24	5.28	3.21	4.58	4.62
Treponema 2	5.12	2.91	3.55	3.86	3.69
Pseudobutyrivibrio	4.52	3.53	4.28	4.11	4.16
Ruminococcus 1	3.56	3.17	2.99	3.24	3.12
Oribacterium	3.38	1.99	2.06	2.47	2.21
Bacteroidales UCG-001 unclassified	3.12	3.47	5.65	4.08	4.02
Rikenellaceae RC9 gut group	3.08	2.21	2.72	2.67	3.07
Anaeroplasma	1.94	0.74	0.74	1.14	1.33
Butyrivibrio 2	1.89	1.33	2.23	1.81	1.34
Bacteroidales S24-7 group unclassified	1.79	0.13	1.27	1.06	0.90
Bacteroidetes unclassified	1.57	0.06	0.06	0.56	0.38
Streptococcus	1.47	0.99	1.82	1.43	1.22
Erysipelotrichaceae UCG-004	1.45	0.83	1.05	1.11	1.03
Erysipelotrichaceae unclassified	1.35	0.06	0.05	0.49	0.37
Lachnospiraceae unclassified	1.16	1.33	0.48	0.99	0.71
Escherichia-Shigella	1.05	0.56	1.34	0.98	1.09
Probable genus 10	0.98	2.08	1.78	1.61	1.52
Roseburia	0.92	1.01	0.63	0.85	0.73
Lachnospiraceae NK4A136 group	0.72	1.63	1.33	1.23	1.15
Pyramidobacter	0.42	0.43	1.41	0.75	0.48
Candidatus Saccharimonas	0.20	1.05	0.01	0.42	0.37
Ruminococcaceae UCG-005	0.04	1.19	0.41	0.55	0.63

[a] Present at a relative abundance > 1% in at least one sample

3.3.4 Identifying a difference in bacterial community composition between two treatments

To determine whether the sequencing pipeline was capable of identifying differences between two treatments, DNA was extracted from faecal samples from two groups of piglets fed either a therapeutic, high zinc oxide diet (2500 ppm; Z) or a standard control zinc oxide diet (100 ppm; C) for twenty days post weaning . A total of 12,272,450 reads were obtained with an average of 511,352 ± 99,774 reads per group. After all filtering and clustering steps a total of 1,356,937 unique, high quality sequences remained with an average of 56,543 ± 17,824 per group. Average coverage was 96.3 ± 1.13 %.

There were found to be nine phyla with a relative abundance greater than 1% in at least one sample, namely Bacteroidetes, Firmicutes, Tenericutes, Proteobacteria, Actinobacteria, Spirochaetes, Epsilonbacteraeota, Kirimatiellaeota and Planctomycetes. Bacteroidetes was significantly higher in samples from animals fed therapeutic levels of zinc oxide alongside significantly lower abundances of Firmicutes, Spirochaetes, Planctomycetes and Kirimatiellaeota compared to the control (Table 3-2). The Firmicutes to Bacteroidetes ratio was significantly lower in samples containing therapeutic levels of ZnO compared to the control levels of ZnO (1.02 ± 0.31 *vs* 1.45 ± 0.30; t = -3.510, df =22, p = 0.002). Other phyla remained unchanged.

Table 3-2 The average (± SD) relative abundance (%) of each phylum that was present at a minimum of 1% abundance in at least one sample from piglets fed either a diet containing a high (High ZnO; 2500 ppm) or a control concentration of zinc oxide (Control; 100 ppm)

Phyla	High ZnO	Control	p value
Bacteroidetes	48.2 ± 6.96	38.3 ± 4.69	< **0.001**
Firmicutes	47.3 ± 6.32	54.5 ± 5.05	**0.005**
Tenericutes	1.84 ± 1.80	1.19 ± 0.86	0.276
Proteobacteria	1.60 ± 1.56	1.69 ± 1.59	0.671*
Actinobacteria	0.70 ± 0.45	0.59 ± 0.53	0.443*
Epsilonbacteraeota	0.16 ± 0.25	0.18 ± 0.36	0.052*
Spirochaetes	0.11 ± 0.14	1.93 ± 2.85	**0.012***
Planctomycetes	0.0008 ± 0.003	0.46 ± 0.65	**0.020***
Kirimatiellaeota	0.00	0.60 ± 1.01	**0.001***

* Denotes a non-parametric test was performed.

Beta diversity analysis revealed a significant effect of therapeutic levels of ZnO on the bacterial community composition, with a clear shift on the NMDS plot (Figure 3-3; PERMANOVA; $F_{1, 23} = 5.3151$, $P < 0.001$). No effect of sex was observed ($F_{1, 23} = 0.8238$, $p = 0.705$). Across three measures of alpha diversity, no difference between Z and C samples was seen (Chao 1 - 8501 ± 4216.4 *vs* 7077 ± 1796.9, Shannon - 4.94 ± 0.289 *vs* 5.22 ± 0.624, Simpson's - 0.96 ± 0.019 *vs* 0.98 ± 0.016 for Z and C respectively; $p > 0.05$; Figure 3-4).

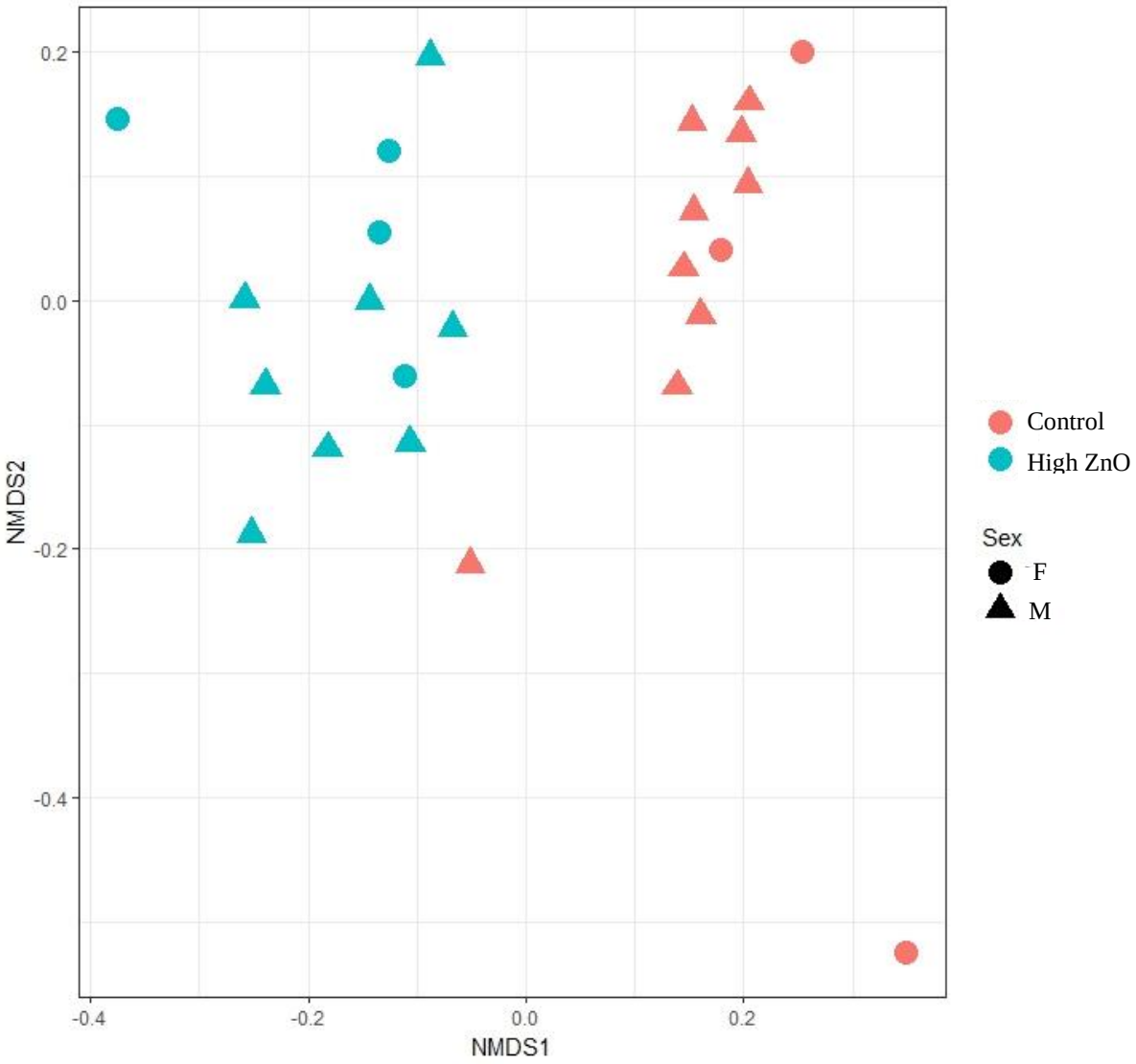

Figure 3-3 Non-metric multi-dimensional scaling (NMDS) plot of the bacterial community composition of faecal samples from male and female piglets fed either a therapeutic (2,500 ppm) or control (100 ppm) level of dietary zinc oxide (ZnO)

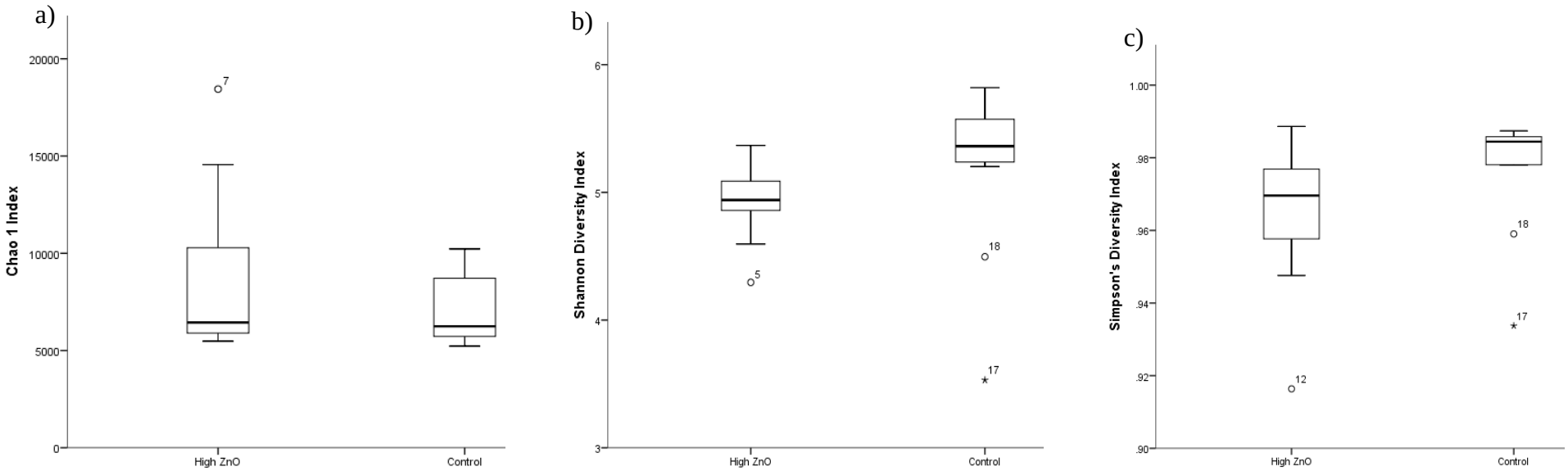

Figure 3-4 Alpha diversity indices of the bacterial community composition from faecal samples collected from piglets fed either a high (2,500 ppm) or control (100 ppm) level of dietary zinc oxide (ZnO). No difference in alpha diversity was observed for any of the three measures a) Chao 1 index $F_{1, 22} = 0.9352$, $p = 0.3445$, b) Shannon diversity index $F_{1, 22} = 1.7546$, $p = 0.1995$ and c) Simpson's diversity index $F_{1, 22} = 2.9409$, $p = 0.1011$

DeSeq2 analysis was performed to determine which OTUs showed the largest difference in relative abundance between the Z and C group. A total of 230 OTUs differed significantly between the samples with 50 OTUs increasing relative to the control diet and 180 OTUs decreasing. Table 3-3 presents the 10 OTUs that showed the greatest increase and decrease in the Z samples relative to the control.

Table 3-3 DeSEQ2 analysis of the operational taxonomic units (OTUs) in the high zinc treated samples that showed the greatest change in abundance relative to the control samples over the experimental period. OTUs are classified to the Genus level. The p values shown are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold change represents Log2 fold change.

OTU Number	Genus	Fold change	p value
Increase with High ZnO			
OTU 185	*Ruminococcaceae UCG-014*	24.21	< 0.001
OTU 103	*Parabacteroides*	7.48	<0.001
OTU 209	*Roseburia*	7.64	< 0.001
OTU 26	*Ruminococcaceae unclassified*	6.07	< 0.001
OTU 118	*Ruminococcaceae UCG-014*	8.77	< 0.001
OTU 222	*Flavonifractor*	7.30	< 0.001
OTU 275	*Roseburia*	7.44	< 0.001
OTU 346	*Ruminococcaceae unclassified*	6.27	< 0.001
OTU 99	*Roseburia*	5.91	< 0.001
OTU 298	*Ruminococcaceae unclassified*	6.32	< 0.001
Decrease with High ZnO			
OTU 281	*Lachnospiraceae AC2044 group*	-25.04	< 0.001
OTU 309	*Treponema 2*	-24.78	< 0.001
OTU 115	*Treponema 2*	-23.60	< 0.001
OTU 67	*Oribacterium*	-8.77	< 0.001
OTU 2778	*Prevotella 9*	-21.21	< 0.001
OTU 53	*Megasphaera*	-7.17	< 0.001
OTU 177	*Ruminococcaceae UCG-010*	-9.54	< 0.001
OTU 49	*Christensenellaceae R-7 group*	-10.76	< 0.001
OTU 133	*Christensenellaceae R-7 group*	-9.73	< 0.001
OTU 114	*Christensenellaceae R-7 group*	-9.23	< 0.001

3.4 Discussion

3.4.1 Comparison of two commercial DNA extraction kits

The first thing of note when comparing the two DNA extraction kits was that the DNA yield from the microbial standard was much higher for the ZymoBIOMICS kit (ZK) than for the QIAamp DNA Stool Mini Kit (QK; 22.7 ± 6.21 *vs* 1.23 ± 0.40 ng/µl). The amount of starting material was 75 µl (1.4 x 10^{10} cells per ml) as stated in the manufacturer's instructions. The QK, is designed for stool samples and would usually require *ca* 200 mg of starting material, resulting in a much larger amount of starting material than observed for the microbial standard. As the volume of buffers for QK cannot be reduced during the extraction process, it is likely that the sample was much more dilute when compared to ZK. When the bacterial profile of the two kits was compared they were found to cluster together on an NMDS plot with no significant effect (PERMANOVA analysis) of kit used. There was also found to be no difference in alpha diversity when measured with Shannon or Simpson diversity indices. Both measures of diversity suggest that the amplified community was the same from both kits despite the much lower extracted DNA concentration from the QK kit.

Both *Listeria monocytogenes* and *Enterococcus faecalis* were underrepresented in extractions from both kits. The aforementioned species are both Gram-positive bacteria which are considered more difficult to lyse than Gram-negative bacteria due to the increased thickness and strength of the peptidoglycan layer in their cell wall (20 - 80 nm vs 1 - 7 nm respectively) (Cabeen and Jacobs-Wagner, 2005). The thicker cell wall of Gram-positive bacteria has been found to cause an under representation of these species in environmental samples (Hermans et al., 2018). Despite this, other Gram-positive species within the microbial community standard extracted and amplified as expected (*L. fermentum, S. aureus, B. subtilis*). The protocol for the QK kit recommended an increase of the first lysis temperature to 95°C from 70°C to improve lysis of Gram-positive bacteria. The increased lysis temperature was used alongside a bead-beating step to maximise lysis of Gram-positive bacteria. The inclusion of a bead beating step has been shown to increase the relative abundance of Gram-positive bacteria four-fold (Albertsen et al., 2015) and freezing samples prior to DNA extraction has been found to improve the extraction of Gram-positive bacteria through disruption of the cell wall due to freeze-thaw (Bahl et al., 2012; Wesolowska-Andersen et al., 2014). The relative abundance of

Firmicutes (Gram-positive phyla) extracted across this thesis from rumen samples is comparable to other published literature. To ensure a more thorough extraction of Gram-positive bacteria from environmental samples when using deeper sequence analysis than the genus level, it may be necessary to include an incubation with lysozyme prior to DNA extraction to maximise cell wall lysis. A combination of lysozyme, mutanolysin and lysostaphin have been found to improve lysis of Gram-positive bacteria from both human and environmental samples with the caveat that the extraction procedure takes longer to perform (Bag et al., 2016).

The DNA from a rumen sample was extracted using both kits, which was then sequenced and the resulting bacterial profiles were compared. The profile produced showed some variation in the relative abundance of both phyla and genera, however, the top three most abundant genera were found to be the same in DNA samples extracted using both methods (*Prevotella 1*, *F082 ge* and *Rikenellaceae RC9 gut group* at 31.55, 7.65 and 3.62 % for QK and 27.09, 9.38 and 4.72 % for ZK respectively) and the Firmicutes to Bacteroidetes ratio was similar (0.66 *vs* 0.65 for QK and ZK respectively). Therefore, both kits appeared to produce a similar bacterial profile from a complex bacterial community. In a study by Wagner Mackenzie et al. (2015), five different extraction methods were used and it was shown that biological variation was greater than any variation introduced via the kit used, therefore, small differences in abundance should not result in different experimental outcomes.

Although both kits were comparable, a consideration when using ZK is that there is a possibility of greater risk of contamination of samples through the extraction processes. Each of the spin column types contained within the kit required a tab to be snapped off the bottom on three separate occasions during the protocol. The risk of introducing environmental contamination through contact with gloves is possible if proper care is not taken. Although the handling time was slightly longer, QK was preferred for its simplicity of use and has been used throughout the thesis.

3.4.2 Repeatability of the experimental ‘pipeline’

DNA extracted from a randomly chosen rumen fermentation sample was amplified via PCR and purified independently three times. Each purified product underwent library preparation and 16S sequencing on the MiSeq platform. The bacterial profile obtained was shown to be highly similar between the three samples, with generally good

coefficients of variation (< 10 %) confirming that the PCR step through to bioinformatics output produced highly similar bacterial community composition. PCR can introduce bias into community analysis due to the fact that not all fragments within a community are amplified with the same efficiency resulting in a different mean relative abundance compared to the original community composition (Pinto and Raskin, 2012; van Dijk et al., 2014). Therefore, it was important to ensure the PCR steps (initial amplification and library preparation) were producing the same amplified community from DNA extracted from the same complex rumen bacterial community each time and not introducing sample-dependant biases which may result in different experimental outcomes. As well as PCR bias, there is scope for error in the sequencing reads resulting in different OTU assignment, as read length increases on the MiSeq platform. As read length increases so too does error rate due to an accumulation cluster interference particularly for the reverse read (Schirmer et al., 2015). However, as the relative abundances in Appendix A-3 show, each sample showed a very similar bacterial composition. The largest coefficient of variation (CV) was observed for the phylum Lentisphaera (24.3 %) and the genus *Erysipelotrichaceae UCG-004* (16.7%). For both of these, the mean relative abundance values were low (1.09 and 0.97 respectively). Although the CV is usually independent of the mean, values at the extremes of a given range can be associated with higher CV values (Reed et al., 2002).

3.4.3 Repeatability of *in vitro* fermentation bottes

The selection pressures on the microbial community within a fermentation bottle of a batch culture *in vitro* rumen model are different to those experienced in the rumen, and consequently, as has been demonstrated in this thesis (see Chapters 4, 5 and 7), the microbial community within a fermentation bottle changes over time. It is important to ensure that the selection pressures within replicate fermentation bottles are similar. For experimental samples throughout this thesis, DNA was extracted from each replicate fermentation bottle (n = 3) and pooled to provide one DNA sample for each treatment at each time point. To confirm that each bottle provided a similar bacterial profile, the DNA from three replicate fermentation bottles of the same treatment underwent independent analysis. The community composition was found to be similar across the three bottles, however, some variation was observed, most noticeably for the phylum Fibrobacteres for which the relative abundances were 12.5, 23.2 and 21.4 % across the three experimental bottles. The relative abundance of Fibrobacteres in a sample has been shown to be

affected by bead beating duration (Henderson et al., 2013), however, as these samples were processed in the same batch this does not explain the difference observed in the relative abundance of this phylum. Pooling of the three fermentation bottles as opposed to random selection of one bottle for sequence analysis is preferable in order to capture the full variation in microbial profile that is observed.

The bacterial composition (relative abundance) of the pooled sample was compared to the mean relative abundance (predicted) values of the three replicate fermentation bottles to confirm that the pooled sample was an accurate representation of the three individual fermentation bottles. The lack of significant difference between the pooled and theoretical values at both the level of the phylum and genus supports the use of a pooled DNA extract as a proxy for individual replicate bottles in sequencing experiments based on the use of an *in vitro* model.

3.4.4 The sequencing pipeline was capable of detecting differences between two treatments

To ensure that the sequencing pipeline was capable of detecting differences between treatments, bacterial DNA was extracted from faecal samples obtained from two groups of piglets that were fed either a diet containing a high concentration of zinc oxide (2,500 ppm; therapeutic concentration) or a standard control diet containing 100 ppm ZnO for the first twenty days post-weaning. At therapeutic levels, ZnO has previously been shown to modulate the intestinal microbiota (Yu et al., 2017) and faecal sampling has been shown to be a useful proxy for the distal gut (Muiños-Bühl et al., 2018). Therapeutic ZnO has been shown to reduce the incidence of post-weaning diarrhoea in piglets and improve growth rate (Poulsen, 1995; Hill et al., 2001; Walk et al., 2015).

A clear shift in the microbial community composition was observed with a significantly higher relative abundance of Bacteroidetes in samples containing therapeutic levels of dietary zinc oxide compared to the control (48.2 ± 6.96 *vs* 38.3 ± 4.69 % respectively). No difference in alpha diversity was observed between the two groups across three different measures, suggesting that the number of species present remains similar, but ZnO is applying a selection pressure resulting in shifts in the relative abundance at both the phyla and genus level. The ratio of Firmicutes to Bacteroidetes (F:B), the two main phyla found in the gut of pigs (Kim and Isaacson, 2015), was significantly lower in piglets fed a therapeutic level of ZnO. The F:B ratio has also been shown to decrease in

faecal samples of Bama minipigs when orally treated with salbutamol, a ß-agonist that has been used as an illegal growth promoter in livestock (Lu et al., 2017). An increased F:B ratio has been associated with an obese phenotype (Ley et al., 2005; Turnbaugh et al.), inflammation (Pellegrini et al., 2017) and irritable bowel syndrome in humans (Nagel et al., 2016). An increased F:B ratio has also been identified in pigs suffering *Brachyspira* associated colitis and mucohaemorrhagic diarrhoea (Costa et al., 2014).

At the genus level, therapeutic levels of ZnO were found to increase the genera *Roseburia*, *Parabacteroides* and *Ruminococcaceae* all of which have been shown to have a protective effect on the intestinal barrier in human studies through the production of butyrate (Dou et al., 2017; Geirnaert et al., 2017). *Roseburia sp*. have been suggested to increase the expression of genes associated with promoting gut barrier function and innate immunity (Patterson et al., 2017) and members of the genus *Parabacteroides* have been shown to reduce inflammation by modulating the levels of both anti-inflammatory (IL-10) and inflammatory cytokines (IL-17, IL-6 and IFN-γ) in the intestinal tract (Kverka et al., 2011; Li et al., 2016). Therefore, the presence of these species supports the theory that therapeutic levels of ZnO provides a protective effect on the intestinal barrier, reducing incidences of post-weaning diarrhoea.

The clear difference in community composition between the two treatments demonstrated that the sequencing pipeline established for the work presented is capable of identifying differences in bacterial communities between samples.

3.4.5 Conclusion

The aim of this chapter was to determine whether the methodological pipeline used throughout this thesis was capable of reliably and accurately identifying the bacterial community associated with *in vitro* rumen fermentation. It was shown that the DNA extraction kit was able to reproducibly extract a known community from a microbial standard on three occasions. There was shown to be a lower than predicted abundance of some Gram-positive species, but the relative abundance of Gram-positive phyla such as Firmicutes in the rumen samples was shown to be similar to previously published studies. There was found to be no difference in bacterial profiles obtained from the same DNA extract amplified and sequenced three times, and a pooled DNA sample was found to be an accurate representation of the bacterial community shown in replicate fermentation bottles. Finally, the pipeline was able to clearly distinguish a difference in bacterial

composition between two treatments known to affect the bacterial composition of the gut, highlighting the suitability of the pipeline not only for the rumen, but also for other environmental samples.

Chapter 4 Cross inoculation of rumen fluid to improve dry matter digestibility and its effect on rumen bacterial composition using an *in vitro* batch model of rumen fermentation

4.1 Introduction

Rumen fermentation is integral to the performance of ruminant animals and therefore the desire to manipulate fermentation to improve livestock output has long been of interest to both animal scientists and microbiologists alike (Chalupa, 1977). The microbial community that resides within the rumen has been associated with an animal's ability to utilise feed and certain microbial populations have been associated with low residual feed intake (i.e. a more efficient animal) suggesting that a particular microbial profile may be responsible for more efficient digestion (Guan et al., 2008; Carberry et al., 2012). Due to the heritability of only some of the rumen bacterial community (Sasson et al., 2017), selection for animals which have a certain microbial profile has proved difficult, but has shown some success when associating the microbial community with a particular trait e.g. methane emissions (Roehe et al., 2016). The microbial population can be manipulated by diet (Henderson et al., 2015; McDermott, 2014) and although the rumen microbial population is considered one of the most efficient at digesting cellulose-rich biomass (Flint et al., 2008), the variability between individual animals (Jami and Mizrahi, 2012; Shabat et al., 2016) indicates there is scope to manipulate the established rumen community to improve fibre digestion.

By studying the difference between microbial communities that show large differences in their ability to digest fibre, it may be possible to identify the key microbes involved in plant cell wall digestion (Oss et al., 2016). It is plausible that by isolating a bacterial community that confers improved fermentative digestion and introducing this into an animal that shows less successful digestion, fermentative efficiency could be improved. This would therefore reduce the amount of feed required by the animal (and thereby land area), reduce the environmental impact associated with ruminant production and increase productivity. However, as described in the General Introduction, there appears to be an element of host control preventing the introduction of non-native species into its rumen.

Indeed, previous attempts to introduce fibrolytic bacterial species into the rumen to improve fibre digestion have not been positive despite inoculating species that had been

isolated from the rumen and cultured in the laboratory. Whether it be due to dilution and subsequent washing out of the rumen, predation by protozoa or the introduced species becoming outcompeted by the resident community, studies report generally negative results (Weimer, 2015). The well-studied inoculated strains may, having been grown for multiple generations under laboratory conditions, have lost their competitive edge when introduced into a complex ecosystem. To increase the likelihood of success, cross-inoculation studies have been designed using mixed ruminal communities sourced directly from the rumen.

As described in the General Introduction (section 1.4.8.3), when rumen content was swapped between a pair of dairy cows, it was found that the host re-established its pre-transfer pH and VFA levels within 24 hours and the bacterial composition was found to revert back to that of the original community within the rumen of the host animal with varying levels of success (Weimer et al., 2010).

A similar study was performed by Zhou et al. (2018). Rumen content from 16 steers identified as either efficient (low residual feed intake; LRFI) or inefficient (high residual feed intake; HRFI) was exchanged between animals such that LRFI received rumen content from either another LRFI animal (n = 4) or a HRFI animal (n = 4) and *vice versa* for the HRFI animals. There was found to be large individual variation in bacterial and archaeal profiles pre and post transfer and similarly to Weimer et al. (2010), the microbial composition after transfer was unlike that of the donor animal despite adding in three washing steps after removing rumen content to minimise re-seeding due to any remaining epimural microorganisms.

Rumen fluid inoculations between species of ruminant have also been attempted. Due to bison's superior ability to digest highly cellulolytic feedstuff (Richmond et al., 1977; Hawley et al., 1981a; Hawley et al., 1981b), Oss et al. (2016) hypothesised that the bison ruminal community would improve fermentative digestion of forage when combined with rumen fluid from cattle. Using a semi-continuous *in vitro* culture of rumen fluid (Rusitec), bison inoculum alone did not show improved fibre digestibility (measured by total dry matter (DM) and acid detergent fibre digestibility) when compared with cattle. When combined, however, the two showed a synergy to improve disappearance of straw DM and neutral detergent fibre (aNDF). *In situ* partial replacement of cattle rumen fluid with that from bison imparted no improvement to fibre digestibility of barley straw, canola straw or timothy hay. The extent of degradation of alfalfa hay showed small improvements compared with cattle alone (Griffith et al., 2017). Repeated inoculation of

bison rumen content (*ca* 70%) into the rumen of Angus x Hereford heifers 14 days apart showed no improvement to fibre digestibility. The microbial community was found to differ from pre-transfer composition, but tended to shift back to pre-transfer composition at 27 days after the second transfer (Ribeiro et al., 2017).

Co-inoculation of anaerobic industrial fermenters with ruminal content has also been investigated as a potential mechanism to improve cellulose hydrolysis (Chapleur et al., 2014). However, ruminal species were unable to establish and therefore it was unsurprising that cellulose digestion was not improved. An accumulation of propionate in the system when ruminal contents were added was thought to indicate process instability through disruption of degradation pathways due to cross-inoculation (Barnes and Keller, 2003). After 16 days, none of the operational taxonomic units initially present in the rumen inoculum were identified in the anaerobic digester.

Therefore, this chapter is a proof of concept study designed to establish whether manipulation of the mature rumen through cross inoculation is possible and if this is something that should be pursued further. As the host animal is thought to have a controlling effect over its residing microbiota, an *in vitro* batch culture model was used to allow manipulation of the bacterial community in the absence of influence from the host animal. Therefore, any differences in performance should be microbial in origin. The chapter aimed to establish whether cross inoculation of rumen fluid could be used to manipulate fermentation to improve dry matter digestibility and associated fermentation parameters. It was hypothesised that when exposed to the same environment, the microbiota and fibre digesting ability of cross-inoculated rumen fluid would rapidly equal that of the superior rumen fluid due to the advantages in energy harvest conferred by the superior rumen fluid.

4.2 Materials and methods:

Rumen fluid was collected at time of slaughter from 11 Holstein-Friesian steers at a commercial abattoir (ABP York, UK). Animals were selected from the same farm, from which all animals were of the same breed and sex and of similar age (656 ± 70.9 days) to reduce variation due to environmental factors. To identify rumen fluids that were at the extremes of performance within the model (best and worst), to be used in the main experiment, each rumen fluid was used to inoculate an *in vitro* batch model as described in the General Methods (Chapter 2.1.5). Fermentations were run for 24 hours with six bottles per rumen fluid. From these bottles, three were used for digestibility analysis and

the remaining three were used for sample collection. Due to the number of bottles, three 24 hour fermentations were performed, with each rumen fluid randomly assigned to a run. Four rumen fluids were used in Runs 1 and 2 and the remaining three in the final fermentation (Run 3). An additional rumen fluid ('standard') was included in each run to control for any differences in *in vitro* performance due to day. Blank bottles were included to account for any fermentation due to organic matter in the inoculum. *In vitro* dry matter digestibility (IVDMD) was measured at the end of the experiment. Gas pressure was recorded after 6.5 hours and at the end of the fermentation and pH was recorded as soon as bottles were uncapped. Samples were collected for VFA, NH_3-N and MCP analysis.

Using the results from the experiment above, two rumen fluids were selected that showed the best (Good) and worst (Bad) performance. Fluids were ranked based on their ability to digest fibre, total VFA production, MCP and NH_3-N concentration and acetate to propionate ratio. Each rumen fluid was given a score (1-11) for each parameter with the best given 1 and the worst 11. Scores were summed for each rumen fluid across the five parameters and the two fluids with the lowest and highest overall score were then used for the cross inoculation experiment. The 'Good' and 'Bad' rumen fluid were used to inoculate the *in vitro* model alongside a mix of the two ('1:1 Mix') using a consecutive batch culture (CBC) approach. The 1:1 Mix bottles were prepared by combining an equal volume of the Good and Bad rumen fluids, mixing by swirling and then 5 ml of this was transferred to each fermentation bottle.

The experimental period lasted for 16 days. An initial 24-hour fermentation was followed by seven 48-hour fermentations and ended with a final 24-hour fermentation (Figure 4-1). At the end of each fermentation (24 or 48 hours) a subset of bottles were uncapped and 5 ml was used to inoculate new bottles containing fresh feed and buffer under CO_2. A total of 162 bottles were used with 18 bottles per batch culture and 6 bottles per fluid per time point. Digestibility and sampling was performed as for the pre-experiment above with an additional aliquot collected for bacterial community sequencing (1.5 ml). Bacterial community composition was compared between the end of the first 24-hour fermentation (Day 1) and the final 24-hour fermentation (Day 16). The bacterial community associated with the dried grass substrate was also extracted.

4.2.1 Statistical analysis

Fermentation data from rumen fluid collected from the 11 Holstein-Friesian steers was analysed as a linear mixed model in R with run as a random factor using the package lme4. Models were reduced and compared using lmerTest. All data was tested for normality (Kolmogorov Smirnoff) and homogeneity (Levene's test) prior to any statistical analysis. Where data did not fit a normal distribution, a generalised linear mixed model was performed with a penalised quasi likelihood error distribution using the packages MASS and car. If the random effect was shown to have no effect in the model (no difference from zero) it was removed.

Correlations were performed on abattoir data against IVDMD. Pearson's correlation was used in all cases. A general linear model was fitted to data from the cross inoculation experiment with Time and Group included as main factors (IBM SPSS Statistics 21). When not significant, interactions were removed and the models were re-run. Tukey's *post-hoc* test was used to identify significant differences within a factor when significant within the model.

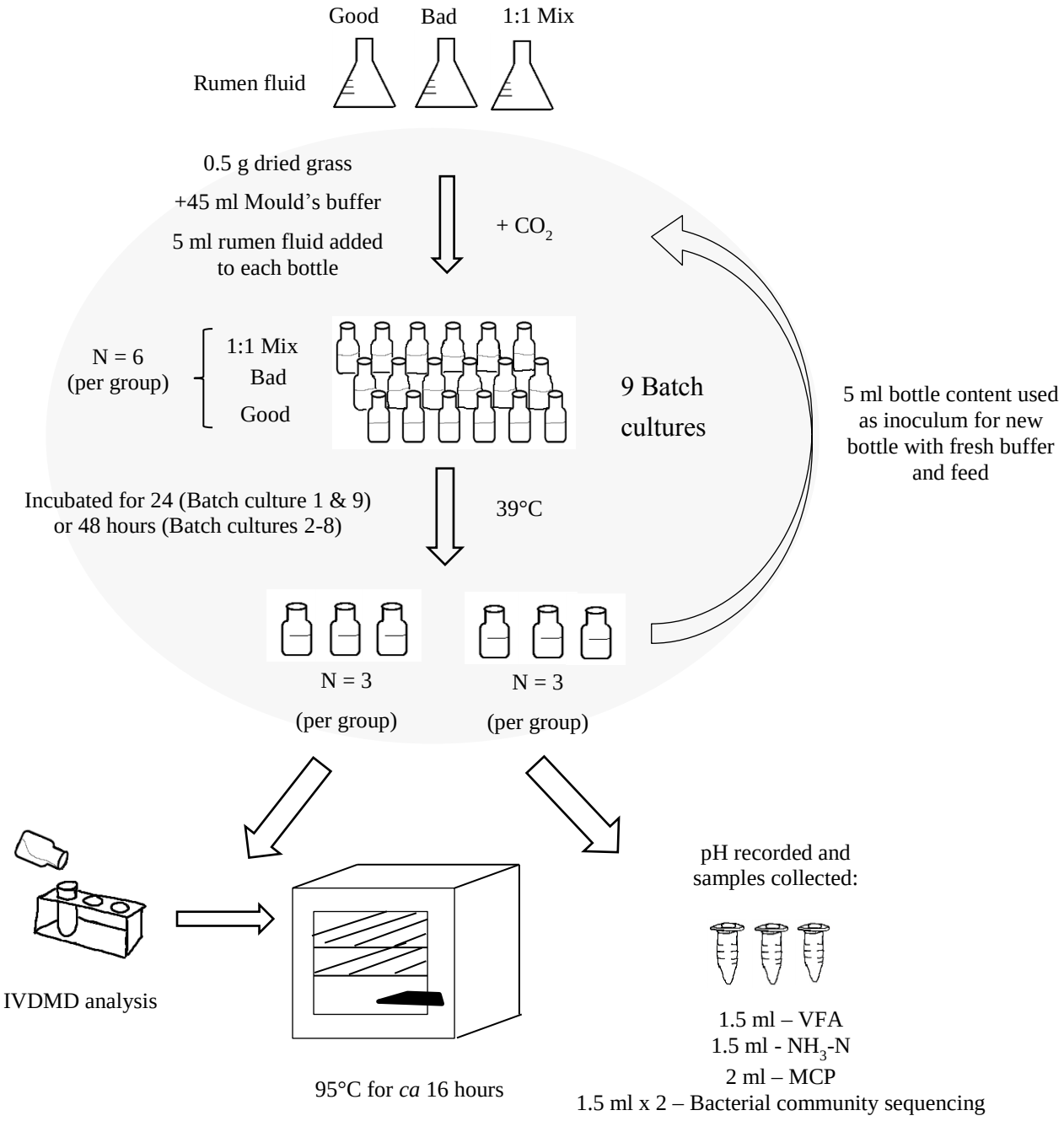

Figure 4-1 Schematic methods for Experiment 1 A good, bad and 1:1 mix of the two rumen fluids were used to inoculate an *in vitro* batch culture model of rumen fermentation. To each fermentation bottle containing *ca* 0.5 g of dried grass (n = 6 per group), 45 ml of salivary buffer and 5 ml of rumen fluid was added. Bottles were fermented for either 24 or 48 hours and the end contents used to inoculate new bottles containing fresh buffer and substrate. Samples were collected for volatile fatty acid (VFA), ammonia nitrogen (NH_3-N), microbial crude protein (MCP) analysis and bacterial community sequencing and the pH was recorded. Half of the bottles were used to calculate *in vitro* dry matter digestibility by gravimetric difference.

4.3 Results

4.3.1 Cattle performance and *in vitro* fermentation data

Each of the rumen fluids collected was run through the *in vitro* model of rumen fermentation. There was a wide variation in dry matter digestibility between the rumen fluids with differences up to 62% (range 0.22 – 0.37). Variation was seen across all rumen parameters measured (Table 4-1). Rumen fluid 1 was removed from further analysis due to experimental error during volatile fatty acid preparation. The 10 rumen fluids (2-11) were ranked from best to worst based upon IVDMD, MCP, NH_3-N, total [VFA] and propionate:acetate ratio (Figure 4-2). The best and worst performing rumen fluids in terms of fibre digestion efficiency were identified for use in the cross inoculation experiment. Rumen Fluid 2 was selected as the 'Good' fluid and due to a lack of fluid for Rumen Fluid 9, the second worst, Rumen Fluid 10, was selected as the 'Bad'.

Table 4-1 Variation between parameters of *in vitro* fermentation of rumen fluid from 11 Holstein Friesian cross steers collected at time of slaughter. Values shown represent the mean standardised per gram dry matter.

	Rumen fluid												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**SEM[1]**	**p value**
Gas volume (ml)	118.7	109.2	120.8	116.6	108.2	111.9	116.2	120.3	98.3	103.9	130.3	0.829	< **0.001**
IVDMD	0.312	0.358	0.343	0.369	0.283	0.313	0.334	0.297	0.22	0.265	0.299	0.005	< **0.001**
pH	6.53	6.54	6.52	6.55	6.62	6.6	6.6	6.56	6.58	6.59	6.56	0.004	< **0.001**
NH_3-N (mg/ml)	1.05	1.05	1.09	0.99	1.04	1.08	1.02	1	1.03	0.99	1.08	0.020	0.360
MCP (mg/ml)	0.54	0.51	0.51	0.38	0.39	0.54	0.17	0.24	0.61	0.24	0.54	0.032	0.088
Total VFA (mM)	NA[2]	51.9	52.9	58.2	43	52.5	50.9	56.1	48.9	49.4	60.9	2.264	**0.010**

[1]SEM = standard error of the mean, NH_3-N = Ammonia nitrogen, MCP = Microbial crude protein, VFA = volatile fatty acid
[2]Experimental error resulted in no VFA data for this rumen fluid

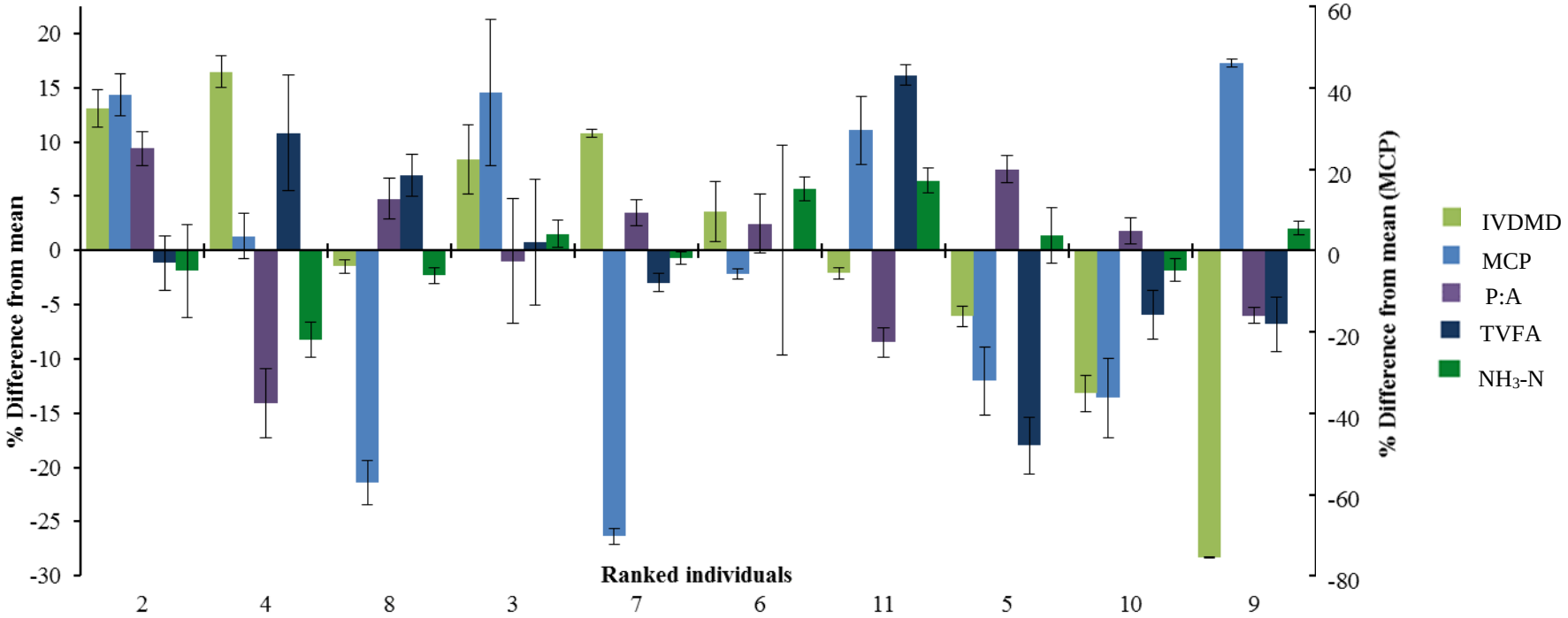

Figure 4-2 Ranking of rumen fluids based upon 5 measures of *in vitro* performance. Rumen fluid was ranked based upon *in vitro* dry matter digestibility (IVDMD), microbial crude protein (MCP), propionate to acetate ratio (P:A), total volatile fatty acid (TVFA) and ammonia-nitrogen (NH_3-N) with the rumen fluid that showed the best overall performance within the *in vitro* model on the left hand side. Due to larger variation, MCP data is shown on the right hand axis. Error bars show SE. Values shown are average distance from the mean for each measure. With the exception of NH_3-N, positive values were considered to be good.

4.3.1.1 No correlation between abattoir data and *in vitro* performance

Information available from the abattoir on price paid for the carcass, age of the animal at time of slaughter and cold weight were correlated with performance *in vitro*. The price paid for the carcass (r = -0.495, N = 9, p = 0.176), age at time of slaughter (r = -0.506, N = 11, p = 0.113) and the cold weight of the carcass (r = -0.424, N = 11, p = 0.194) showed no significant correlation with IVDMD (Figure 4-3).

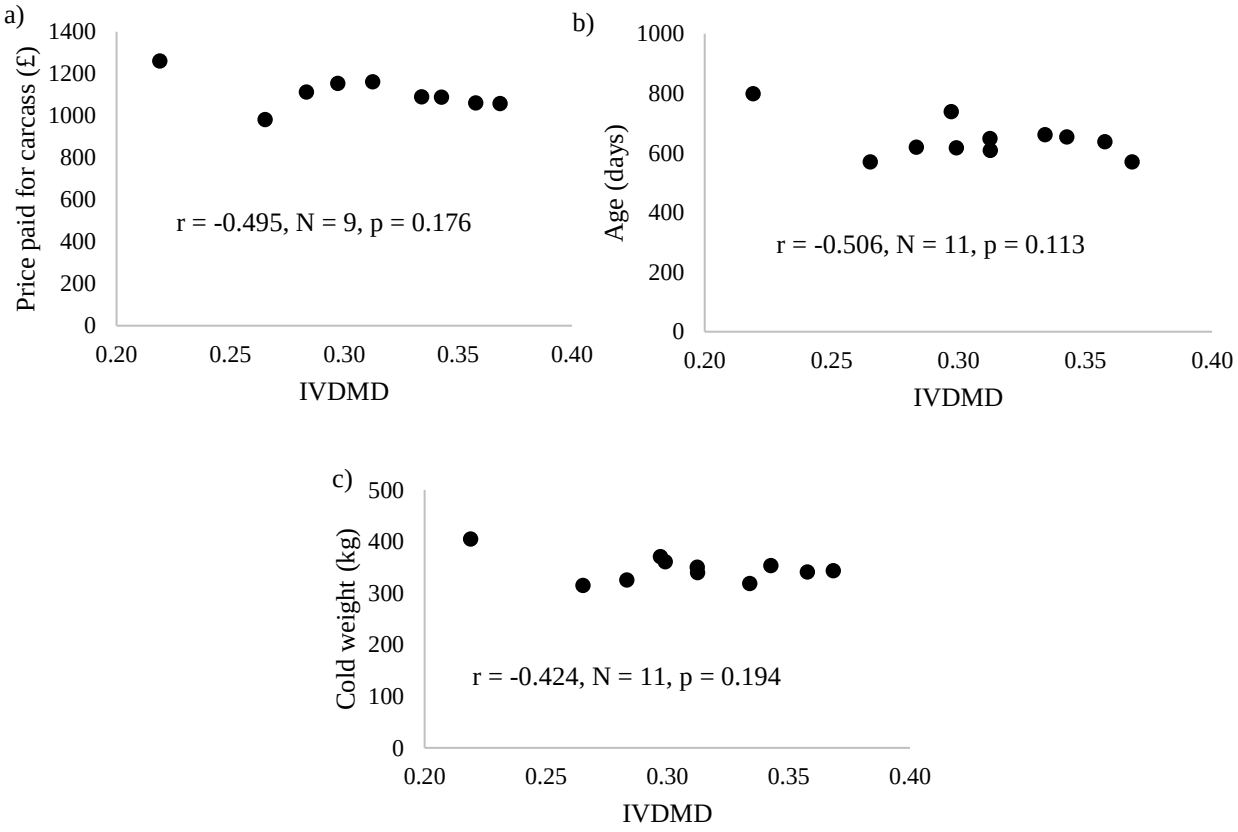

Figure 4-3 Correlations between *in vitro* dry matter digestibility and data received from the abattoir. a) price paid for carcass (£), b) age of the animal (days), c) cold weight (kg) showed no correlation with *in vitro* dry matter digestibility (IVDMD). R^2 is shown for goodness of fit.

4.3.2 The effect of cross inoculation on *in vitro* dry matter digestibility and fermentation parameters

The best and worst performing rumen fluid identified from Figure 4-2 (Rumen Fluid 2 and 10) were selected for use in this experiment. Rumen fluids (RF) were combined in equal ratio to identify whether cross-inoculation could be used to improve *in vitro* dry matter digestibility (IVDMD) over a 16 day period. IVDMD significantly increased ($t = -8.237$, $df = 6.34$, $p < 0.001$) from the start (Day 1) to the end (Day 16) of the experimental period, with an average increase of 45, 142 and 63% for the Good, Bad and 1:1 Mix RF respectively (Figure 4-4a). Cross inoculating rumen fluid resulted in an intermediate IVDMD for the 1:1 Mix RF at 24 hours compared to the Good and Bad RFs (29 compared to 34 and 20 g of digested DM per 100g DM respectively; $F_{2,6} = 351.461$, $p < 0.001$), but differences between the fluids were lost with consecutive culturing. IVDMD increased with each 48 hour consecutive batch culture up to Day 9. Following this, no further improvement was observed, reaching a maximum digestibility of 64g per 100g DM for Good and Bad and 63 g per 100g DM for 1:1 Mix RFs (Figure 4-4b).

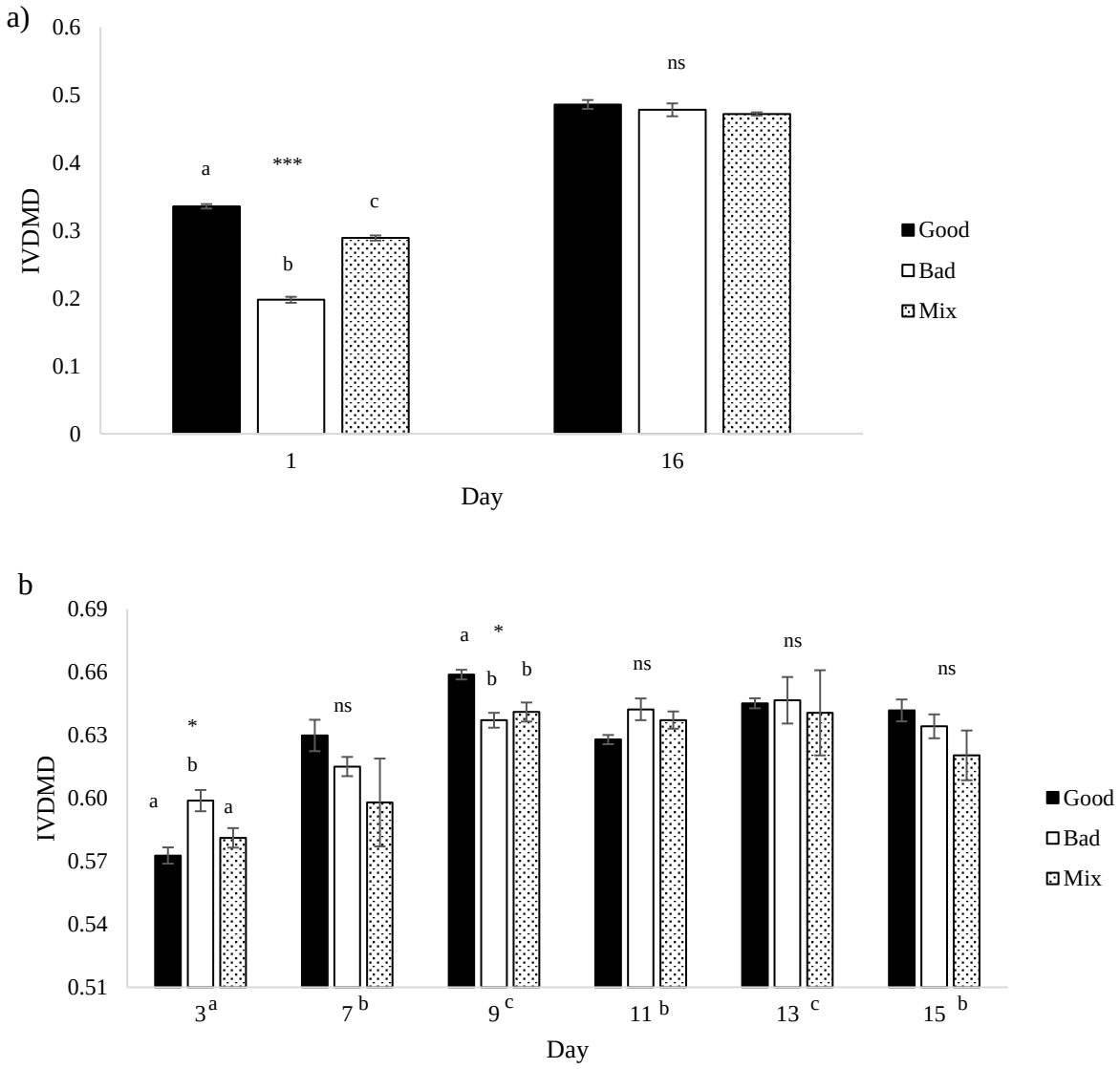

Figure 4-4 *In vitro* dry matter digestibility analysis for the Good, Bad and cross inoculated (1:1 Mix) rumen fluid. a) 24 hour fermentations (Day 1 & Day 16) and b) 48 hour fermentations (Days 3, 7, 9, 11 & 15). Bars show the mean value at each time point with standard error bars. Significant differences between RFs within a batch culture are shown by different superscript letters above the columns (Figure 4-4a and 4-4b) and differences between batch cultures are shown by different superscript letters next to the x-axis day labels (Figure 4-4b). *** $p < 0.001$, * $p < 0.01$, ns, no significant difference.

Parameters for the 24 hour fermentations on Days 1 and 16 are summarised in Table 4-2. Gas volume showed a similar pattern to IVDMD, with total gas produced over 24 hours increasing over the experimental period. A significant difference in gas volume was observed between the groups on Day 1 ($F_{2,15} = 85.370$, $p < 0.001$) with both the Good and 1:1 Mix RFs producing a higher volume than the Bad (151 and 148 ml *vs* 130 ml

respectively; $p < 0.001$). No difference was seen between the three fluids at Day 16 (160 vs 163 vs 159 ml for Good, Bad and 1:1 Mix respectively; $F_{2, 17} = 1.114$, $p = 0.354$).

The concentration of total volatile fatty acids (tVFA; mM) produced over 24 hours revealed a significant interaction between Group and Time ($F_{2,17} = 10.615$, $p < 0.01$) with tVFA decreasing between Day 1 and Day 16 for both the Good RF (80.93 *vs* 71.18; $t = 4.747$, $df = 4$, $p < 0.01$) and 1:1 Mix RF (79.84 *vs* 72.08; $t = 6.654$, $df = 4$, $p = 0.003$), but showing no statistically different change for the Bad RF (69.26 *vs* 70.97; $t = -0.762$, $df = 4$, $p = 0.489$). The difference between the three groups at Day 1 (80.9 *vs* 69.3 *vs* 79.8; $F_{2, 8} = 26.643$, $p = 0.001$) was not seen at Day 16 (71.2 *vs* 71.0 *vs* 72.1; $F_{2,8} = 0.178$, $p = 0.841$). The concentration of acetate, propionate and butyrate were also examined (Table 4-3). There was no difference in acetate production between the three groups on Day 1 or Day 16 ($F_{2, 17} = 2.086$, $p = 0.161$) with no effect of Day ($F_{1, 17} = 0.672$, $p = 0.426$) and no interaction ($F_{2, 17} = 1.533$, $p = 0.255$). For propionate, there was an interaction term between Day and Group ($F_{2, 17} = 4.844$, $p = 0.029$) with a significant difference between all three groups at Day 1 ($p < 0.001$), but no difference seen at Day 16 ($p = 0.873$). Only an effect of Day was seen for butyrate ($F_{1, 17} = 50.497$, $p < 0.001$) with the concentration decreasing from Day 1 to Day 16 (38.2 vs 21.7 mM). The acetate to propionate ratio (A:P) showed an interaction between Day and Group ($F_{2, 17} = 8.239$, $p = 0.006$) with significant differences between the three groups at Day 1 (2.0 vs 2.7 vs 2.3 for Good, Bad and 1:1 Mix respectively; $p < 0.001$) but not at Day 16 (2.2 vs 2.1 vs 2.1 respectively; $p = 0.764$).

There was a significant effect of time ($p < 0.001$) on the pH of the fermentation liquor with the pH after the second 24 hour fermentation (Day 16) lower than after the first (Day 1), but no difference was observed between the groups at both Day 1 (6.64 *vs* 6.68 *vs* 6.64; $F_{2,8} = 2.750$, $p = 0.142$) and Day 16 (all 6.55; $F_{2,8} = 2.750$, $p = 0.914$). Microbial crude protein (MCP) decreased between Days 1 and 16 (537.1 ± 18.83 *vs* 392.0 ± 36.97 µg/ml; $F_{1, 16} = 6.765$, $p < 0.05$), but no Group effect was observed ($F_{2, 16} = 0.085$, $p = 0.919$). Ammonia nitrogen concentration remained the same across the two 24 hour fermentations (1.15 ± 0.04 *vs* 1.14 ± 0.02) with no effect of Group ($F_{2, 17} = 2.072$, $p = 0.163$) or Time ($F_{1, 17} = 0.043$, $p = 0.839$) over the 24 hour fermentations.

Table 4-2 Fermentation parameters for 24 hour fermentations on Day 1 and Day 16 of Mixing Experiment 1. Mean values shown are corrected per g DM.

		Day 1	Day 16	SEM[1]	Time	Group	Time*Group
Gas volume (ml)	**G**	150.5	160.1	0.700	**< 0.001**	**< 0.001**	**< 0.001**
	B	130.3	163.3				
	M	148.1	159.3				
pH	**G**	6.64	6.55	0.000	**< 0.001**	0.132	0.204
	B	6.68	6.55				
	M	6.64	6.55				
tVFA (mM)	**G**	80.93	71.18	1.627	**< 0.001**	**0.001**	**0.002**
	B	69.26	70.97				
	M	79.84	72.08				
NH_3-N (mg/mL)	**G**	1.13	1.16	0.032	0.839	0.163	0.351
	B	1.2	1.15				
	M	1.12	1.12				
MCP (µg/mL)	**G**	540.9	351.6	59.161	**0.016**	0.908	0.747
	B	516.7	424.1				
	M	553.8	400.4				

[1]SEM = standard error of the mean, tVFA = total volatile fatty acids, NH_3-N = ammonia nitrogen, MCP = microbial crude protein, G = good, B = bad, M = 1:1 Mix rumen fluid

Table 4-3 VFA analysis for the 24 hour fermentations on Day 1 and 16. Mean values are shown and values are standardised per g DM. All concentrations shown are in mM.

	1			16				P value		
	Good	**Bad**	**Mix**	**Good**	**Bad**	**Mix**	**SEM[1]**	**Time**	**Group**	**Day*Time**
Acetate	87.0	80.7	90.4	87.2	87.6	88.7	1.05	0.426	0.161	0.255
Propionate[2]	43.9[a]	30.4[b]	38.7[c]	40.6	42.7	42.9	1.02	0.051	0.100	**0.029**
Butyrate	39.5	35.7	39.4	23.1	21.1	20.8	1.24	**< 0.001**	0.611	0.807
A:P[1]	2.0[a]	2.7[b]	2.3[c]	2.2	2.1	2.1	0.04	**0.020**	0.057	**0.006**

[1]A:P, Acetate to propionate ratio, SEM, standard error of the mean

[2]Different superscript letters within a row, within a day represent a significant difference between the groups ($p < 0.05$)

The parameters for the 48 hour fermentations are summarised in Table 4-4. A Time*Group interaction was observed for gas volume ($F_{12, 125} = 2.520$, $p < 0.01$) with a reduction in gas production from Day 3 to Day 7, an increase in from Day 7 – 11 and a final decrease to Day 15 for all groups, although changes were generally small (< 30 ml). The total VFA concentration (tVFA) increased with each consecutive batch culture from 88.1 ± 1.4 mM on Day 3 to 98.8 ± 2.4 mM on Day 15, but no difference was seen between the three fluids ($F_{2,44} = 2.767$, $p = 0.076$). The pH of the rumen liquor was significantly affected by both Time ($F_{3, 35} = 4.591$, $p < 0.05$) and Group ($F_{2, 35} = 21.458$, $p < 0.001$). Numerically, the pH of the Good RF was higher than that of the Bad and the 1:1 Mix at all time points. A significant interaction between Time and Group was identified for MCP ($F_{8, 34} = 2.790$, $p < 0.05$) with the concentration of microbial protein fluctuating in all three groups over the course of the experimental period. NH_3-N concentration was affected only by Time ($F_{4, 44} = 32.537$, $p < 0.001$) reaching a maximum value of 1.51 ± 0.03 mg/ml at Day 9.

The breakdown of individual VFAs can be seen in Table 4-5. Briefly, there was only a Time effect for acetate, generally increasing with each consecutive batch culture ($F_{4, 44} = 29.419$, $p < 0.001$). Days 3 and 7 showed no significant difference in acetate production, nor did days 9, 11 and 15. For propionate, there was a significant interaction between Time and Group ($F_{8,44} = 2.274$, $p = 0.049$) with both main effects also significant ($F_{4, 44} = 8.572$, $p < 0.001$ and $F_{2, 44} = 18.331$, $p < 0.001$ for Time and Group respectively). Differences between the groups were seen only for days 7 and 9. Butyrate had main effects of both Time ($F_{4, 44} = 8.386$, $p < 0.001$) and Group ($F_{2, 44} = 17.117$, $p < 0.001$). The Good rumen fluid showed significantly higher concentrations of butyrate than both the Bad ($p < 0.001$) and the 1:1 Mix ($p < 0.001$). There was no difference between the Bad and Mix ($p = 0.979$). The A:P showed a significant interaction between Time and Group ($F_{8, 44} = 2.492$, $p = 0.033$) with both main effects also significant ($F_{4, 44} = 5.462$, $p = 0.002$ and $F_{2, 44} = 17.978$, $p < 0.001$ for Time and Group respectively). There was a difference between the groups at days 7 and 9 as for propionate above.

Table 4-4 Fermentation parameters for the 48 hour consecutive batch culture fermentations on days 3, 7, 9, 11 and 15. Mean values are shown. Significant values are shown in bold.

		Day						p value		
		3	**7**	**9**	**11**	**15**	**SEM[1]**	**Time**	**Group**	**Time*Group**
Gas volume (ml)	**G**	210.4	204.0	215.5	217.4	211.9	1.341	< **0.001**	0.114	**0.006**
	B	216.0	199.6	215.3	224.0	208.5				
	M	211.1	190.8	215.5	224.4	210.0				
pH	**G**	6.55	6.53	6.54	6.52	6.42	0.00	**0.009**	< **0.001**	0.473
	B	6.50	6.49	6.51	6.49	6.41				
	M	6.52	6.52	6.51	6.49	6.40				
tVFA (mM)	**G**	86.55	87.2	95.84	96.34	101.54	1.377	< **0.001**	0.076	0.605
	B	89.21	89.06	95.74	98.6	97.85				
	M	88.53	85.23	92.13	94.91	97.02				
NH3-N (mg/mL)	**G**	1.2	1.4	1.5	1.43	1.45	0.032	< **0.001**	0.103	0.071
	B	1.11	1.27	1.49	1.48	1.35				
	M	1.16	1.29	1.54	1.51	1.28				
MCP (µg/mL)	**G**	422.65	381.15	431.57	459.05	646.09	25.054	< **0.001**	**0.042**	**0.03**
	B	460.69	379.81	507.35	563.65	453.81				
	M	585.4	541.57	494.73	374.11	488.7				

[1]SEM = standard error of the mean, tVFA = total volatile fatty acids, NH_3-N = ammonia-nitrogen, MCP = microbial crude protein, G = good, B = bad, M = 1:1 Mix rumen fluid

Table 4-5 Individual VFA analysis for the 48 hour consecutive batch culture fermentations on days 3, 7, 9, 11 and 15. Mean values are shown and significant values are highlighted in bold. All concentrations shown are in mM .Values are corrected per g DM.

	Day																		
	3			7			9			11			15				p value		
	Good	Bad	Mix	Good	Bad	Mix	Good	Bad	Mix	Good	Bad	Mix	Good	Bad	Mix	SEM[1]	Time	Group	Time*Group
Acetate	98.4	101.7	100.8	103.5	104.1	102.3	119.2	118.5	113.0	118.8	125.5	121.8	127.4	121.6	123.5	0.94	**< 0.001**	0.654	0.790
Propionate[2]	45.3	47.2	48.3	41.0[a]	54.6[b]	48.5[b]	49.8[ac]	56.7[b]	49.9[c]	48.5	54.6	51.0	51.5	55.1	54.5	0.44	**< 0.001**	**< 0.001**	**0.049**
Butyrate	40.1	39.5	37.8	40.2	29.7	29.4	34.3	27.4	32.3	37.2	30.2	28.9	36.2	30.0	27.1	0.47	**< 0.001**	**< 0.001**	0.087
A:P[1,2]	2.2	2.2	2.1	2.5[a]	1.9[b]	2.1[b]	2.4[a]	2.1[b]	2.3[a]	2.5	2.3	2.4	2.5	2.2	2.3	0.02	**0.002**	**< 0.001**	**0.033**

[1]A:P, Acetate to propionate ratio, SEM, standard error of the mean

[2] Different superscript letters within a row, within a day represent a significant difference between the groups ($p < 0.05$)

4.3.3 The effect of cross inoculation on the microbial profile

The microbial profile of the rumen fluid from the fermenters after Day 1 and Day 16 was examined to identify whether the differences seen at Day 1, in terms of digestibility, and the lack of difference at Day 16 could be explained by the microbial composition. A total of 2,757,257 sequences were obtained with an average of 459,543 ± 50,724 sequences per group. After all filtering and clustering steps, a total of 68,123 unique, high quality sequences remained with an average of 11,353 ± 2,096 per group.

In total, nine phyla had a relative abundance greater than 1%: Bacteroidetes, Firmicutes, Fibrobacteres, Spirochaetae, Tenericutes, unclassified bacteria, Proteobacteria, Actinobacteria and Synergistetes. The most abundant phyla on Day 1 were Bacteroidetes (38.0 ± 0.84 %), Firmicutes (30.2 ± 0.36 %) and Fibrobacteres (17.5 ± 2.72 %). The relative abundance of Bacteroidetes and Firmicutes was very similar across the three rumen fluids, however, Fibrobacteres showed more variation, with a 5 % difference between the Good (20.4 %) and the Bad RF (15.0 %) with the 1:1 Mix RF intermediate (17.3 %). The Bad RF at the end of Day 1 also had a higher percentage of Tenericutes (5.9 %) and unclassified bacteria (1.4 %) compared with the Good (2.1 and 0.8% respectively). The 1:1 Mix had similar levels of Tenericutes (4.63 %) and unclassified bacteria (1.31 %) as the Bad.

By the end of the 24 hour fermentation on Day 16, Bacteroidetes (35.6 ± 0.88 %), Firmicutes (28.6 ± 1.43 %) and Fibrobacteres (16.8 ± 3.71 %) were again the most abundant. In contrast to Day 1, the relative abundance of Fibrobacteres was 5% higher in the Bad rumen fluid sample than the Good (20.9 *vs* 15.8 %) and this time lowest in the 1:1 Mix (13.6 %). The relative abundance of Tenericutes was still found to be much higher in the Bad fluid when compared with the Good (9.6 vs 3.7%), and again similar to that in the 1:1 Mix (9.3 %).

From the Phyla identified above, 39 Genera had a > 1% abundance across the samples. *Prevotella 1* (22.1 ± 1.16 %) was the most abundant at Day 1, followed by *Fibrobacter* (17.5 ± 2.72 %) and *Treponema 2* (7.3 ± 1.54 %). The same three genera were still the most abundant after Day 16, with *Fibrobacter* becoming the most abundant (16.8 ± 3.71 %) followed by *Prevotella 1* (12.2 ± 2.36 %) and *Treponema 2* (6.1 ± 1.99 %).

The relative abundance of all phyla and genera can be seen in Appendix B.

4.3.4 Alpha and beta diversity

Chao 1, Shannon and Simpson's diversity analysis were performed to compare alpha diversity between the samples. Alpha diversity is a measure of the abundance (Chao1) and evenness (Shannon, Simpson) of species within a sample. A significant reduction in alpha diversity was seen between Day 1 and Day 16 with Chao1 values almost halving between the two fermentations (Table 4-6). No difference in alpha diversity was observed between the Good, Bad and 1:1 Mix RFs for any measure used (Chao1 p = 0.6191, Shannon p = 0.5952, Simpson's p = 0.9186).

Table 4-6 Chao 1, Shannon and Simpson's indices of alpha diversity for Mixing Experiment I Statistical difference is shown by values in bold.

		Time			p value		
	Group	**Day 1**	**Day 16**	**SEM[1]**	**Time**	**Group**	**Time*Group**
Chao1	Good	3553.7	1870.5	167.87	**0.001**	0.6191	0.4492
	Bad	3264.5	1805.5				
	Mix	3982.3	1756.3				
Shannon	Good	5.7	4.7	0.07	< **0.001**	0.5952	0.6063
	Bad	5.9	4.6				
	Mix	5.8	4.7				
Simpson's	Good	0.987	0.975	0.00	**0.0203**	0.9186	0.7166
	Bad	0.987	0.958				
	Mix	0.989	0.971				

[1]SEM = standard error of the mean

Beta diversity is a measure of differences in community composition between samples. Similarly to alpha diversity above, only time was found to have a significant effect on beta diversity ($F_{2, 6} = 6.13$, $p = 0.011$). No difference was observed between the groups ($F_{2, 6} = 0.96$, $p = 0.639$) with the points for Good, Bad and 1:1 Mix RFs overlapping within a time point on a non-metric multi-dimensional scaling plot (NMDS; Figure 4-5) all of which were clustered separately from the bacterial community associated with the grass substrate.

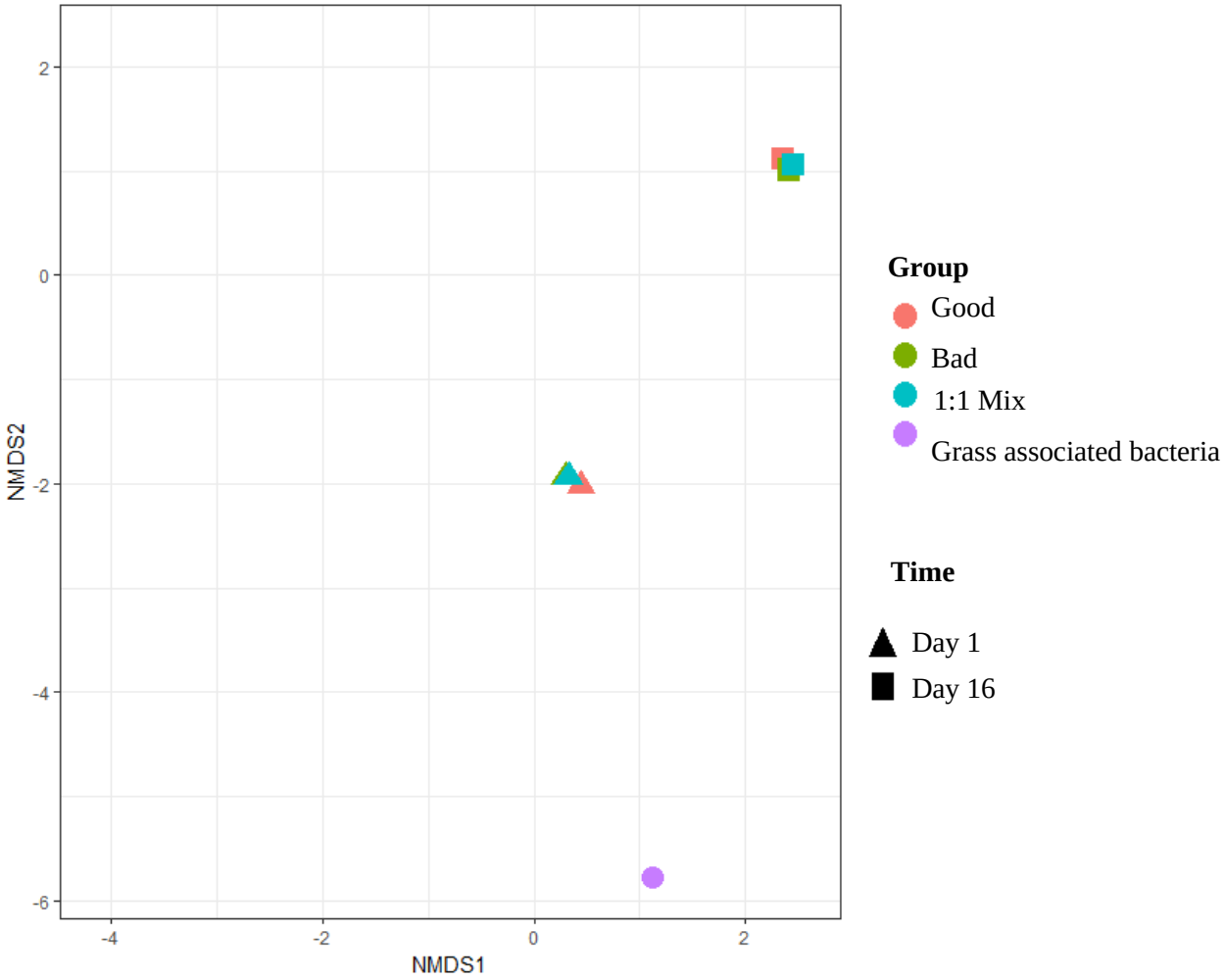

Figure 4-5 Non-metric multi-dimensional scaling (NMDS) plot for the Mixing Experiment I samples and the bacterial profile associated with the dried grass substrate using Bray-Curtis distances. PERMANOVA analysis showed that there was a significant time effect (p = 0.011), but no effect of group (p =0.639).

DESeq2 analysis was performed to identify which operational taxonomic units (OTUs) were responsible for the significant effect of time seen in the PERMANOVA above. The 10 OTUs with the largest increase and decrease from Day 1 to Day 16 are shown in Table 4-7 below

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Table 4-7 Operation taxonomic units that show the most significant changes in abundance for Mixing I. A) The OTUs that increased from Day 1 to Day 16 B) the OTUs that decreased from Day 1 to Day 16. OTUs are classified to the Genus level. The p values shown are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold change represents Log2 fold change.

A)	**Increased from Day 1 to Day 16**		
	Genus	**Fold change**	**p value**
OTU 11	*Bacteroidales UCG001* unclassified	10.04	< 0.001
OTU 24	*Ruminococcus 1*	9.67	< 0.001
OTU 19	*Bacteroidales S24-7 group* unclassified	9.30	< 0.001
OTU 15	*Streptococcus*	7.46	0.002
OTU 42	*Escherichia-Shigella*	8.56	0.002
OTU 60	*Pyramidobacter*	8.37	0.002
OTU 76	*Prevotella 1*	8.53	0.002
OTU 38	*Prevotella 1*	7.88	0.003
OTU 49	*Bacteroidales UCG-001* unclassified	8.42	0.003
OTU 7	*Ruminococcus 1*	7.51	0.004

B)	**Decreased from Day 1 to Day 16**		
	Genus	**Fold change**	**p value**
OTU 27	*Fibrobacter*	-11.59	< 0.001
OTU 64	*Fibrobacter*	-9.51	< 0.001
OTU 170	*Prevotella 1*	-8.90	0.002
OTU 18	*Fibrobacter*	-6.78	0.002
OTU 139	*Bacteroidales UCG-001* unclassified	-8.42	0.002
OTU 149	*Treponema 2*	-8.81	0.002
OTU 166	*Ruminococcaceae NK4A214 group*	-8.65	0.002
OTU 176	*Bacteroidales S24-7 group* unclassified	-8.60	0.002
OTU 219	*Prevotella 7*	-8.49	0.002
OTU 112	*Fibrobacter*	-7.70	0.003

4.4 Discussion

4.4.1 Cross inoculation of two rumen fluids yields an IVDMD of dried grass that is the average of the two after 24 hours of incubation

The host animal is thought to exert a controlling effect on the microbiota that reside within the rumen, resulting in a community that is resilient to perturbation (Weimer et al., 2010; Fouhse et al., 2017). Understanding the mechanisms by which the microbial composition is maintained is imperative to allow manipulation of complex communities as engineering the rumen community is of great interest to improve rumen efficiency and thus improve animal performance and reduce environmental pollution (Santra and Karim, 2003; Martinez-Fernandez et al., 2016; Guyader et al., 2017)

In this Chapter, it was hypothesised that by removing the direct effect of the host animal through the use of an *in vitro* batch model of rumen fermentation, cross inoculation of two rumen fluids in a 1:1 ratio would improve dry matter digestibility of dried grass. Indeed, cross inoculation of two rumen fluids ('Good' and 'Bad') resulted in a mixed rumen fluid that performed at an average level between the two after 24 hours of fermentation (34 vs 20 vs 29 g/100g DM for Good, Bad and 1:1 Mix respectively). In the absence of host control, the rumen microbial community was successfully manipulated to enhance *in vitro* performance. It appears that cross inoculation was able to mostly ameliorate the IVDMD of the poorer performing rumen fluid over 24 hours of fermentation, through the introduction of a rumen microbial community that had a superior ability to harvest energy from the dried grass substrate that was provided to the model. This is in partial agreement with Oss et al. (2016) who found a synergistic effect of cross inoculating rumen fluid from cattle and bison over 48 hours in the RUSITEC system. Although there was no initial difference in terms of fibre digestion between the two starting fluids in their paper (cattle and bison) the authors showed that cross inoculation could improve fermentative digestion *in vitro* which supports the work presented here.

As the response for the cross inoculated fermentation bottles was an average between the initial Good and Bad rumen fluids, this suggests that although the addition of a microbial community that is better able to harvest energy improved the performance of the Bad rumen fluid, there may be factors within the Bad fluid which prevented the full establishment and performance of the microbial community associated with the Good. Factors such as bacteriophages, bacterioicins, fungi and a lack of protozoal survival may

prevent the success of microbial establishment and these are explored further in Chapter 8. Alternatively, bacterial number may affect inoculations and this is explored further is Chapter 7. The ability to manipulate the microbial community to increase IVDMD within an *in vitro* model of rumen fermentation is a step towards understanding the effect of host specificity as this adds weight to the hypothesis that the host animal has a direct effect on the microbiota that reside within the gastrointestinal tract.

4.4.2 Each rumen inoculum improved its ability to digest dry matter over time and differences between the fluids were lost

The initial 24 hour batch culture demonstrated that cross inoculation *in vitro* could improve the fermentative digestion of dried grass of a poorer performing rumen fluid through the introduction of a microbial community that showed greater performance. Over the course of the experiment, it was clear that when the constraints of the host animals were removed, the microbial population adapted to the new environment provided within the model. This was suggested by the improved dried grass IVDMD in all three rumen fluids such that after the third consecutive batch culture (Day 9) there was no difference between the three fluids. In the absence of host control, the community within the model was able to freely adapt to the substrate and environment provided. The initial consecutive batch cultures appear to indicate a transitionary period as bacteria were adapting to the substrate and new environment provided by the *in vitro* model.

Due to the adaptation of the microbial community to the *in vitro* model, each of the rumen inoculums used (Good, Bad and 1:1 Mix) improved their ability to digest the dried grass substrate with time and after 9 days, no difference between the three was observed. Due to this fact, it is unclear to what extent the improvement in the 1:1 mix was due to the introduction of a microbial community that was better able to harvest energy. The improvement seen could be wholly or partly attributable to the natural adaptation of the microbial community to the substrate provided within the model. Future cross inoculation experiments should provide the same substrate to the model as the animal was fed prior to slaughter to minimise changes in microbial community structure and fermentation due to feed.

4.4.3 Time had the greatest effect on fermentation parameters

Across the course of the experimental period, Time was found to have the greatest effect on the fermentation parameters measured. Looking first at the 24 hour fermentations on Day 1 and Day 16, Time was shown to significantly affect each of the parameters measured (gas production, pH, total VFA production, individual VFAs and MCP concentration) with the exception of NH_3-N which remained stable (1.15 ± 0.04 at Day 1 and 1.14 ± 0.02 at Day 16). Only the volume of gas produced and tVFA were affected by the rumen inoculum used with a Time*Group interaction for both. In both cases, the differences observed between the fluids at Day 1, the Good and 1:1 Mix showed higher gas volume and tVFA than the Bad, were lost by Day 16 (Table 4-2). The average performance shown by the 1:1 Mix for IVDMD was reflected in both gas and total VFA production.

It was interesting that for the 24 hour fermentations no difference was observed in MCP and NH_3-N between the rumen inoculums despite initial differences in the ability of the three rumen fluids to digest dry matter *in vitro*. It was expected that the better performing rumen fluid ('Good') would more efficiently utilise protein and that cross inoculation would result in improved protein utilisation in the 1:1 Mix compared to the Bad. The findings suggest that bacterial turnover and protein utilisation were the same despite differences in fermentative digestion. It was noted that although the three groups showed improved IVDMD across the course of the experiment, the average MCP concentration was found to decrease by around 20% (100 µg/ml). After the first 24 hours of fermentation there was no difference in MCP between the three fluids which raises the question as to whether there was a difference to begin with. Future experiments should also measure parameters in the neat rumen fluid used to inoculate the *in vitro* model.

A lack of difference in protein utilisation across a cross-inoculation experiment has also been reported by Griffith et al. (2017) who investigated the effect of near total exchange of rumen content between beef cattle and bison on rumen nitrogen digestion. The group found no improvement in the efficiency of microbial nitrogen synthesis (g/kg of digested organic matter) of ruminal ammonia-N concentration before feeding. However, they did show that the total N digestibility was improved (68.3 *vs* 70.4 %).

Across the 48 hour consecutive batch cultures from Day 3 to Day 15, Time was found to significantly affect all parameters measured with interactions between Time and Group for gas volume and MCP. A group effect was seen for pH. Interestingly, the pH for the

Good fluid was higher than that of the Bad and 1:1 Mix throughout the experiment (albeit marginally) and after Day 7, the 1:1 Mix pH more closely resembled that of the Bad than the Good despite no difference in VFA production between the three fluids. MCP was shown to decrease across the course of the experiment, increasing again in concentration at Day 9 for Good and Bad and later at Day 15 for the 1:1 Mix.

4.4.4 The effect of cross inoculation on the microbial population

Due to the nature of the *in vitro* model, any differences in performance that were observed between the rumen inoculums were assumed to be microbial in origin. To determine the effect of cross inoculation on microbial community composition, the bacterial community was sequenced at both the end of Day 1 (where cross inoculation improved IVDMD of a poorer performing rumen fluid to an average level) and at the end of Day 16 where no difference was seen between the three rumen inocula in terms of IVDMD (49 *vs* 48 *vs* 47 g/100g DM for Good, Bad and 1:1 Mix RFs respectively) and fermentation parameters were also largely similar.

Time was found to have the greatest effect on bacterial community composition, with no significant effect of rumen inoculum used (i.e. Good, Bad or 1:1 Mix) for both alpha and beta measures of diversity. Alpha diversity was shown to decrease over the course of the experiment with variation also decreasing between the three groups (3600 ± 361.1 *vs* 1811 ± 57.3 for Chao 1 and 5.8 ± 0.10 *vs* 4.7 ± 0.06 for Shannon diversity at Day 1 and Day 16 respectively). The reduction of alpha diversity in the model over time is reflective of a simpler microbial profile. A study of the development of ruminal microbiota in different *in vitro* models inoculated with goat's rumen liquor also found a decrease in alpha diversity (Shannon and Pielou eveness) when using a batch culture model of rumen fermentation after a 24-hour incubation (Soto et al., 2013).

In both this Chapter and the study by Soto et al. (2013), the substrate provided to the *in vitro* model(s) was not the same as had been fed to the animal used as a source of inoculum. In Soto et al. (2013), goats were provided with alfalfa hay *ad libitum* and the model (Wheaton Bottle; WB, which was most similar to the one used in this Chapter) was incubated with alfalfa hay, cereal straw, sunflower cake, wheat and variable proportions of barley and vegetable wastes. In this Chapter, the model was provided with dried grass (see General Methods 2.1.3) and the diet provided to the animals prior to slaughter was unknown. Possible explanations for the reduction in alpha diversity over time are that the substrate selected for a different, less diverse, microbial community or

that the environment of the *in vitro* model caused the decline in alpha diversity that was observed. Belanche et al. (2016) showed that feeding grass hay instead of fresh grass actually increased the diversity of the microbial population when fed with 20% concentrate *in vitro*. Therefore, it is important to know the composition of the diet fed to the animals used as rumen fluid donors in order to determine the possible causes of variation in microbial population within the *in vitro* model.

The reduction of alpha diversity in the model over time may also be reflective of a simpler microbial profile which supports the findings of Shabat et al. (2016). Shabat et al. (2016) examined the taxonomic composition, gene content, microbial activity and metabolomic composition of 78 animals at the extremes of feed efficiency. Animals that showed higher feed efficiency were found to have lower richness of both gene content and microbial taxa. When compared with the least efficient animals, a lower number of metabolic pathways were used when compared with inefficient cattle and these pathways were more targeted to meet the energy needs of the animal. When applied to the *in vitro* work presented here, the model may be selecting for a community most suited to the substrate and as the direct controlling effects of the host have been removed this is possible.

Similar to alpha diversity, only Time had a significant effect on beta diversity. Beta diversity is reflective of differences in community composition between samples, such as the type and quantity of OTUs present (McMurdie and Holmes, 2013). Results showed that only Time significantly affected the bacterial genera present and no difference in bacterial composition between rumen inocula were seen as demonstrated by the overlap of points within a time point on the NMDS plot in Figure 4-5. It was also observed that the community composition of each rumen inoculum (Good, Bad and 1:1 Mix) changed in the same way from Day 1 to Day 16, supporting the idea that the *in vitro* model was selecting for a bacterial community suited to both the substrate and environment it provided. The grass associated bacterial community (or 'epiphytic' bacteria) were also included on the beta diversity plot in Figure 4-5. The community associated with the dried grass is discussed elsewhere (Chapter 6).

The lack of difference in bacterial composition at Day 1 despite large differences in IVDMD highlights that it may be of greater importance to consider what the microbial community are doing through additional 'omics techniques rather than solely identifying “who” is there. Transcriptomics, proteomics and metabolomics have recently been used

to identify which genes are being expressed, which proteins are being produced and which metabolites are found in the rumen, and are reviewed by Wallace et al. (2017).

DeSeq2 analysis (Table 4-5) was performed to identify the operational taxonomic units (OTUs) that showed the largest change across the experimental period. OTUs assigned to the genera *Bacteroidales*, *Ruminococcus* and *Prevotella 1* increased from Day 1 to Day 16, and four OTUs assigned to the genus *Fibrobacter* decreased. It is interesting that *Fibrobacter* species decreased when provided with a dried forage substrate in the model as *Fibrobacter succinogenes* is a major rumen bacterial species usually found in high quantity when the animal is provided with cellulose-rich feed (Forano et al., 2008). However, when considering the genus as a whole, there was no significant difference in *Fibrobacter* abundance (17.5 ± 2.71 *vs* 16.8 ± 3.71 for Day 1 and Day 16 respectively). In some cases e.g. *Prevotella 1*, the genus was shown to both increase (OTUs 76, 38) and decrease (OTU 170) over the experiment. Sequencing of 16S rRNA cannot accurately resolve down to the species level (Janda and Abbott, 2007; Jovel et al., 2016). In addition, different bacteria hold different copy numbers of the 16S gene, each of which can differ in their sequence. An example of this is *Aeromonas veronii* which has 6 copies of the 16S gene, each of which differ by ca 1.5 % (Janda and Abbott, 2007). The species and/or strains within a particular genus are changing with time and this is reflected in the DeSeq2 tables.

4.4.4.1 Tenericutes

Tenericutes is a Phylum consisting of the class Mollicutes. Tenericutes (from the Latin *tener* 'tender' and *cutis* 'skin'), which are distinct in the fact that they lack a cell wall (Brown, 2015). This phylum is a common gut inhabitant of ruminants and has been found in the gastrointestinal tract of many species including humans (Kim et al., 2013), dogs (Suchodolski et al., 2010), mice (Robertson et al., 2017) and termites (Tai et al., 2015). It is also a member of the microbial population found in anaerobic digesters and at landfill sites (Li et al., 2015b; Song et al., 2015; Cibis et al., 2016).

The relative abundance of Tenericutes was consistently higher in the Bad and 1:1 Mix inoculum when compared with the Good. Whilst this may explain difference in performance seen between the fluid groups at Day 1 (Tenericutes - 2.1 *vs* 5.9 *vs* 4.6 % for Good, Bad and 1:1 Mix respectively), the difference was still present at the end of the experiment (3.7 *vs* 9.6 *vs* 9.3 % for Good, Bad and 1:1 Mix) where performance (IVDMD) of the three fluids did not differ. Tenericutes appears to be an opportunistic

phylum that flourishes in times of dysbiosis. Experiments that introduce flavanoids (Zhan et al., 2017) and polyphenols (De Nardi et al., 2016) into the rumen, which both have antimicrobial properties, results in an increase in Tenericutes. Tenericutes has further been shown to increase in soils which are treated with insecticides where many other phyla decrease (Fu et al., 2015). Individuals considered susceptible to subacute ruminal acidosis show higher relative abundance of Tenericutes compared with those at low risk (Li et al., 2017). A diet deficient of n-3 polyunsaturated fatty acids (n-3 PUFA) is thought to cause gut disturbances in the caecum of mice. When fed an n-3 PUFA deficient diet, Tenericutes was more abundant than in mice fed a control or n-3 PUFA supplemented diet (Robertson et al., 2017). It may be that the rumen fluid obtained from the 'Bad' individual showed some dysbiosis in its microbial community, which may be a reason for the lower IVDMD initially. At Day 16, where no difference in IVDMD was observed, it may be that the presence of Tenericutes did not affect *in vitro* performance, but a larger relative abundance was able to establish due to dysbiosis in the initial Bad inoculum and through cross inoculation in the 1:1 Mix.

4.4.5 Correlation of IVDMD to abattoir data

Rumen fluid for use in this experiment was collected at time of slaughter from a group of animals sent to a commercial abattoir. These animals were selected as they were a large group (11) of same sex (steer), breed (HFX) and similar age (656 ± 70.9 days) animals finished on the same farm. It was expected that the animals would show intra-herd variation in their ability to digest fibre and that this would be reflected in terms of IVDMD within the model as correlations between *in vivo* and *in vitro* estimates of fibre digestion have been previously identified (Jancik 2011). As these animals were sourced from the same farm prior to slaughter, the microbial population was likely to have been subjected to the same environmental factors (e.g. diet) prior to slaughter.

No correlation was observed between IVDMD measured within the *in vitro* model and parameters obtained from the abattoir such as price paid for carcass, cold weight and the age of the animal. While the animals were raised on different farms they were all finished on the same farm and therefore it was assumed they had all received the same diet and management prior to slaughter, although the actual diet fed was unknown. If the diet was concentrate based, it could be that the rumen fluids identified as 'Good' and 'Bad' for their ability to digest dried grass in the model may have had microbial profiles that were the most and least adaptable to a change in the substrate rather than a reflection on how well

the community was able to digest fibre *in vitro*. For future work it would be of interest to compare animals on a measure such as residual feed intake (RFI) or daily live weight gain of animals raised on a forage based diet to provide a better idea of how well the animal converts feed into growth. This could then be compared to *in vitro* performance.

4.4.6 Conclusion

This chapter was a proof of concept study designed to establish whether manipulation of the mature rumen through cross inoculation was possible through the use of an *in vitro* batch model which removes the direct controlling effect of the host animal. Cross inoculation of two rumen fluids that differed in their ability to digest dry matter *in vitro* (Good and Bad) resulted in a mixed fluid (1:1 Mix) that showed an average performance of the two. *In vitro* dry matter digestibility of the 1:1 Mix was improved when compared with the Bad along with an increase in both gas production and total volatile fatty acid concentration over a 24 hour fermentation.

Over the following consecutive batch cultures, differences between the three fluids were lost as each fluid improved its ability to digest dry matter within the model. Time had the greatest effect on both fermentation parameters and bacterial community composition. No difference in alpha or beta diversity between the three rumen fluids were observed within a time point, suggesting that community structure was highly similar. Bacterial composition changed over the course of the 16 day experiment due to adaptation of the microbial community to both the substrate and the environment that the model provided and alpha diversity was reduced. For future experiments, it will be of importance to know the diet fed to the animals prior to slaughter to obtain a deeper understanding of the interactions occurring within the model. The rumen fluid(s) used as inoculum should also be sequenced alongside experimental bottles to provide a starting reference point for microbial composition. In the absence of host control, manipulation of rumen fermentation was shown to be possible over a 24 hour period, however, due to the adaptation of the microbial community to the model, it was not possible to determine whether this improvement was maintained.

Chapter 5 The effect of cross inoculation of rumen fluid from genetically similar animals raised on a forage diet using an *in vitro* batch model of fermentation

5.1 Introduction

Rumen fluid contains a complex microbial community consisting of bacteria, fungi, protozoa, archaea and viruses (Tapio et al., 2017). These microorganisms rely upon metabolites from each other to survive, rendering classical culturing techniques extremely difficult for many species (Kim et al., 2017). The rumen microbial community is dynamic in early life and is thought to have multiple sources of origin including the mother, the environment and feed (Ziolecki and Briggs, 1961; Yáñez-Ruiz et al., 2015). At birth the rumen is thought to be mostly sterile and the accumulation of microorganisms occurs after parturition (Fonty et al., 1987; Yáñez-Ruiz et al., 2015). Heritability of rumen microorganisms is moderate, with some members of the microbial community more likely to establish than others through direct and/or indirect effects of the animal's genetic makeup (Hernandez-Sanabria et al., 2013; Sasson et al., 2017). From weaning the complexity of the rumen community expands rapidly and sequentially until the mature rumen community is formed and in the mature animal, the microbial community has proved difficult to manipulate (Weimer et al., 2010).

Animals that are raised in a similar environment are thought to share similar microbial species and strains due to the method by which the microorganisms are acquired (Laukens et al., 2016). Despite this, the rumen bacterial community has been shown to differ between cows that were co-housed and co-fed which is thought to be due to the strong influence of the host animal (Jewell et al., 2015). The gross microbial composition has been shown to differ in cattle that vary in feed utilisation due to animal factors (Guan et al., 2008; Carberry et al., 2012). However, it follows that these animals may be better suited to cross inoculation experiments as they share similar species and strains, albeit at different abundances. There is a redundancy of bacterial species within the rumen (Weimer, 2015) and it has been suggested that communities that are similar to one another may simply substitute upon mixing (Rillig et al., 2015) and perturbation may also be reduced.

As described in the General Introduction (Section 1.4.9), the host animal is believed to have a controlling effect over the microorganisms that reside within its gastrointestinal tract through mechanisms that have only recently begun to be explored (Fouhse et al., 2017). It is possible to remove the direct, controlling effect of the host animal using an *in vitro* model. By removing the host influence, fermentation and the microbial profile can be studied and manipulated. In the previous chapter, it was shown that cross-inoculation of 'good' and 'bad' performing rumen fluids resulted in a mixed fluid that performed at an average level between the two 24 hours after inoculation into the *in vitro* model. Differences between the three rumen fluids were lost with time as each of the rumen fluids increased their ability to digest the substrate. Both alpha and beta diversity measures showed a clear divergence in the microbial population with time away from that of the initial inoculum. The animals used as a source of inoculum in Chapter 4 had been raised on multiple different holdings before the one they were finished on and the diet they were fed was unknown. The substrate provided in the model was likely different to that fed to the animal prior to slaughter and the abrupt change in substrate may have caused unnecessary perturbation to the microbial community. Perturbation not only disrupts normal activity, but allows opportunistic microorganisms to flourish (Brown et al., 2012; Grazul et al., 2016).

This chapter is a follow-on study from the cross-inoculation experiment described in Chapter 4. The aim of this chapter was to identify rumen fluids from cattle of the same breed and sex raised in a common environment, i.e. same feed, same management, which showed different *in vitro* performances when provided with a forage substrate (dried grass). Due to the common environmental factors, differences in *in vitro* performance were assumed to be due to different microbiotas which must be due to animal factors. Attempts to manipulate the rumen microbiota *in vivo* via mixing and exchanging rumen fluids have been unsuccessful (Chapter 1.4.8); animal factors have been implicated in this. If true, then it should be possible to manipulate the rumen microbiota *in vitro* where animal factor(s) are essentially absent.

5.2 Materials and Methods

Rumen fluid was collected at time of slaughter from 11 Charolais-cross steers raised on a forage-based diet, either high sugar grass pasture (HSG), permanent pasture (PP) or high clover pasture (HC), at the North Wyke Farm Platform (NWFP, Okehampton, Devon, UK) Animals were of similar age (676.5 ± 26.02 days) and weight (666.9 ± 31.35 kg) and were sired by the same bull. Bimonthly live weight data of these animals was available allowing their daily live weight gain (DLWG) to be calculated by linear regression. The weights used were recorded between 20 May 2015 and 25 January 2016 during the linear growth phase. Each rumen fluid was used to inoculate the *in vitro* model to allow identification of the best and worst performing as described for the cross inoculation study of Chapter 4. Six bottles were used for each animal, with the rumen fluids run in a random order across two fermentations (six animals in Run 1, five animals in Run 2) with three bottles used for IVDMD analysis and three sample collection for fermentation parameters. Fermentations were performed for 24 hours.

The 'Good' and 'Bad' rumen fluid were identified by their ability to digest dry matter within the model and were used to inoculate a consecutive batch culture fermentation along with a combination of the two fluids in a 1:1 ratio ('1:1 Mix'; 2.5 mL of each). Four, 48-hour fermentations were used with sampling at 6, 12, 18, 24, 30, 36, 42 and 48-hours for Consecutive Batch Culture 1 (CBC1), 6, 12, 18, 24, 36 and 48-hours for CBC2, 12, 24, 36 and 48-hours for CBC3 and 24 and 48-hours for CBC4. To allow inoculation of new bottles containing fresh buffer and feed at the end of the 48 hour period, extra bottles were included for each run as a source of inoculum. No-substrate blanks were also included. A total of 93, 72, 51 and 27 bottles were used for CBC 1, 2, 3 and 4 respectively.

Sampling (Chapter 2.2.1 – 2.2.6) and digestibility analysis (Chapter 2.2.3) was performed for each time point. Bacterial community analysis was performed on samples taken from the neat fluids used as inoculum (Good, Bad and 1:1 Mix) samples from the end of CBC1 (48 hours) and the end of CBC4 (192 hours) and the dried grass substrate. DNA extraction through to sequence analysis was performed as described in the General Methods (Chapter 2.3)

5.2.1 Statistical analysis

Differences in IVDMD and fermentation parameters between the 11 Charolais cross steers were analysed individually for each parameter as linear mixed models in R with run as a random factor using the package lme4. Models were reduced and compared using lmerTest. All data was tested for normality (Kolmogorov Smirnoff) and homogeneity (Levene's test) prior to any statistical analysis. Where data did not fit a normal distribution, a generalised linear mixed model was performed with a penalised quasi likelihood error distribution using the packages MASS and car. If the random effect was shown to have no effect in the model (no difference from zero) it was removed.

Abattoir data (live weight, cold weight, price paid for carcass and age) including DLWG data, was correlated against IVDMD of the 11 CHX steers. Pearson's correlation was used in all cases except for age, for which a Spearman's rank was performed.

For the cross inoculation experiment, a general linear model was fitted to data with time and group (Good, Bad or 1:1 Mix) included as main factors. When not significant, interactions were removed and the models were re-run. *Post-hoc* differences were identified using Tukey's test with $p < 0.05$ as significant. Analysis of sequencing reads was performed as described in the General Methods (Chapter 2.3.6)

5.2.2 Curve Fitting

The use of frequent sampling during the fermentations allowed for model fitting of IVDMD using nonlinear regression. IVDMD data for CBC1 and CBC2 were fitted to a right handed Gompertz sigmoidal curve using GenStat (12th Edition):

$$Y = A + C * EXP^{(-EXP^{(-B*(X-M))})}$$

where Y is IVDMD (g/ 100g DM), A the lower asymptote, A+C the upper asymptote (maximal IVDMD, g/100g DM), B the slope i.e. the rate of DM digestibility (g per 100g DM per hour, M the inflection point which represents the lag time and X time (hours).

As the nonlinear parameters (B and M) were not significantly different between fluids, they were used to transform time, enabling data to be analysed by simple linear regression with groups.

5.3 Results

5.3.1 Cattle performance and *in vitro* rumen fermentation data

Each of the 11 rumen fluids were used as an inoculum in the *in vitro* model to identify the best and worst performing fluid in terms of *in vitro* dry matter digestibility (IVDMD). There was a significant difference in IVDMD between the fluids ($F_{10, 32} = 6.135$, $p < 0.001$), which ranged from 36 to 40 g of DM digested per 100 g of DM. The parameters from the fermentation can be seen in Table 5-1. *Post-hoc* tests revealed that the all of the rumen fluids showed the same IVDMD with the exception of Rumen Fluid 7 which had a significantly lower IVDMD compared to all other fluids with the exception Rumen Fluid 10 and 11. Rumen fluid 5 was selected as the Good rumen fluid and Rumen fluid 7 as the Bad for use in the cross inoculation experiment (5.3.2).

Table 5-1 *In vitro* dry matter digestibility, gas volume and pH values for the rumen fluids collected from 11 Charolais cross cattle grazed on three pasture types

	Rumen fluid												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**SEM[1]**	**p value**
Pasture type	HSG	HSG	PP	HC	HSG	HC	HSG	PP	PP	PP	PP		
IVDMD	0.393[a]	0.395[a]	0.393[a]	0.386[a]	0.400[a]	0.389[a]	0.364[b]	0.391[a]	0.391[a]	0.383[ab]	0.381[ab]	0.004	< **0.001**
Gas volume (ml)	137.78	131.19	126.55	134.99	121.47	124.71	116.21	135.94	127.33	127.57	131.80	2.57	0.112
pH	6.56[a]	6.58[b]	6.58[bc]	6.54[d]	6.58[b]	6.56[a]	6.58[b]	6.54[d]	6.57[ac]	6.55[a]	6.54[d]	0.002	< **0.001**

[1] SEM standard error of the mean, HSG high sugar grasses PP permanent pasture, HC high clover

[a-d] Means within a row that do not share a common superscript are significantly different $p < 0.05$

The correlations of abattoir and DLWG data against IVDMD are presented in Figure 5-1. Only age was significantly correlated with IVDMD (ρ = 0.785, N = 11, p = 0.004).

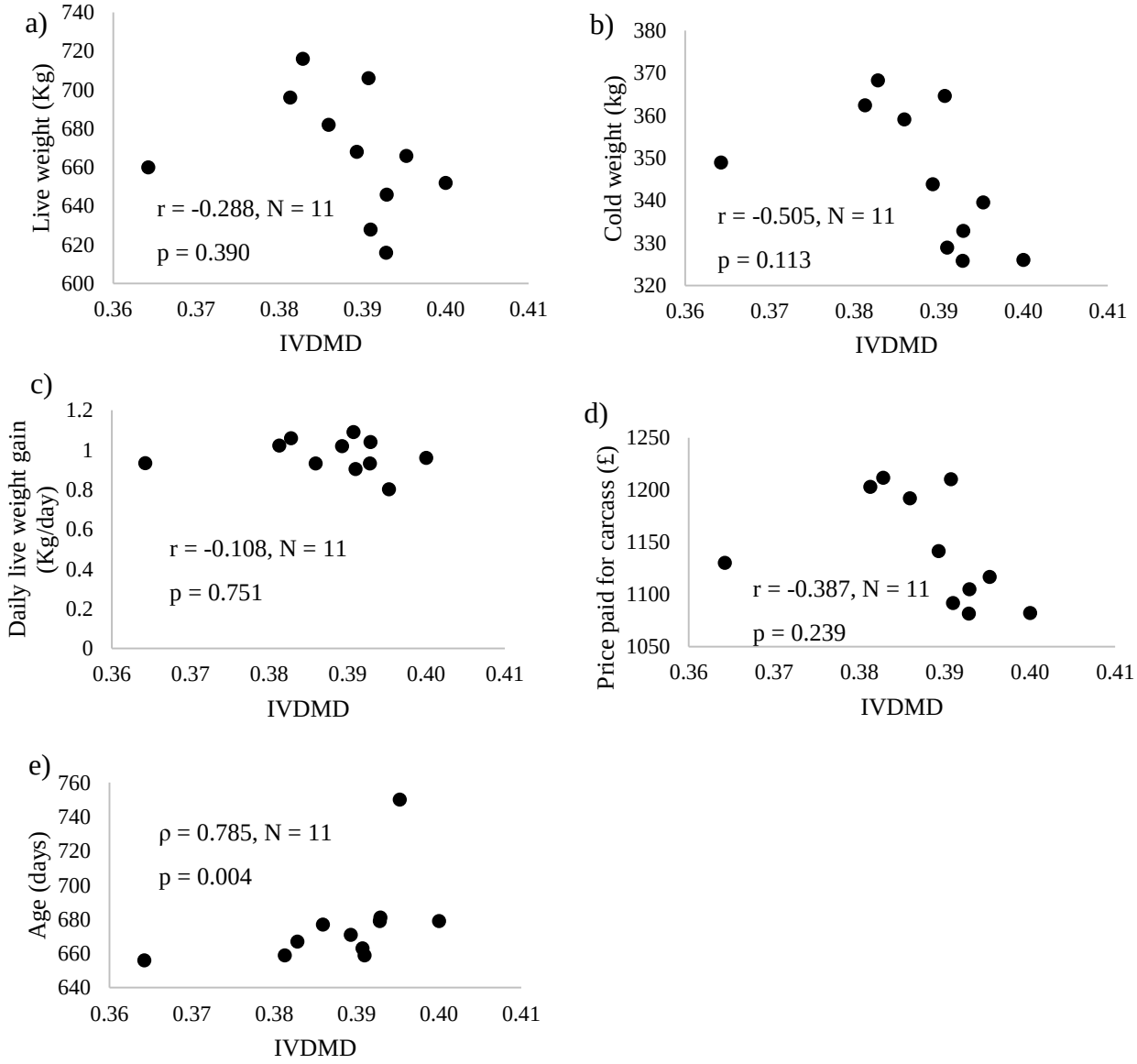

Figure 5-1 Correlation of abattoir parameters and daily live weight gain against *in vitro* dry matter digestibility. The correlation coefficient (r or ρ), number of data points (N) and significance (p value) are given for each graph where a) live weight (kg), b) cold weight (kg), c) daily live weight gain (kg per day), d) price paid for carcass (£) and e) age at slaughter (days). Only age showed a significant correlation with IVDMD.

5.3.2 The difference in IVDMD between a Good, Bad and cross-inoculated (1:1 Mix) rumen fluid

Gompertz curves were fitted to IVDMD values for CBC1 and 2. The models showed that there was no significant difference in the rate of digestion (g of digested DM per 100 g DM per hour) or the lag time (hours) between the Good, Bad and 1:1 Mix RF for CBC1 (7.7 g per 100 g DM per hour; lag time 23.2 hours) or CBC2 (7.1 g per 100g DM; lag time 17.5 hours). Time was transformed by these parameters and the data was re-fitted to a general linear model (Figure 5-2 and 5-3).

Between CBC1 and CBC2, the amount of DM digested at the end of each incubation increased for each RF: Good (59.7 to 71.6 g/100 g DM), Bad (57.7 to 70.2 g/100g DM) and 1:1 Mix (57.9 to 67.8 g/100g DM). There was no favourable effect of cross inoculating rumen fluid on *in vitro* dry matter digestibility. On this occasion, no intermediary effect was observed for the 1:1 Mix RF group at 24 hours as was observed for the cross inoculation experiment in Chapter 4. The 1:1 Mix RF bottles performed most similarly to the Bad RF at all time points (Figures 5-2, 5-3 and 5-4).

For Consecutive Batch Cultures 1-3, there was found to be a significant effect of both Time (CBC1 $F_{6, 60} = 201.962$, $p < 0.001$; CBC2 $F_{5, 49} = 297.738$, $p < 0.001$; CBC3 $F_{3, 35} = 2530.36$; $p < 0.001$) and Fluid on IVDMD (CBC1 $F_{2, 60} = 10.845$, $p < 0.001$; CBC2 $F_{2, 49} = 38.319$; $p < 0.001$; CBC3 $F_{2, 35} = 29.815$; $p < 0.001$), but no interaction between the two (CBC1 $F_{12, 60} = 1.925$, $p = 0.060$; CBC2 $F_{10, 49} = 1.865$, $p = 0.089$; CBC3 $F_{6, 35} = 0.841$, $p = 0.551$). IVDMD was shown to increase significantly with each time point and the Good RF showed significantly higher average IVDMD than both the Bad and 1:1 Mix RF across each 48 hour fermentation ($p < 0.001$; CBC1 47.3 vs 45.7 and 45.6; CBC2 56.6 vs 49.9 vs 52.3; CBC3 56.3 vs 54.2 vs 54.2 g digested DM per 100g DM for Good, Bad and 1:1 Mix RF respectively). No difference in average IVDMD was observed between the Bad and 1:1 Mix RF ($p = 0.343$, $p = 0.929$ and $p = 0.965$ for CBCs 1to 3 respectively).

In the final consecutive batch culture (CBC4) there was a significant effect of both Time ($F_{1, 17} = 3953.558$, $p < 0.001$) and Fluid ($F_{2, 17} = 3.911$, $p = 0.045$), but no interaction ($F_{2, 17} = 1.545$, $p = 0.253$). Although Fluid showed a significant effect in the model, there was no *post-hoc* differences between the three fluids when Tukey's was used (Good *vs* Bad $p = 0.998$, Good *vs* 1:1 Mix $p = 0.075$, Bad *vs* 1:1 Mix $p = 0.058$), but the *post-hoc* tests LSD and Duncan's indicated that the IVDMD of the 1:1 Mix RF was significantly lower than that of both the Good and Bad. The difference between the fluids however was small (1.1 and 1.2 % respectively).

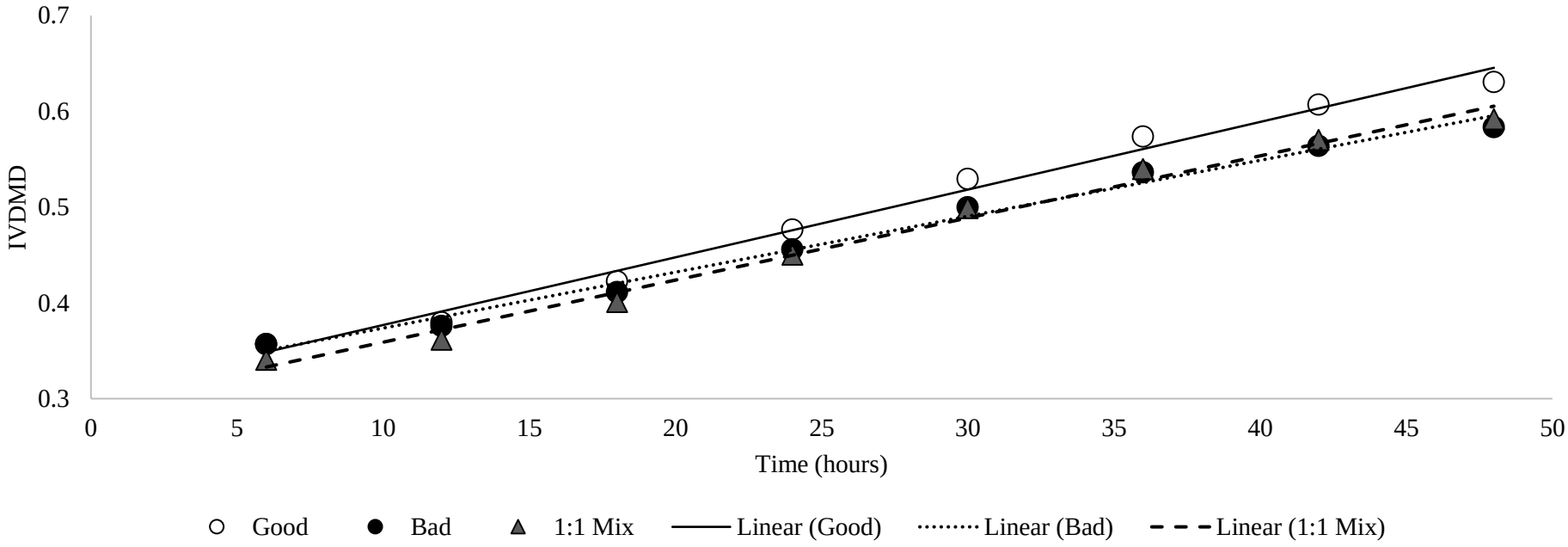

Figure 5-2 Fitted values from a simple linear regression with groups for the *in vitro* dry matter digestibility (IVDMD) during Consecutive Batch Culture 1 The values at 48 hours have been extrapolated

Equations for the lines are as follows:

$$Good = 0.0071x + 0.3063$$
$$Bad = 0.0058x + 0.3153$$
$$1{:}1\ Mix = 0.0065x + 0.2942$$

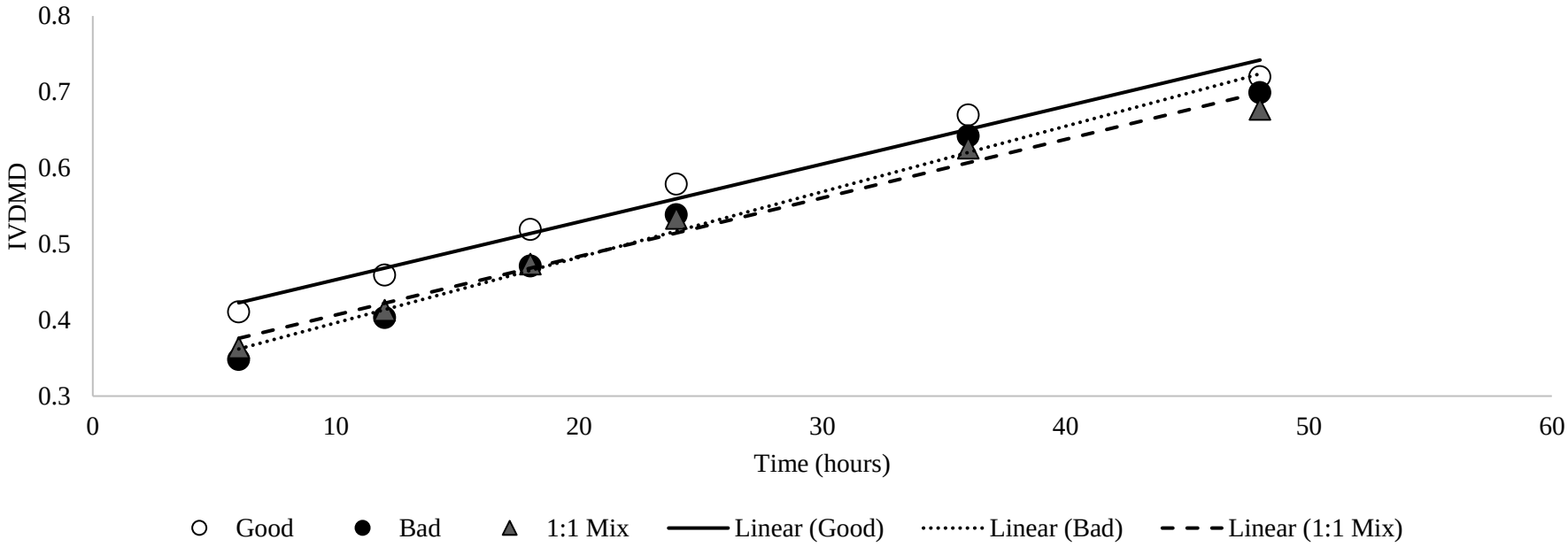

Figure 5-3 Fitted values from a simple linear regression with groups for the *in vitro* dry matter digestibility (IVDMD) during Consecutive Batch Culture 2 Equations for the lines are as follows:

$$Good = 0.0076x + 0.3771$$

$$Bad = 0.0086x + 0.3103$$

$$1{:}1\ mix = 0.0077x + 0.3298$$

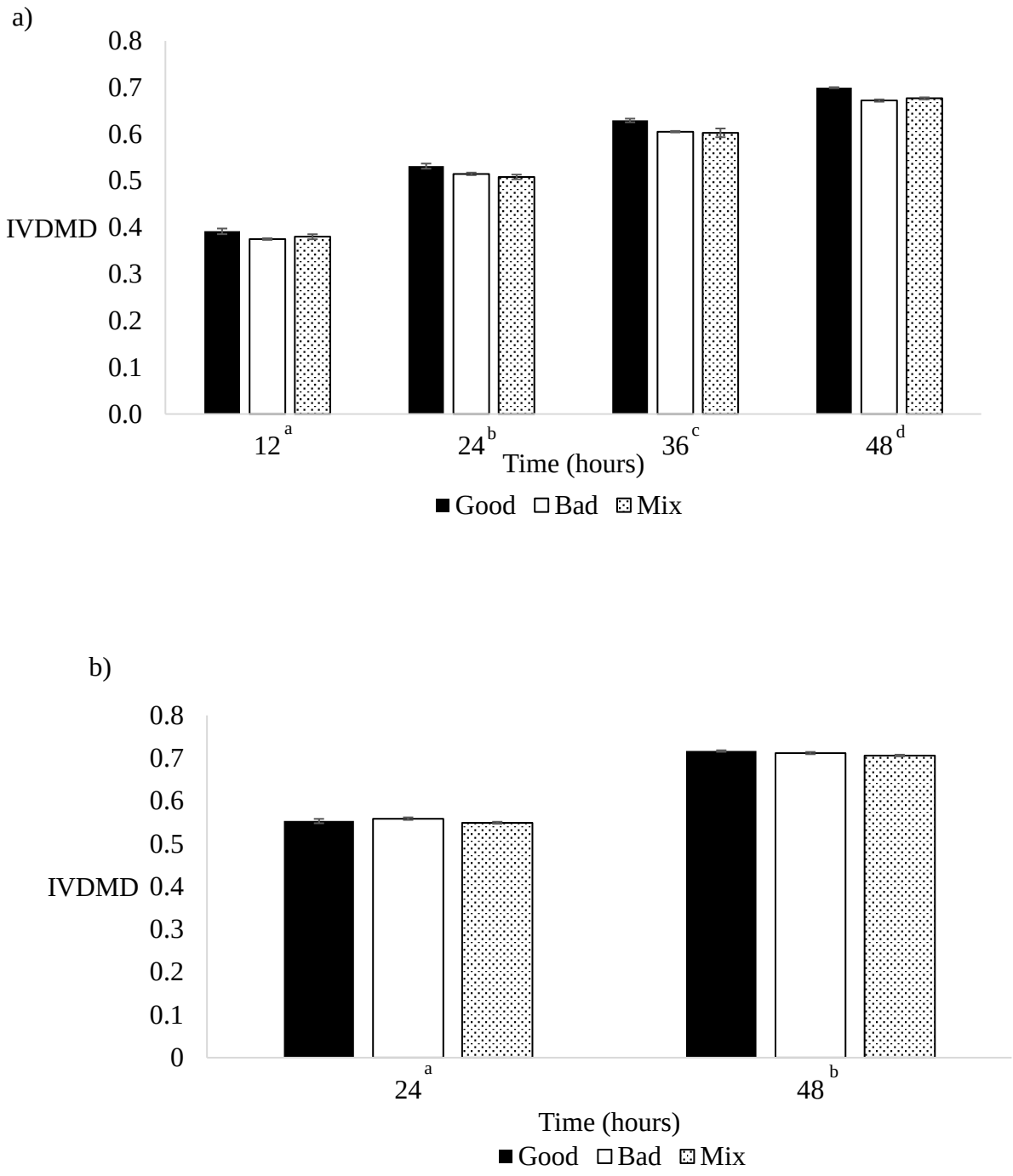

Figure 5-4 ***In vitro*** **dry matter digestibility (IVDMD) analysis for CBC3 (a) and CBC4 (b)** Bars show the mean value at each time point with standard error bars. Significant differences ($p < 0.05$) are shown between time points by different superscript letters.

5.3.3 Effect of cross inoculation on fermentation parameters

The effect of cross inoculation on fermentation parameters is summarised in Tables 5-2 to 5-5. Significant time effects were observed for all four CBCs ($p < 0.001$) with gas production increasing across each fermentation. There was a significant interaction between Time and Fluid (Time*Fluid) in CBC1 ($F_{14, 71} = 2.729$, $p = 0.005$), with significant differences between the three fluids seen only for the first 24 hours of fermentation. After this total gas volume was similar for all three fluids with cumulative gas volumes of 212, 199 and 212 ml for Good, Bad and 1:1 Mix respectively after 48 hours of fermentation. There was no interaction ($p > 0.05$) between Time and Fluid in CBC2 and 3 for gas volume. While there was no effect of Fluid ($p > 0.05$) on gas volume in CBC2, there was in CBC3 ($F_{2, 35} = 7.114$, $p = 0.003$) where the Good fluid produced a significantly higher average volume of gas in comparison to the 1:1 Mix (158.7 ml vs 141.6 ml ; $p < 0.01$).

For total VFA concentration (mM) there was found to be a significant effect of Time for each of the consecutive batch cultures ($p < 0.001$). However, in CBC1 and 2 this effect of time on total VFA concentration was only significant between samples up to 24 hours of incubation. In CBC1 total VFA concentration increased from 58.3 mM at 6 hours to 156.2 mM at 24 hours (Table 5-2). There was an effect of fluid only in CBC3 with differences between Good and 1:1 Mix (103.7 mM *vs* 98.6 mM; $p < 0.05$). There was no interaction between Time and Fluid in any of the consecutive batch cultures.

An interaction between Time and Fluid was observed in CBC1 for pH ($F_{14, 71} = 2.635$, $p = 0.006$). The pH initially increased up to 18 hours for the Good and 24 hours for the Bad and 1:1 Mix before gradually decreasing to a final pH of 6.57 for the Good and Bad and 6.58 for the 1:1 Mix. Significant differences between the fluids were seen at all time points in CBC1, with the exception of 18 and 48 hours, with the pH of the Good fluid lower than both the Bad and 1:1 Mix (Table 5-2). A significant Time effect was observed for CBC2 and significant main effects of both Time ($p < 0.001$) and Fluid ($p < 0.05$) were seen for CBC3 and CBC4. For CBC3, differences in pH were seen between both Good and Bad (6.50 *vs* 6.54; $p = 0.017$) and Good and 1:1 Mix (6.50 *vs* 6.54; $p = 0.011$). A difference was seen only between Good and 1:1 Mix for CBC4 (6.50 vs 6.49; $p = 0.015$).

Only Time had a significant effect on MCP ($p < 0.001$), with concentration increasing with time of fermentation in each consecutive batch culture (see Tables 5-2, 5-3, 5-4 and 5-5).

The concentration of ammonia-N increased ($p < 0.001$) with fermentation time in all four CBCs, although in CBC1 this was not significant ($p = 0.072$). There was a significant effect of fluid ($F_{2, 61} = 5.992$; $p = 0.05$) on ammonia-N concentration in CBC1 with the

fermentation fluid inoculated with the Bad rumen fluid having a higher concentration of ammonia-N than that inoculated with the 1:1 Mix rumen fluid (1.44 *vs* 1.38; $p < 0.001$).

Table 5-2 Fermentation parameters for consecutive batch culture 1 (CBC1)

	Time (hours)									p value		
	6	**12**	**18**	**24**	**30**	**36**	**42**	**48**	**SEM[1]**	**Fluid**	**Time**	**Time * Fluid**
Gas volume (ml)												
Good	18.33[a]	56.26[a]	85.79[a]	132.60[a]	153.03	176.88	185.73	211.65				
Bad	13.41[b]	52.63[a]	79.75	116.58[b]	150.20	166.89	186.89	199.28	18.17	**< 0.001**	**< 0.001**	**0.005**
1:1 Mix	7.82[c]	44.56[b]	75.73[b]	118.21[b]	146.88	178.13	195.63	213.00				
pH												
Good	6.60[a]	6.64[a]	6.69	6.66[a]	6.66[a]	6.63[a]	6.59[a]	6.57				
Bad	6.63[b]	6.67[b]	6.69	6.69[b]	6.66[a]	6.66[b]	6.63[b]	6.57	0.00	**< 0.001**	**< 0.001**	**0.006**
1:1 Mix	6.65[c]	6.69[b]	6.69	6.71[b]	6.69[b]	6.65[ab]	6.62[b]	6.58				
Total VFA (mM)												
Good	61.43	101.74	135.30	177.46	165.96	171.24	195.96					
Bad	57.10	97.92	139.66	153.69	214.68	199.23	195.76		257.12	0.080	**< 0.001**	0.053
1:1 Mix	56.49	87.44	126.09	137.40	153.13	199.51	193.20					
Ammonia nitrogen (mg/ mL)												
Good [ab]	1.44	1.41	1.36	1.37	1.38	1.45	1.47					
Bad [a]	1.45	1.45	1.46	1.43	1.41	1.50	1.45		0.002	**0.005**	0.072	0.595
1:1 Mix [b]	1.38	1.31	1.37	1.43	1.45	1.42	1.37					
Microbial crude protein (µg / mL)												
Good	92.86	166.41	282.26	456.00	501.79	595.01	699.61					
Bad	87.78	232.06	254.79	323.85	479.91	405.58	589.26		41.22	0.091	**< 0.001**	0.172
1:1 Mix	109.77	154.26	203.67	381.35	432.96	630.55	637.59					

[1]SEM standard error of the mean; VFA volatile fatty acid

[a-c] Means within a row that do not share a common superscript are significantly different ($p < 0.05$). When a fluid effect was observed, fluid type names that do not share a common superscript are significantly different ($p < 0.05$).

Table 5-3 Fermentation parameters for consecutive batch culture 2 (CBC2)

	Time (hours)							p value		
	6	**12**	**18**	**24**	**36**	**48**	**SEM[1]**	**Fluid**	**Time**	**Time * Fluid**
Gas volume (ml)										
Good	33.31	79.60	116.99	155.76	196.13	218.15				
Bad	31.82	75.76	114.24	150.92	200.96	218.36	31.46	0.355	< **0.001**	0.226
1:1 Mix	30.46	77.90	120.25	149.24	180.07	219.93				
pH										
Good	6.67	6.67	6.66	6.61	6.52	6.44				
Bad	6.7	6.67	6.66	6.64	6.53	6.43	0.00	0.128	< **0.001**	0.377
1:1 Mix	6.66	6.67	6.65	6.62	6.53	6.41				
Total VFA (mM)										
Good	39.88	74.12	96.3	130.26	159.43	171.49				
Bad	36.98	68.51	96.33	135.11	165.44	172.2	39.36	0.982	< **0.001**	0.976
1:1 Mix	42.64	71.13	96.7	127.34	161.87	172.91				
Ammonia nitrogen (mg/ mL)										
Good	1.51	1.59	1.71	1.67	1.83	1.82				
Bad	1.57	1.62	1.58	1.55	1.73	1.79	0.003	0.189	< **0.001**	0.120
1:1 Mix	1.49	1.67	1.63	1.7	1.77	1.74				
Microbial crude protein (µg / mL)										
Good	9.93	143.53	279.28	420.90	498.84	532.22				
Bad	41.65	116.05	145.05	325.40	601.67	576.29	30.19	0.531	< **0.001**	0.087
1:1 Mix	76.91	145.36	297.29	514.04	486.30	489.65				

[1] SEM standard error of the mean; VFA volatile fatty acid

Table 5-4 Fermentation parameters for consecutive batch culture 3 (CBC3)

	Time (hours)					p value		
	12	**24**	**36**	**48**	**SEM[1]**	**Fluid**	**Time**	**Time * Fluid**
Gas volume (ml)								
Good [a]	62.18	144.48	196.14	232.13				
Bad [ab]	55.47	134.27	184.49	216.69	75.18	**0.003**	< **0.001**	0.545
1:1 Mix [b]	49.92	129.98	164.51	222.23				
pH								
Good [a]	6.58	6.53	6.45	6.43				
Bad [b]	6.61	6.57	6.51	6.46	0.006	**0.018**	< **0.001**	0.644
1:1 Mix [b]	6.61	6.56	6.55	6.44				
Total VFA (mM)								
Good [a]	50.08	94.69	125.92	144.19				
Bad [ab]	47.86	94.83	115.34	136.31	15.13	**0.010**	< **0.001**	0.540
1:1 Mix [b]	46.66	87.82	116.36	137.90				
Ammonia nitrogen (mg/ mL)								
Good	1.68	1.69	1.81	1.91				
Bad	1.70	1.77	1.87	1.95	0.001	0.077	< **0.001**	0.800
1:1 Mix	1.71	1.69	1.87	1.92				
Microbial crude protein (µg / mL)								
Good	129.25	341.79	1088.34	576.01				
Bad	114.23	414.80	824.19	754.38	95.572	0.891	< **0.001**	0.941
1:1 Mix	81.74	315.78	921.01	598.79				

[1]SEM standard error of the mean; VFA volatile fatty acid

[a-c] Fluid type names that do not share a common superscript are significantly different ($p < 0.05$).

Table 5-5 Fermentation parameters for consecutive batch culture 4 (CBC4)

	Time (hours)			p value		
	24	**48**	**SEM[1]**	**Fluid**	**Time**	**Time * Fluid**
Gas volume (ml)						
Good	157.02	240.96				
Bad	157.80	232.98	28.01	0.642	< **0.001**	0.531
1:1 Mix	157.00	239.23				
pH						
Good [a]	6.51	6.48				
Bad [ac]	6.51	6.48	3.07×10^{-5}	**0.031**	< **0.001**	0.274
1:1 Mix [bc]	6.49	6.48				
Total VFA (mM)						
Good	73.80	101.09				
Bad	73.63	103.28	3.2	0.65	< **0.001**	0.455
1:1 Mix	75.56	101.71				
Ammonia nitrogen (mg/ mL)						
Good	1.26	1.66				
Bad	1.39	1.58	0.006	0.893	< **0.001**	0.184
1:1 Mix	1.35	1.63				
Microbial crude protein (µg / mL)						
Good	174.99	312.56				
Bad	201.19	431.57	36.202	0.288	< **0.001**	0.472
1:1 Mix	208.65	335.55				

[1] SEM standard error of the mean; VFA volatile fatty acids

[a-c] Fluid type names that do not share a common superscript are significantly different ($p < 0.05$)

Individual VFAs were also examined (Tables 5-6 to 5-9). For CBC1, there was only a Time effect for acetate ($F_{6, 62}$ = 753.606, $p < 0.001$) with the concentration increasing with each consecutive time point. Propionate, butyrate and A:P each showed an interaction between Time and Group ($F_{12, 62}$ = 4.344, $p < 0.001$; $F_{12, 62}$ = 5.024, $p < 0.001$ and $F_{12, 62}$ = 2.262, $p = 0.026$ for propionate, butyrate and A:P respectively) with the majority of the differences between the groups observed between 24 – 30 hours of fermentation. Where there was a significant group effect, this tended to be between the Good and Mix fluid, with concentrations of propionate and butyrate generally higher in the Good.

For CBC2, there was only an effect of Time for both acetate and propionate ($F_{5, 53}$ = 197.929, $p < 0.001$ and $F_{5, 53}$ = 623.730, $p < 0.001$ respectively) again with concentration increasing with each time point. There was a significant interaction between Time and Group for butyrate ($F_{10, 53}$ = 3.392, $p = 0.003$). Butyrate concentration was initially greater in the Good up to 24 hours, after which butyrate concentration was greatest in the Bad fluid. Finally, for the A:P ratio, there was a significant main effect of both Time ($F_{5, 53}$ = 128.249, $p < 0.001$) and Group ($F_{2, 53}$ = 3.339, $p = 0.044$) where the difference was seen between the Good and Mix ($p = 0.034$), with a lower A:P in the good compared to the mix until 36 hours of fermentation after which no difference was seen.

For CBC3 there was an effect of both Time ($F_{3, 35}$ = 451.811, $p < 0.001$) and Group ($F_{2, 35}$ = 3.402, $p = 0.047$) on acetate concentration with the concentration increasing with each time point. There was a significant difference between the Good and Bad only ($p = 0.041$), with the concentration of acetate generally higher in the Good than the Bad. There was only a Time effect for propionate ($F_{3, 35}$ = 673.756, $p < 0.001$). There was an interaction for butyrate between Time and Group ($F_{6, 35}$ = 2.670, $p = 0.040$) with the Good, generally the same as the Bad; both of which were higher than the Mix except at the start where all three groups were different (8 *vs* 7 *vs* 6 mM respectively). There was also an interaction for the A:P ratio ($F_{6, 35}$ = 4.013, $p = 0.006$).

Finally at CBC4, there was a Time effect only for acetate concentration and A:P ($F_{1, 17}$ = 558.364, $p < 0.001$ and $F_{1, 17}$ = 73.128, $p < 0.001$ respectively). For both propionate and butyrate, there was significant main effects for both Time ($F_{1, 17}$ = 316.548, $p < 0.001$ and $F_{1, 17}$ = 197.921, $p < 0.001$) and Group ($F_{2, 17}$ = 9.653, $p = 0.002$ and $F_{2, 17}$ = 92.942, $p < 0.001$ respectively). For propionate, the Good had a significantly lower concentration than both the bad and mix ($p = 0.014$ and $p = 0.003$ respectively). There was no difference between the bad and mix ($p = 0.654$). For butyrate, all three groups were significantly different to each other ($p < 0.001$) with the highest butyrate concentration seen in the Good rumen fluid, then the bad and the lowest in the mix (11.2, 9.8 and 8.1 mM ± 0.165 SEM respectively)

Table 5-6 Volatile fatty acid analysis for consecutive batch culture 1 (CBC1). Mean values are presented and corrected per g DM. All concentrations shown are in mM. Significant values are shown in bold

Time (hours)	Acetate			Propionate			Butyrate			A:P[1]		
	Good	**Bad**	**Mix**	**Good^{ab}**	**Bad^{a}**	**Mix^{b}**	**Good^{a}**	**Bad^{a}**	**Mix^{b}**	**Good**	**Bad**	**Mix**
6	36.8	42.0	36.9	16.7	8.1	7.6	13.0	7.0	7.0	2.1	5.2	5.0
12	51.7	55.1	49.3	31.4	33.9	29.2	18.6	18.1	16.3	1.6	1.6	1.6
18	67.3	68.4	69.5	43.2	45.1	44.0	24.8	26.2	20.9	1.6	1.5	1.6
24	89.4	89.2	91.3	66.5^{a}	26.4^{ab}	26.7^{b}	39.0^{a}	21.4^{b}	19.4^{b}	1.3^{a}	3.5^{ab}	3.4^{b}
30	105.2	101.3	99.6	32.9^{a}	77.5^{b}	30.1^{a}	27.9^{a}	50.1^{b}	23.4^{c}	3.2^{a}	1.3^{b}	3.3^{c}
36	109.5	113.4	114.1	35.2	35.0	36.3	26.5	26.2	23.9	3.1	3.3	3.2
42	125.1	127.4	127.0	40.6	39.7	40.3	30.2^{a}	28.6^{a}	25.9^{b}	3.1^{a}	3.2^{b}	3.2^{b}
SEM[1]		0.434			1.426			0.648			0.108	
Time		**< 0.001**			**< 0.001**			**< 0.001**			**< 0.001**	
Group		0.284			**0.030**			**0.001**			0.112	
Time*Group		0.275			**< 0.001**			**< 0.001**			**0.026**	

[1] SEM standard error of the mean, A:P acetate to propionate ratio

[a-c] Means within a row that do not share a common superscript are significantly different ($p < 0.05$). When a group effect was observed, fluid type names that do not share a common superscript are significantly different ($p < 0.05$).

Table 5-7 Volatile fatty acid analysis for consecutive batch culture 2 (CBC2). Mean values are presented and corrected per g DM. All concentrations shown are in mM. Significant values are shown in bold

Time (hours)	Acetate			Propionate			Butyrate			A:P[1]		
	Good	Bad	Mix	Good	Bad	Mix	Good[ab]	Bad[b]	Mix[c]	Good[a]	Bad[ab]	Mix[b]
6	26.2	25.0	29.9	7.4	6.9	7.6	6.2	5.0	5.1	3.5	3.6	3.9
12	46.3	44.3	46.2	15.7	14.7	15.1	12.0[a]	9.5[b]	9.8[b]	2.9	3.0	3.1
18	55.6	57.1	58.0	22.4	22.6	22.7	18.4	16.6	16.0	2.5	2.5	2.6
24	67.7	75.1	70.2	34.9	32.1	31.9	27.7	27.9	25.2	1.9	2.4	2.2
36	87.3	85.7	88.6	41.8	42.8	42.1	30.3[a]	37.0[b]	31.2[a]	2.1	2.0	2.1
48	94.9	92.3	96.3	45.5	45.2	45.4	31.1[a]	34.7[b]	31.3[a]	2.1	2.0	2.1
SEM	0.742			0.248			0.237			0.023		
Time	**< 0.001**			**< 0.001**			**< 0.001**			**< 0.001**		
Group	0.544			0.612			**0.005**			**0.044**		
Time*Group	0.929			0.904			**0.003**			0.215		

[1] SEM standard error of the mean, A:P acetate to propionate ratio

[a-c] Means within a row that do not share a common superscript are significantly different ($p < 0.05$). When a fluid effect was observed, fluid type names that do not share a common superscript are significantly different ($p < 0.05$).

Table 5-8 Volatile fatty acid analysis for consecutive batch culture 3 (CBC3). Mean values are presented and corrected per g DM. All concentrations shown are in mM. Significant values are shown in bold

Time (hours)	Acetate			Propionate			Butyrate			A:P		
	Good[a]	**Bad[b]**	**Mix[ab]**	**Good**	**Bad**	**Mix**	**Good[a]**	**Bad[b]**	**Mix[c]**	**Good[a]**	**Bad[b]**	**Mix[b]**
12	31.0	29.5	29.2	11.1	11.4	11.5	8.0[a]	7.0[b]	6.0[c]	2.8[a]	2.6[b]	2.5[b]
24	53.3	53.5	50.6	23.3	23.5	23.2	18.1[a]	17.8[a]	14.1[b]	2.3	2.3	2.2
36	74.3	67.1	71.3	30.5	28.9	29.1	21.1[a]	19.3[a]	16.0[b]	2.4[a]	2.3[b]	2.4[a]
48	86.8	81.4	84.9	34.0	33.1	34.4	23.4[a]	21.8[a]	18.5[b]	2.6	2.5	2.5
SEM		0.556			0.188			0.145			0.01	
Time		**< 0.001**			**< 0.001**			**< 0.001**			**< 0.001**	
Group		**0.047**			0.588			**< 0.001**			**< 0.001**	
Time*Group		0.410			0.642			**0.04**			**0.006**	

[1] SEM standard error of the mean, A:P acetate to propionate ratio

[a-c] Means within a row that do not share a common superscript are significantly different ($p < 0.05$). When a fluid effect was observed, fluid type names that do not share a common superscript are significantly different ($p < 0.05$).

Table 5-9 Volatile fatty acid analysis for consecutive batch culture 4 (CBC4). Mean values are presented and corrected per g DM. All concentrations shown are in mM. Significant values are shown in bold

Time (hours)	Acetate			Propionate			Butyrate			A:P[1]		
	Good	Bad	Mix	Good[a]	Bad[b]	Mix[b]	Good[a]	Bad[b]	Mix[c]	Good	Bad	Mix
24	40.2	40.2	42.3	23.9	25.0	26.3	9.8	8.4	6.9	1.7	1.6	1.6
48	57.6	59.5	60.3	30.7	32.6	32.2	12.8	11.2	9.2	1.9	1.8	1.9
SEM[1]	0.386			0.19			0.095			0.013		
Time	**< 0.001**			**< 0.001**			**< 0.001**			**< 0.001**		
Group	0.064			**0.002**			**< 0.001**			0.168		
Time*Group	0.633			0.171			0.403			0.531		

[1] SEM standard error of the mean, A:P acetate to propionate ratio

[a-c] Fluid type names that do not share a common superscript are significantly different ($p < 0.05$).

5.3.4 The bacterial composition

The bacterial profiles of the rumen fluids used as inoculum and of fermentation fluids at the end of CBC1 and CBC4 were examined to identify whether the differences in IVDMD between fluids at the end of CBC1 and the lack of difference at the end of CBC4 could be explained by the bacterial community composition. A total of 4,649,394 sequences were obtained with an average of 516,599 ± 71,166 sequences per sample. After all filtering and clustering steps, a total of 106,154 unique, high quality sequences remained with an average of 11,974 ± 1850 per group.

5.3.4.1 Bacterial community composition in the experimental fermenters at the end of consecutive batch culture 1 (48 hours) and CBC4 (192 hours).

In total, 11 phyla had a relative abundance greater than 1%: Firmicutes, Bacteroidetes, Fibrobacteres, Tenericutes, Spirochaetae, unclassified bacteria, Proteobacteria Planctomycetes, Lentisphaerae, Candidate division SR1 and Candidate division TM7 (in decreasing abundance). The most abundant phyla at the end of CBC1 across all three rumen fluid were Firmicutes (38.9 ± 1.10%), Bacteroidetes (34.7 ± 0.35%) and Fibrobacteres (13.5 ± 0.31 %). The relative abundance of Firmicutes, Bacteroidetes and Fibrobacteres was very similar across the three rumen fluids.

Similar to results shown in Chapter 4, the Bad RF at the end of CBC1 had a higher percentage of Tenericutes (8.30 %) when compared with the Good RF (6.78 %). However, in this experiment, the Bad fluid also had higher levels of Tenericutes than the 1:1 Mix (6.52%).

At the end of CBC4, Firmicutes (47.7 ± 2.34 %) and Bacteroidetes (37.8 ± 0.85 %) followed by Spirochaetae (3.9 ± 0.87 %) were the most abundant Phyla across all three RFs with Fibrobacteres decreasing to 2.8 ± 0.79 %. The relative abundance of Tenericutes in the Bad was comparable to the Good and 1:1 Mix (2.52, 2.37 and 2.82 % respectively).

From the Phyla identified above, 33 Genera had a relative abundance greater than 1% across the experimental fermentation samples. *Prevotella 1* (15.8 ± 1.51 %) was the most abundant for CBC1, followed by *Fibrobacter* (13.5 ± 0.31 %) and *Ruminococcus 1* (7.2 ± 0.34 %) across all three rumen fluids.

At the end of CBC4, the most abundant genus was *Rikenellaceae RC9 gut group* (13.8 ± 1.00 %) followed by *Prevotella 1* (7.1 ± 2.52 %) *and Bacteroidales BS11 gut group unclassified* (6.1 ± 0.67%). Interestingly, these were also the three most abundant genera in the neat rumen fluids used as inoculum (Section 5.3.4.2).

The relative abundance of all phyla and genera can be seen in Appendix C.

5.3.4.2 The bacterial composition of the original 'neat' rumen fluids

The rumen bacterial community of the Good, Bad and 1:1 Mix fluids used as inoculum in the experiment were also sequenced.

A total of 8 phyla had a relative abundance greater than 1% in the neat fluids: Bacteroidetes (46.6 ± 1.20 %), Firmicutes (33.5 ± 2.70 %), unclassified bacteria (4.5 ± 0.76 %), Lentisphaerae (3.4 ± 0.99 %), Candidate division SR1 (3.2 ± 0.60 %), Planctomycetes (2.2 ± 0.38 %), Tenericutes (2.7 ± 0.27 %) and Candidate division TM7 (0.9 ± 0.18%). On the whole the fluids were very similar (Appendix C). The most obvious difference between the fluids was a higher (4-5 %) relative abundance of Firmicutes for the Bad and 1:1 Mix when compared with the Good (34.48 and 35.62 % *vs* 30.48 % respectively). The Good fluid had a higher relative abundance of Lentisphaerae when compared with the Bad and 1:1 Mix (4.56 *vs* 2.82 *vs* 2.87 % respectively).

Tenericutes, which was shown to have a higher relative abundance in the 'Bad' RF experimental samples in both this chapter, at the end of CBC1 (8.3, 6.8 and 6.5% for Bad, Good and 1:1 Mix respectively) and in the previous chapter after both Day 1 (5.9, 2.1 and 4.6 % for Bad, Good and 1:1 Mix respectively) and Day 16 (9.6, 3.7 and 9.3 % for Good, Bad and 1:1 Mix respectively) showed little difference between the Good and Bad RF used as inoculum for the *in vitro* model (2.5 and 2.8 % for Good and Bad RF respectively).

In the neat fluid inocula, 27 genera had a relative abundance, in at least one of the fluids, greater than 1%. The three most abundant genera were, as seen for CBC4 above: *Prevotella 1* (19.7 ± 0.83 %), *Rikenellaceae RC9 gut group* (8.8 ± 0.14 %) and *Bacteroidales BS11 gut group unclassified* (6.4 ± 0.29 %). There was very little difference in the relative abundance of each genera between the three fluid types (Appendix C).

5.3.4.3 Alpha diversity

Alpha diversity decreased significantly with Time for both Chao1 and Shannon alpha diversity measures ($p < 0.001$; Table 5-10). Alpha diversity was highest in the neat rumen fluid samples prior to fermentation (Chao1 4481.8 ± 132.36 Shannon 7.3 ± 0.05, Simpson's 0.998 ± 0.0005), had decreased by the end of CBC1 (48 hours; Chao1 2731.9 ± 14.82, Shannon 5.8 ± 0.00, Simpson's 0.982 ± 0.0005), and had decreased further by the end of the experimental period for the Chao1 measure of alpha diversity only (192 hours; Chao1 2037.5 ± 79.76). Interestingly, there was no difference in alpha diversity between the Good and Bad rumen fluids used to inoculate the *in vitro* model, suggesting that the number of OTUs that were present in each sample were similar. No effect of Fluid was identified across the experimental period.

Table 5-10 Chao 1, Shannon and Simpson's indices of alpha diversity obtained from neat rumen inoculum (Good, Bad and 1:1 Mix), experimental samples at 48 hours (CBC1) and 192 hours (CBC4).

		Time			p value		
		Neat	**CBC1**	**CBC4**	**Time**	**Fluid**	**Time*Fluid**
Chao1	**Good**	4436.3	2711.4	2048.1	**< 0.001**	0.6843	0.8441
	Bad	4347.3	2746.7	1934.9			
	Mix	4661.8	2738.2	2129.4			
Shannon	**Good**	7.3	5.8	5.8	**< 0.001**	0.9352	0.9985
	Bad	7.2	5.8	5.7			
	Mix	7.3	5.8	5.9			
Simpson's	**Good**	0.997	0.983	0.990	0.1001	0.9569	0.9236
	Bad	0.998	0.982	0.987			
	Mix	0.998	0.982	0.989			

5.3.4.4 Beta diversity

Community composition did not differ between the Good, Bad and 1:1 Mix RFs across the experimental period ($F_{2, 8} = 1.223$, $p = 0.240$) demonstrated by the overlap of points on the NMDS plot in Figure 5-5. PERMANOVA analysis determined a significant effect only of Time ($F_{2, 8} = 5.015$, $p = 0.003$; Figure 5-5). The community composition of the dried grass substrate was very dissimilar to that of the rumen and fermentation fluid.

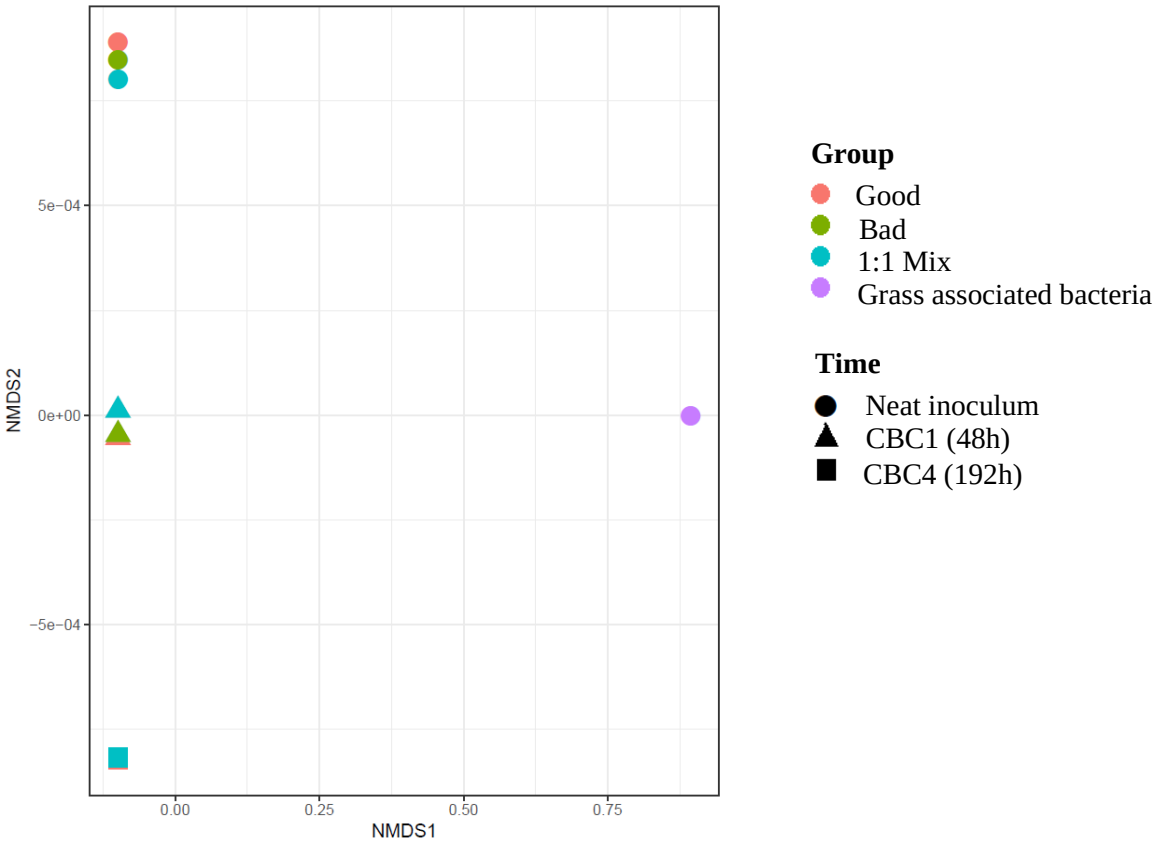

Figure 5-5 Non-metric multi-dimensional scaling (NMDS) plot of the bacterial community obtained from neat rumen inoculum (Good, Bad and 1:1 Mix), experimental samples at 48 hours (CBC1) and 192 hours (CBC4) across four consecutive batch cultures and the epiphytic bacterial community associated with the substrate using Bray-Curtis distances. PERMANOVA analysis showed a significant effect of Time ($F_{2, 8} = 5.015$, $p = 0.003$), but not rumen inoculum (Group; $F_{2, 8} = 1.223$, $p = 0.240$) on bacterial community composition.

DeSEQ2 analysis was used to identify which OTUs were responsible for the significant effect of Time seen in Figure 5-5 and the closest genera was assigned. The 10 OTUs that showed the greatest change in abundance are shown in Table 5-11 below. Shown are the 10 OTUs that both increase and decrease significantly from the neat inoculum to the end of CBC1 and from the end of CBC1 to the end of the experiment at CBC4. *Prevotella 1* OTUs were shown to decrease in abundance from both the neat rumen fluids to the end of CBC1 and also from CBC1 to CBC4. The *Bacteroidales S24-7 group unclassified* OTUs that increased from the neat rumen fluid to CBC1 were found to decrease to CBC4 (OTU 19 and OTU 47).

Table 5-11 DeSEQ2 analysis of the operational taxonomic units that showed the greatest change in abundance over the experimental period.

OTUs were classified to the Genus level. The p values shown are adjusted for multiple testing using the Benjamin-Hochberg correction. Fold change represents Log2 fold change relative to the neat inoculum.

OTU number	Genus	Fold change	p value	OTU number	Genus	Fold change	p value
Increased from inoculum to CBC1 (48 hours)				**Decreased from inoculum to CBC1 (48 hours)**			
OTU 2	*Fibrobacter*	9.79	< 0.001	OTU 35	*Candidate division SR1 unclassified*	- 5.09	< 0.001
OTU 7	*Ruminococcus 1*	6.63	< 0.001	OTU 127	*Prevotella 1*	- 5.41	< 0.001
OTU 9	*Anaeroplasma*	6.18	< 0.001	OTU 119	*[Eubacterium] coprostanoligenes group*	- 4.65	< 0.001
OTU 4	*Pseudobutyrivibrio*	5.45	< 0.001	OTU 130	*Prevotella 1*	- 4.45	< 0.001
OTU 19	*Bacteroidales S24-7 group unclassified*	9.01	< 0.001	OTU 196	*Candidate division SR1 unclassified*	- 4.29	0.002
OTU 1	*Fibrobacter*	6.54	< 0.001	OTU 125	*Prevotella 1*	- 4.76	0.002
OTU 47	*Bacteroidales S24-7 group unclassified*	6.56	< 0.001	OTU 328	*Prevotella 1*	- 5.17	0.003
OTU 28	*Prevotella 1*	4.83	< 0.001	OTU 226	*Bacteroidales BS11 gut group unclassified*	- 4.31	0.003
OTU 6	*Oribacterium*	5.30	< 0.001	OTU 320	*p-1088-a5 gut group*	- 4.99	0.003
OTU 61	*Ruminococcus 1*	5.20	< 0.001	OTU 277	*WA aaa01f12 unclassified*	- 4.41	0.004

Table continued…

OTU number	Genus	Fold change	p value	OTU number	Genus	Fold change	p value
Increased from CBC1 to CBC4 (192h)				**Decreased from CBC1 to CBC4 (192h)**			
OTU 30	Ruminococcaceae UCG-005	8.13	< 0.001	OTU 1	Fibrobacter	-7.56	< 0.001
OTU 57	Prevotella 1	8.42	< 0.001	OTU 137	Ruminococcus 1	-8.62	< 0.001
OTU 115	Family XIII unclassified	8.60	< 0.001	OTU 120	Prevotella 1	-8.02	< 0.001
OTU 70	Bacteroidales BS11 gut group unclassified	4.31	< 0.001	OTU 87	Prevotella 1	-6.93	< 0.001
OTU 133	Bacteroidales RF16 group unclassified	4.77	< 0.001	OTU 47	Bacteroidales S24-7 group unclassified	-4.88	< 0.001
OTU 107	Rikenellaceae RC9 gut group	3.89	0.001	OTU 143	Prevotella 1	-6.07	< 0.001
OTU 12	Rikenellaceae RC9 gut group	3.05	0.001	OTU 144	Probable genus 10	-5.37	< 0.001
OTU 72	Rikenellaceae RC9 gut group	4.19	0.001	OTU 19	Bacteroidales_S24-7 group unclassified	-4.48	< 0.001
OTU 158	Bacteroidales BS11 gut group unclassified	4.77	0.001	OTU 140	Bacteroidales UCG-001 unclassified	-6.88	< 0.001
OTU 171	Ruminococcus 1	5.11	0.001	OTU 274	Saccharofermentans	-7.10	< 0.001

5.4 Discussion

The aim of the work presented here was to first identify two rumen fluids from genetically and environmentally similar forage-fed cattle that were dissimilar in terms of IVDMD, to compare their microbiota and to mix them and study the *in vitro* fermentation performance and microbiota of the mix relative to the two unmixed rumen fluids.

5.4.1 Each rumen fluid improved its ability to digest dry matter, but cross inoculation was unable to improve the performance of a poorer performing rumen fluid

Each of the rumen microbial communities (Good, Bad and 1:1 Mix) significantly improved their ability to digest dry matter of the dried grass substrate across the course of the experimental period (19, 24 and 21% for Good, Bad and 1:1 Mix RFs respectively) presumably as the microorganisms adjusted to the substrate and environment within the model. The performance (IVDMD) of each rumen fluid improved with each consecutive batch culture, more so for the Bad than the Good such that the differences in IVDMD between the fluids had disappeared by CBC4 (72, 71 and 71 g/ 100g DM respectively for Good, Bad and 1:1 Mix). Across CBCs 1-3, the Good fluid showed a continued ability to digest more dry matter than both the Bad and 1:1 Mix.

In agreement with the previous chapter, after 8 days of consecutive batch culture fermentation, the microbial community appeared to have adapted to the substrate and environment reaching a final IVDMD of *ca* 72 g / 100 g DM. This is similar to the adaptation period granted to the semi-continuous RUSITEC. The microorganisms are generally allowed around 8-10 days to adapt to the model and its substrate prior to experimental sample collection starting (Belanche et al., 2016; Mateos et al., 2017).

Curves were fitted to the data for CBC1 and CBC2 to try and explain the difference in DM digestibility between the three fluids. The rate of digestion was moderately lower for CBC2 when compared with CBC1, however, the initial IVDMD at 6 hours was higher and the lag time was lower. This may indicate that the microbial population was able to quickly establish during CBC2 and begin digestion of both the soluble and insoluble fractions prior to the first IVDMD recording at 6 hours, which is supported by the higher gas volumes recorded after 6 hours of fermentation (13.2 *vs* 31.9 ml for CBC1 and CBC2 respectively).

The IVDMD of the mixed fluid was shown previously to be an average of the Good and Bad rumen fluids for the first 24 hours of fermentation and thereafter differences between the fluids were lost as the microbial community presumably adapted to the substrate and environment provided within the model (Chapter 4). In the current chapter, the performance (IVDMD) of the cross inoculated bottles was shown to be similar to that of the Bad, reducing the performance of the Good fluid that was inoculated into the bottles. It is possible that there were elements present within the Bad RF that prevented the microbial community from the Good RF to establish and flourish. Bacteriophages and bacteriocins, which are involved in structuring the microbial community (Koskella and Meaden, 2013) and niche defence (Yang et al., 2014) respectively, may be at play and these are discussed in more detail in the General Discussion (Chapter 8).

The difference observed between the data presented here and the previous chapter may be due to the rumen fluid used to inoculate the fermentation bottles. The IVDMD of the two rumen fluids used as inoculum in this cross inoculation experiment were less different than the two rumen fluids used in the previous chapter (3.6 *vs* 14.9 % difference in IVDMD between Good and bad rumen fluids respectively) and the bacterial community was found to be very similar between the Good and Bad RF. This is likely a reflection of the similar genetic and environmental factors of the cattle from which the rumen fluid was sourced. The similarity of the two rumen fluids may have led to a more harmonised amalgamation of the microbial communities.

The lack of a favourable response to cross-inoculation on IVDMD may also have been due to the diet that the animals were fed prior to slaughter. The rumen fluid for this experiment was sourced from animals that were raised on a forage diet. As the substrate provided within the *in vitro* model was also forage, the microorganisms present were putatively pre-adapted to digest fibre effectively. Although not the same substrate as the animals were fed prior to slaughter (dried grass *vs* fresh grass), the diet provided in the fermenters was potentially less of a ‘shock’ to the microbial community, reducing perturbation experienced by the microorganisms transferred to the *in vitro* model in contrast to the previous chapter where the diet was thought to cause perturbation, therefore allowing cross-inoculation to improve IVDMD to an average of the two rumen fluids, albeit only for the first 24 hours of the experiment. However, the diet provided to the animals in the previous chapter was unknown, so this is only a hypothesis.

Overall, cross-inoculated rumen fluid did not provide a favourable increase to performance (IVDMD) of a poorer performing rumen fluid when mixed in a 1:1 ratio. Each rumen inoculum increased IVDMD across the experimental period and differences between the fluids were lost by the final consecutive batch culture.

5.4.2 Fermentation parameters were not modulated by cross inoculation

As well as improving the degradative ability of a rumen fluid through cross inoculation it may also be possible to improve the fermentation parameters associated with a particular substrate. Overall, there was found to be little difference between the fluids in terms of fermentation parameters measured across each of the consecutive batch cultures and as to be expected, time had a significant effect on the parameters measured.

Similar to IVDMD above, cross inoculation was unable to improve the fermentation parameters associated with the poorer performing rumen fluid inoculum (i.e. maximise the partition of dietary organic matter into microbial protein, increase VFA production and reduce ammonia nitrogen concentration). Where there was an interaction or a fluid effect, it was found that, in general, the Good fluid was different to both the Bad and the 1:1 Mix with higher gas production (CBC1 and 3), lower pH (CBC1 and 3), higher VFA production (CBC3) and generally lower ammonia-nitrogen concentration (CBC1). However, differences between fluids were not consistent across fermentations. Despite this, the differences seen between the Good fluid in comparison to the Bad and 1:1 Mix further confirmed that cross-inoculation was unable to manipulate fermentation of a poorer performing rumen fluid.

For the first consecutive batch culture, the pH for all three fluids increased initially and then declined, but the timing of the decline was dependent upon the fluid used. The Good fluid showed a decline 6 hours earlier than the Bad and 12 h earlier than the 1:1 Mix and this coincided with an increase in VFA production. This earlier VFA production may demonstrate a more effective colonisation and digestion of the substrate, however, no significant difference in the lag phase was observed, which may suggest that this instead represents digestion of the soluble fraction of the substrate.

The same pattern was also seen for CBC3 and no difference in pH was seen between the rumen fluids for CBC 2 or 4. It is unclear as to why the pH shows different patterns across the four consecutive batch cultures. Both MCP and NH_3-N concentration were affected only by Time, in general increasing over the course of a fermentation. No fluid

effect was observed demonstrating no difference in these measured parameters of N metabolism between the three groups.

It was interesting to note that even though digestibility of the substrate improved over the course of the four consecutive batch cultures, the total VFA and microbial crude protein concentration decreased (195.0 *vs* 102.0 mM and 642.2 *vs* 359.9 µg/ml at CBC1 and CBC4 for total VFA and MCP concentration respectively). This may be indicative of a smaller, more efficient microbial population and is something that should be explored further through molecular quantification of the microbial population (qPCR).

Modulation of fermentation parameters within an *in vitro* batch culture through the introduction of rumen isolated bacterial species has been shown previously (Fraga et al., 2015). They introduced a large dose of one bacterial strain (10^6 cells/ml) into each fermentation bottle over a 96 hour fermentation with seven different strains of bacteria tested (*Butyrivibrio hungatei* 63C, *B. hungatei* 79C, *B. hungatei* 58C, *Pseudobutyrivibrio ruminis* 50C, *P. ruminis* 55C, and two unclassified *Lachnospiraceae* strains, 21C and 56C). Differences were observed in gas production, VFA concentration and pH between control bottles and those dosed with native rumen bacterial strains. The authors also showed a modulation to the microbial community at the family and genus level within the fermenters and speculated that they had probably enhanced the fermentation of non-soluble carbohydrates, shown by an increase in gas production during the slow phase of fermentation through the introduction of their probiotics. The study did not introduce the strains in combination and although they used qPCR to quantify methanogenic microorganisms, they did not quantify the concentration of the introduced strain remaining within the fermenter bottles at the end of the 96 hour fermentation. It would have been of interest to know whether their probiotic strains were maintained within the *in vitro* model.

5.4.3 Community composition was significantly affected only by time

As differences in performance between the rumen fluids within the *in vitro* model were expected to be microbial in origin, bacterial community composition of the rumen fluids was explored. Samples were taken from the neat rumen inocula prior to the beginning of fermentation (neat), after the first batch culture (CBC1), where a significant difference between the fluids was observed, and at the end of the fourth consecutive batch culture

(CBC4) where performance (IVDMD and fermentation parameters) of the rumen fluids were found to converge.

To understand the differences in performance between the three rumen fluids, community composition was determined through both alpha and beta diversity. The community was found to differ significantly in terms of both richness within a sample (alpha diversity) and community composition between samples (beta diversity) but both alpha and beta diversity were significantly affected by Time only and not by the rumen inoculum used, as was also seen for the previous chapter. This suggests that the bacterial community composition was not responsible for the differences in performance that were observed. The community composition shows clear divergence from that of the neat inoculum. It is of interest that there was no difference in alpha diversity between samples at the beginning of the experiment, despite significantly different digestive ability within the *in vitro* model. Shabat et al. (2016) showed previously that more efficient animals have a less diverse microbial profile than inefficient animals with fewer metabolic pathways. It will be of interest for future work to identify the activity of the microbial populations to determine whether the similar microbial populations were expressing different gene pathways, therefore producing different metabolites.

Previous studies using an *in vitro* model of rumen fermentation to describe the microbial community have also found a decline in alpha diversity over time (Soto et al., 2013). A reduction in alpha diversity within the batch culture model of the rumen is thought to be an artefact of this type of model, despite supporting the growth of fibrolytic species (Fraga et al., 2015). Alpha diversity was found to decline within 24 hours for the batch culture model, but was still stable after 3 days for continuous culture (Soto et al., 2013). Anderson et al. (2017) reported that diet and not animal had the greatest effect on beta diversity in a cross-over experiment considering the effect of four diets in five steers. In the current study, the rumen inoculum used had no significant effect on the composition of the bacteria. The global rumen census project determined that feed was the largest contributor to the shaping of the rumen bacterial community, playing a larger role than biological factors associated with the host animal such as the immune system, host-derived nutrients and antimicrobial peptides (Henderson et al., 2015).

By using rumen fluid sourced from animals raised on a forage based diet, it was hypothesised that there would be a smaller effect of Time on bacterial community composition due to prior adaptation of the microbial community to a high fibre substrate.

However, Time was still found to have a significant effect on community structure. As well as substrate, variation in the bacterial population may have been caused by buffer composition, pH and temperature. However, previous work in this lab showed that small changes in pH (6.4, 6.7 and 7.0) and temperature (37, 39 and 41°C) had no effect on bacterial community composition despite differences in IVDMD and fermentation parameters when analysed by restriction fragment length polymorphisms (Merrick, 2017).

The *in vitro* model does not appear able to maintain the microbial profile of the inoculating rumen fluid. While efforts were taken to minimise the 'diet/substrate' effect by using rumen fluid from cattle fed forage diets, there was still found to be a difference. Even the same substrate dried or fresh has been shown to invoke a difference in fermentation and presumably also the microbiota (Rymer et al., 2005). The process of drying can increase the dry matter digestibility of a feed stuff when compared to its fresh counterpart due to increasing the surface area available for microbial attachment by roughening the surface and also decreasing digestibility in the case of protein (Lowman et al., 2002). An alternative explanation is that the removal of the controlling effect of the host animal may remove constraints on the population that were previously holding the community in a stable state.

When using an *in vitro* batch model to determine the effect of a treatment on the microbial community, it is important to know the composition of the starting inoculum and how this changes over the course of the fermentation alongside the effect of the treatment to determine whether the changes in microbial composition observed are caused by the treatment or due to adaptation to the model.

5.4.4 Changes in the bacterial community

As concluded in the previous section bacterial community structure was similar between rumen fluids, confirmed by measures of alpha and beta diversity, however, there were changes in bacterial community structure with time that are worth exploring.

In the previous Chapter, Tenericutes was identified as a phyla that differed in abundance between the Good and Bad RFs throughout the experiment with similar levels in the 1:1 Mix as there were in the Bad. In this experiment Tenericutes was again found to be higher in the Bad fluid than that of the Good (8.3 *vs* 6.78 %) at the end of CBC1, emphasising the potential role of this phylum in an animal's performance. However, in this

experiment, the 1:1 Mix had similar levels of Tenericutes to the Good (6.52 %) and by the end of the experiment (CBC4), the level was similar across all three groups (2.52 *vs* 2.37 *vs* 2.82 for Good, Bad and 1:1 Mix respectively). In the neat inoculum, prior to *in vitro* fermentation, the relative abundance of Tenericutes was again similar between the Bad and the Good (2.77 and 2.47 % respectively). If, as described in Chapter 4.4.4.1, Tenericutes is an opportunistic Phyla that increases in times of perturbation, this may suggest that the microbial community residing within the Bad rumen fluid showed greater disturbance upon addition to the *in vitro* model, but this was not the case when mixed with the Good rumen fluid suggesting that the microbiota of the Good RF may have a better resilience to perturbation.

At the end of the experimental period of CBC4, the level of Tenericutes had returned to its baseline level. Interestingly, the genera that were most abundant at the end of the experiment were similar to those observed in the neat inoculum. This, alongside the reduction in Tenericutes may suggest that the community had stabilised and was reflective of the original community at least at the Genus level. In terms of Phyla, Bacteroidetes was the most abundant in the neat rumen inoculum followed by Firmicutes. Within the *in vitro* model, the relative abundance of Bacteroidetes and Firmicutes was reversed. The abundance of Firmicutes increased with time (33.6 ± 2.77, 38.9 ± 1.10 and 47.7 ± 2.34 % for neat inoculum, CBC1 and CBC4 respectively) in agreement with Belanche et al. (2017).

The relative abundance of the genus *Pseudobutyrivibrio* was higher in the Good rumen fluid than both the Bad and 1:1 Mix at both experimental time points (CBC1 5.34, 3.50 and 3.53 %; CBC4 8.39, 4.74 and 4.89 % for Good, Bad and 1:1 Mix respectively). Members of the *Pseudobutyrivibrio* genus have been associated with some of the highest xylanase activities of all rumen bacteria (Zorec et al., 2014) and has been identified as a secondary coloniser of a grass substrate (Belanche et al., 2017; Huws et al., 2016; Mayorga et al., 2016). This genera may therefore be responsible for the improved IVDMD seen for the Good rumen fluid across the experiment within the *in vitro* model despite initial low relative abundance in the neat inoculum (0.28 and 0.25 % for Good and Bad RF respectively).

The relative abundance of *Oribacterium* was shown to be higher in both the Bad and 1:1 Mix bottles at the end of the experiment at CBC4 than the Good (5.04, 4.31 and 2.22 % respectively). *Oribacterium* of the family Lachnospiracheae (Phylum: Firmicutes), is a

common member of the oral cavity and has been previously identified as a member of the rumen community (Huws et al., 2015). Similar to the Phylum Tenericutes, *Oribacterium* may be an opportunistic genus. Treatment with both Monensin and Nisin (a bacteriocin) resulted in an increase in the relative abundance of *Oribacterium* when rumen fluid was incubated in an *in vitro* model (Shen et al., 2017). The presence of *Oribacterium* may therefore indicate that there was more dysbiosis associated with the Bad rumen fluid when incubated within the *in vitro* model that was not observed in the Good. Similarly to *Pseudobutyrivibrio*, relative abundance was low in the neat inoculum (0.08 and 0.15 % respectively for Good and Bad RF).

Similar to the results of the DeSeq2 analysis in the previous chapter, *Prevotella* species were implicated throughout the fermentation with multiple OTUs changing in abundance across the experimental period. *Prevotella* was again found to be the most abundant genera in the samples in agreement with previous studies (Mickdam et al., 2016; Duarte et al., 2017; Darwin et al., 2018). *Prevotella* has been reported to be the predominant rumen genus accounting for 42 – 60 % of bacterial 16S rRNA sequences (Stevenson and Weimer, 2007; Pitta et al., 2010). The large increase in *Fibrobacter* and *Ruminococcus* OTUs from the neat rumen fluid to the end of fermentation in CBC1 showed a rapid growth of solid-attached bacterial species as previously observed (Koike et al., 2003), thought to be due to the vast removal of solid attached bacteria during rumen fluid processing (Soto et al., 2013). The larger increase for *Fibrobacter* compared to *Ruminococcus* is thought to be due to the ability of *F. succinogenes* to attach to both damaged and undamaged fibrous material (Shinkai and Kobayashi, 2007).

Although there were differences in the relative abundance of certain Phyla and Genera, the community structure on the whole was very similar between rumen fluids.

5.4.4.1 How do all the sequencing samples compare?

Beta diversity analysis was performed on bacterial community profiles from the previous chapter (Chapter 4) and this chapter, on the same NMDS plot to explore any relationship between the microbial profiles across the two experiments and to determine whether the communities converged to the same point (Figure 5-6). Both Time ($F_{4, 14} = 6.27$ $p < 0.001$) and Experiment ($F_{1, 14} = 3.81$ $p < 0.001$) caused significant dispersion of the data points. All points cluster away from the grass associated bacterial community shown in

pink. No clear convergence of the data points was seen, however the samples are moving along the same axis (NMDS2) with time.

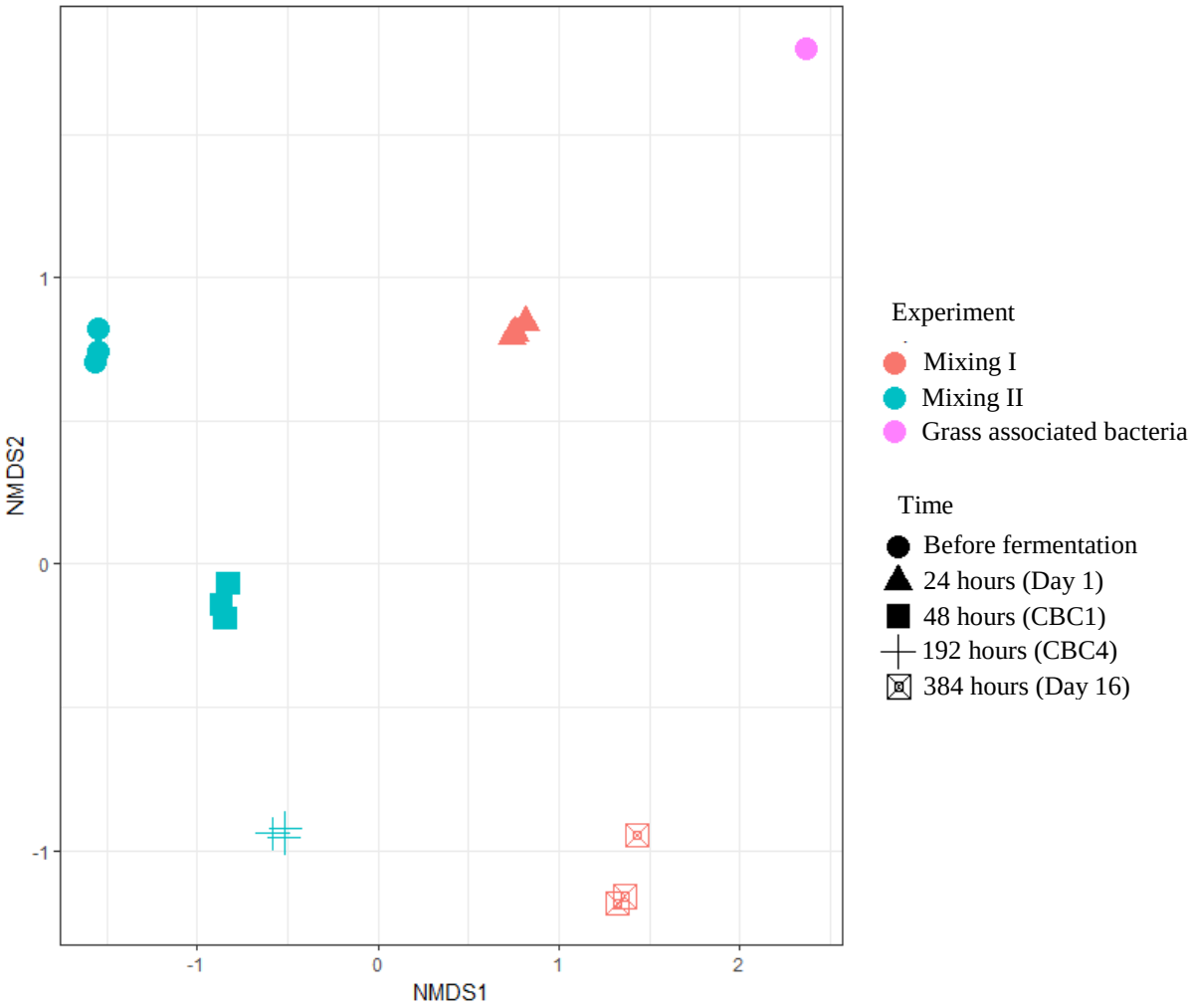

Figure 5-6 Non-metric multi-dimensional scaling (NMDS) plot for both cross inoculation experiments and the bacterial profile associated with the dried grass substrate using Bray-Curtis distances. Data for Mixing Experiment I (Chapter 4) can be seen in red and data from this chapter (Mixing Experiment II) in blue. The grass associated "epiphytic" bacteria is shown in pink. Different shapes represent different time points. Before fermentation represents the samples from the neat rumen fluid used as inoculum

5.4.5 Correlation of abattoir parameters and daily live weight gain to IVDMD

The eleven rumen fluids from which the two rumen fluids used as inocula in this experiment (Good and Bad) were selected were all run through the *in vitro* model to determine their ability to digest the dried grass substrate provided. The observed variation in dry matter digestibility was smaller than that seen in Chapter 4 (4 g/ 100g *vs* 15 g/ 100g), therefore making it more difficult to discern differences in performance associated with mixing the rumen fluids.

Similar to the previous chapter, there was no correlation between the dry matter degradation of each rumen fluid *in vitro* and measures provided from the abattoir with the exception of age. The older the animal at time of slaughter, the higher the *in vitro* dry matter digestibility. Although in Figure 5-1 there appears to be one animal that may be causing this significant effect (age 750 days, IVDMD 0.3953), when removed from the data set, age was still found to significantly correlate with IVDMD. Age has been explored as a factor which may affect fibre digestion and feed retention time in dairy cows, peaking at around 4-6 years of age (Grandl et al., 2016; Grandl et al., 2017), but these studies have been over very large time frames for example up to 10 years of age. The age range of the animals in this study, however, was very narrow with 25 days difference between the oldest and youngest with the exception of the outlying animal which was 94 days older than the youngest steer so it is very unlikely that age is having an effect. The animals used were all raised on three different pasture types: permanent pasture, high sugar grasses and high clover. Animals that were provided with high sugar grasses appeared to show more variation between age and their ability to digest dry matter within the model when compared with those from the permanent pasture and the pastures containing a high proportion of clover. It may therefore be the effect of these animals that is causing the significant correlation between age and IVDMD.

Despite having a microbial community adapted to a high fibre diet, there was also found to be no correlation between daily live weight gain of the animals and their ability to digest dry matter *in vitro* although this may have been impacted by the quality of the forages provided to the animals. A measure such as residual feed intake (RFI) may have been more appropriate as this would allow comparison of the animal's efficiency

independent of the animal's size and growth rate (Herd and Arthur, 2009). Feed intake was not measured in these animals. In addition, despite providing a high fibre, grass-based substrate, the diet of the animal was still different to that provided *in vitro.* The difference between the two feeds may have caused significant shifts in the microbial population and masked differences in performance that may have been seen *in vivo*. It is recommended that to compare the rumen fermentation performance of animals using an *in vitro* model that the model use as substrate the same feed as fed to the animals at the time of rumen fluid collection.

5.4.6 Conclusions

The aim of this chapter was to identify rumen fluids from cattle of the same breed and sex raised in a common environment, i.e. same feed, same management, with different *in vitro* performances when provided with a forage substrate (dried grass). Differences in *in vitro* performance were assumed to be due to different rumen microbiotas, which were assumed to be largely due to animal factors rather than environmental factors which were shared by the animals. Animal factors have been implicated in the unsuccessful attempts to manipulate the rumen microbial community, therefore the aim of this chapter was to determine whether it was possible to manipulate the rumen microbiota *in vitro* where animal factor(s) are essentially absent.

It has been shown that even when using rumen fluid from animals that were genetically similar, raised form birth on the same farm and fed a diet similar to that provided to the *in vitro* model, cross inoculation of rumen fluids with significantly different abilities to digest dried grass DM (IVDMD) did not improve the performance of the less good rumen fluid to that of the better rumen fluid or affect fermentation parameters measured. The bacterial community of the Good and the Bad fluid at the start of the experiment was found to be very similar suggesting that it may be what the two communities are doing, and not necessarily who is there, that determines their ability to digest dry matter *in vitro.* Alternatively, other members of the rumen community (i.e. fungi, protozoa, archaea and bacteriophages) may be responsible for the differences in fermentative digestion observed. Across both cross-inoculation experiments, Tenericutes was identified as a bacterial phylum that appears to flourish in times of perturbation. The relative abundance of this phyla could be used as a biomarker for dysbiosis in the rumen with further investigation.

Overall, when the controlling effect(s) of the host animal were removed through the use of an *in vitro* model, cross inoculation was found to have no effect on bacterial community structure and simply reflected the two communities mixed. Subsequent changes to community structure were due to time as the microbial community adapted to the substrate and environment provided within the *in vitro* model as was also seen for the unmixed communities. This suggests that as well as the effect of the host animal, there is a resilience to change within the community itself that prevents manipulation of the mature rumen community.

No favourable effect of cross inoculation was observed for IVDMD, or fermentation parameters, with the cross inoculated fluid performing most like the Bad across the experimental period. This suggests that cross inoculation of rumen fluid in the mature animal does not appear to be an effective method to manipulate the microbial community to improve productivity and efficiency in the animal, supporting *in vivo* studies.

Chapter 6 The role of the grass associated bacterial community in the fermentative digestion of substrate in an *in vitro* model of the rumen

6.1 Introduction

The *in vitro* model of rumen fermentation has been used for decades to examine the fermentative parameters of feeds prior to feeding to animals e.g. Woodman and Evans (1938), Hino et al. (1993) and Capelari and Powers (2017). The small scale of the *in vitro* model allows multiple feeds to be tested in parallel in a controlled laboratory environment, reducing the number of animals required for trials and reducing the cost associated with animal experiments (Lengowski et al., 2016). As described in the General Introduction (Chapter 1.2.3.4), there are multiple types of *in vitro* model and it is the batch *in vitro* model that will be the focus of this Chapter.

The *in vitro* model allows controlled study of the fermentative digestion of feedstuff such as forage and can also be used to examine the effects of different substrates and supplements on the microbial ecosystem. As part of this thesis, the *in vitro* batch model of rumen fermentation was used to study the performance of rumen inoculate sourced from different animals. There is a growing interest in understanding why one animal can outperform another when raised on the same feed, on the same farm and a correlation between the microbial community and an animal's residual feed intake has been previously observed (Guan et al., 2008; Carberry et al., 2012). Therefore, there is scope to culture these complex ecosystems within the *in vitro* model to determine the mechanisms by which one community better utilises the substrate than another. The composition of the inoculum used for *in vitro* experiments has been indicated to affect the extent of digestion of substrate due to the population of microorganisms present (Muetzel et al., 2001). However, it is important to understand how the model itself affects both fermentation and the microbial population present in order to elicit the differences between animals. If the *in vitro* model is causing a shift in microbial population and fermentation parameters observed then its suitability for this kind of study may be questioned.

Alongside the microbial ecosystem introduced into the *in vitro* model through rumen inoculum, there is a large, complex microbial community associated with the plant phyllosphere (Lindow and Brandl, 2003; Berlec, 2012) despite the somewhat hostile environment associated with the above ground surface of the plant e.g. exposure to sunlight and limited nutrient and water availability (Lindow and Leveau, 2002). As the plant tissue is not sterilised prior to use as a substrate in the *in vitro* model, it is important to determine the potential contribution of these microorganisms to fermentation. Due to the close association of the epiphytic community to the plant substrate, it is possible that these microorganisms could compete with rumen species for binding sites and play a larger role in fermentation than is currently thought.

Due to the between animal differences seen in a rumen fluid's ability to ferment forage *in vitro* (Chapter 4 and 5) it is important to determine whether the difference in performance is due to the microbial community present or a carryover effect from the host animal. Rumen inoculum contains a diverse array of enzymes, many of which are involved in the degradation of polymers in the plant cell wall e.g. cellulases, xylanases, ß-glucanases and pectinases (Wang and McAllister, 2002).

Rumen inoculum is also rich in metabolites. A metabolomics study revealed the composition of rumen inoculum to contain phospholipids, inorganic ions and gases, amino acids, dicarboxylic acids, VFA, diglycerides, triglycerides, carbohydrate and cholesterol esters (Saleem et al., 2013). The study revealed a total of 248 metabolites within the rumen inoculum with variation across the 8 animals studied. Not all of the metabolites were present in all of the animals despite feeding the same diet, therefore variation in rumen fluid composition may influence fermentative digestion within the model, but to a lesser extent than the microbial community.

Therefore, the overall aim of this chapter was to test the *in vitro* model in order to gain a deeper understanding of its workings, allowing a greater assurance in the results obtained from experiments using this platform. This chapter aimed to determine the contribution of the grass associated “epiphytic” community to fermentation within a batch culture *in vitro* model of rumen fermentation and the epiphytic bacterial community was identified via 16S rRNA sequencing. The effect of the *in vitro* model on the bacterial composition is explored further in Chapter 7. It was hypothesised that the epiphytic bacterial community would be able to ferment the substrate due to its close association, however, this would not be to the same extent as when rumen fluid was included. The grass

associated bacterial community was also expected to be distinct from that of the rumen bacterial community.

6.2 Experiment 1

The aim of this experiment was to determine the contribution of the substrate associated microbial community ("epiphytic" bacteria) to an *in vitro* batch culture model of rumen fermentation over a 24 hour period.

6.2.1 Methods

A sample of dried grass substrate (*ca* 200 g) was milled to pass through a 1 mm sieve, separated into 3 subsamples to allow repeated experimentation and sent to the Dalton Cumbrian facility (University of Manchester, UK) for sterilisation using cobalt-60 irradiation at a minimum dosage of 50 kGy to a maximum of 65 kGy. All glassware and additional equipment used in the preparation of the *in vitro* model was autoclaved prior to use. Mould's buffer (2.5 L) was prepared as described in the General Methods (2.1.4) and autoclaved prior to use. To prevent any cross-contamination when weighing out substrate, the sterilised grass was prepared first followed by the un-sterilised feed. All surfaces were wiped down with 70 % ethanol and dried prior to use.

Rumen fluid was sterilised by vacuum filtering through a 0.22µm pore size filter cup (Millipore, UK). The filtrate was then used as inoculum for fermentations. To ensure that the filtrate was sterilised, filtered rumen fluid and standard, un-sterilised rumen fluid (150 µl each) was plated on to Lysogeny Broth (LB) agar and incubated at 39°C for 7 days.

A total of 42 fermentation bottles were prepared with 6 bottles per treatment. The six treatments used were as follows: sterilised grass plus buffer (SGB), grass plus buffer (GB), filtered rumen fluid, sterilised grass plus buffer (FRSGB), filtered rumen fluid, grass plus buffer (FRGB), rumen fluid, sterilised grass plus buffer (RSGB) and rumen fluid, grass and buffer (RGB). Three bottles from each treatment were used for IVDMD analysis and the remaining three were used to collect samples for VFA, MCP and NH_3-N analysis. Due to a limited amount of filtered rumen fluid, one bottle was removed from each of the treatments containing this prior to beginning the experiment (FRSGB, FRGB and FRB). Of the five bottles, three were used for IVDMD and two were used for sample collection. An additional six no-substrate blank bottles were also included (rumen fluid plus buffer (RB), filtered rumen fluid plus buffer (FRB)). Fermentation bottles that did

not contain substrate (RB, FRB) did not undergo IVDMD analysis. The bottles were used only for sample collection and gas production was used as a blank measure for the two rumen fluids. When setting up the fermentation, bottles that did not contain inoculum were prepared first, followed by those containing filtered rumen fluid and finally those with the full inoculum to prevent any cross-contamination. Fermentation was performed for 24 hours.

All statistical analysis was performed in IBM SPSS Statistics 21. Data was tested for normality (Kolmogorov-Smirnoff test) and homogeneity of variance (Levene's test) prior to further statistical analysis. Differences were considered significant if $p < 0.05$. Data were expressed as means with pooled SEM. A one-way ANOVA was performed with Tukey's *post-hoc* test when data was normally distributed, otherwise a Kruskall-Wallis test was performed. When two groups were compared, a t-test was used.

6.2.2 Results and Discussion

To confirm that rumen fluid was sterilised, filtered rumen fluid was plated to identify any microbial growth. Standard rumen fluid was also plated as a control. At the end of the seven day period, there was no growth on the plate seeded with filtered rumen fluid indicating that the sterilisation process had been successful. When checked at 24 hours, a full lawn of microbial growth was seen on plates seeded with neat un-filtered rumen fluid. The use of 0.22 µm pore size filter to sterilise liquids has been reported previously (Bobbitt and Betts, 1992; Fareez et al., 2015; Chiara et al., 2016) and was found to be sufficient to sterilise microbial rich rumen fluid in this case.

Over a 24 hour fermentation, *in vitro* dry matter digestibility (IVDMD) was shown to differ significantly between the treatments ($F_{5, 17} = 270.4$, $p < 0.001$; Figure 6-1). Bottles containing sterilised dried grass were shown to have significantly greater IVDMD than bottles containing standard dried grass (31 *vs* 29 g/100g DM; $t = 12.460$, $df = 4$, $p < 0.001$). This suggests that the epiphytic bacterial community does not play a significant role in *in vitro* rumen fermentation as, in the absence of this community, apparent IVDMD was found to be significantly greater.

As the sterilisation process removed the microbial community associated with the dried grass substrate, it follows that the higher IVDMD associated with the sterilised grass was due to an increased solubility of the dried grass substrate and not microbial digestion. In order to fully remove all microbial life on the surface of the substrate it was subjected to

a high dose of gamma irradiation (minimum of 50 kGy) to ensure absolute sterility (da Silva Aquino, 2012). Gamma irradiation was used instead of heat (e.g. autoclaving) or chemical treatment in order to limit changes to the nutrient composition of the substrate. Irradiation has been shown to effect pectin in the cell wall by increasing the activity of polygalacturonase and pectin methyl esterase (Kovacs and Keresztes, 2002). While pectin concentration is low in grasses (*ca* 2-10%), it is present in the middle lamella which is responsible for adhesion of neighbouring cells (Latarullo et al., 2016). So, by increasing the activity of pectin degrading enzymes the link between cells will be weakened likely making the grass more soluble. Pectins are also thought to be responsible for determining the porosity of the cell wall (Baron-Epel et al., 1988) and in doing so control the size of enzymes that can penetrate the cell (Buckeridge et al., 2016). The sterilisation process may therefore increase the digestibility of the grass substrate by weakening links between neighbouring cells and by increasing the porosity of the cell wall allowing more enzymatic degradation to occur.

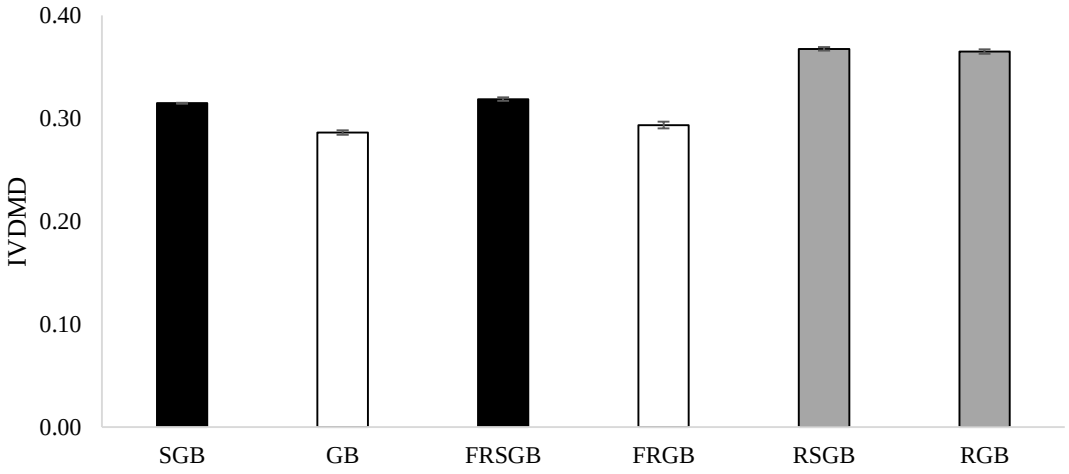

Figure 6-1 Sterilisation of the fermentation substrate and rumen inoculum significantly reduces *in vitro* dry matter digestibility after 24 hours of fermentation. Different colours are used to show significant differences between the treatments. All differences are less than or equal to $p < 0.01$. Error bars show SE. SGB sterilised grass plus buffer, GB grass plus buffer, FRSGB filtered rumen fluid, sterilised grass plus buffer, FRGB filtered rumen fluid, grass plus buffer, RSGB rumen fluid, sterilised grass plus buffer, RGB rumen fluid, grass plus buffer

In the absence of rumen inoculum (SGB, GB), *ca* 30 g/100 g DM was apparently digested in the model following 24 hours of fermentation. The solubility of the dried grass and the sterilised grass was identified by measuring IVDMD at zero hours (6.3.2 Experiment

2 below). It was found that both types of dried grass substrate showed solubilisation upon the addition of buffer at zero hours (41.4 and 40.4 g/100g for sterilised and non-sterilised grass respectively).

The higher values shown for the zero hour sampling in Experiment 2 and the values shown at 24 hours here are most likely due to the method of sampling used. Due to the number of bottles in Experiment 2, IVDMD was performed on the same bottles that samples for NH_3-N, VFA and MCP were collected from. As some of the dried grass substrate was removed during sampling, this increased the IVDMD value obtained. In the literature, IVDMD is usually performed on the same bottles from which samples have also been taken (Meale et al., 2012; Medjekal et al., 2017; Anele et al., 2016; Toral et al., 2016; Pisarcikova et al., 2016), but studies do also use a separate set of fermentation bottles (Tekippe et al., 2012; Molina-Alcaide et al., 2017). As the number of bottles in experiments increases, removing samples from the same bottles as those used for digestibility may be unavoidable. Therefore, it is important to note in the methodology which sampling technique was used, as this may influence results obtained.

When filtered rumen fluid (FRGB) was added to the fermentation, there was no significant difference in IVDMD between these bottles and the fermentation bottles containing only grass and buffer (GB; 31 vs 30 g/ 100g DM for FRGB and GB respectively; $t = -0.65$, $df = 10$, $p = 0.531$) confirming that the filter sterilisation process is suitable to remove all microorganisms from the rumen inoculum, and more importantly confirming that free enzymes and metabolites associated with rumen fluid had an insignificant effect on IVDMD. When comparing the performance of bottles containing filtered rumen fluid with either standard or sterilised dried grass, again, the sterilised grass bottles showed significantly higher IVDMD than their non-sterilised equivalents (0.32 vs 0.29; $t = 6.917$, $df = 4$, $p < 0.01$).

Bottles containing standard, un-filtered rumen inoculum (RSGB, RGB) showed increased digestibility of the substrate when compared to all other bottles (0.37 vs 0.30; $t = 10.05$, $df = 13.2$, $p < 0.001$). In this case, there was no significant effect of sterilising the grass (0.37 vs 0.36; $t = 0.919$, $df = 4$, $p = 0.410$).

Although no difference in IVDMD was observed when filtered rumen fluid (FRGB) was compared with bottles containing only grass and buffer (GB), there was an increase in gas production when filtered rumen fluid was included in the fermentation (84.6 *vs* 50.8 ml/g DM for FRGB and GB respectively; Fig 6.2) indicative of increased fermentative

digestion, although this was not found to be significant. The additional soluble nutrients present in the filtered rumen fluid were presumably the cause of higher gas production by the epiphytic bacteria as the 24 hour incubation showed they appear to ferment the soluble nutrients rather than any of the non-soluble DM. As mentioned in the introduction to this chapter, rumen fluid is a rich source of soluble nutrients and enzymes, which probably provide the grass associated microorganisms extra substrate for growth. As well as the microorganisms digesting the soluble fraction of the plant substrate, the additional dry matter carried over in the rumen fluid was not accounted for in the dry matter content of the bottle which may explain why the digestibility of the two treatments showed no significant difference. There was a significant difference between all the treatments in terms of gas production (KW = 31.06, N =34, $p < 0.001$) which can be seen in Figure 6-2 below.

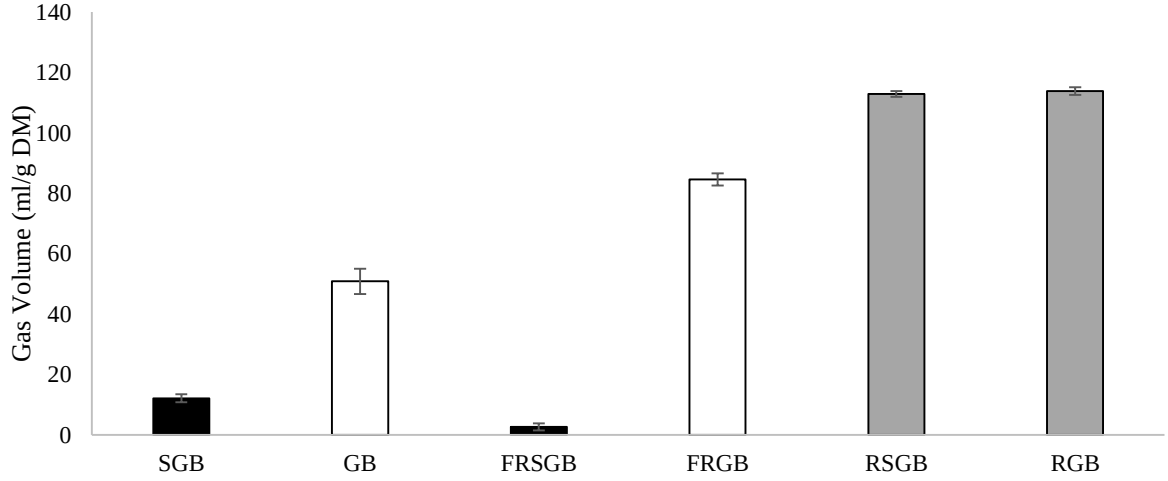

Figure 6-2 Total gas production (ml / g DM) from sterilised substrate and rumen inoculum and their non-sterilised counterparts. Different colours represent significant differences between the treatments. Error bars show SE. SGB sterilised grass plus buffer, GB grass plus buffer, FRSGB filtered rumen fluid, sterilised grass plus buffer, FRGB filtered rumen fluid, grass plus buffer, RSGB rumen fluid, sterilised grass plus buffer, RGB rumen fluid, grass plus buffer

Overall, it was shown that the epiphytic microbial community associated with the dried grass substrate was capable of digesting the substrate (GB) and this was increased in the presence of filtered rumen fluid (FRGB) as demonstrated by the increased gas production indicative of increased fermentation. The DM within the filtered rumen inoculum

provided an additional source of substrate for the epiphytic community to ferment. The sterilised grass was found to be more soluble than the un-sterilised grass due to the use of gamma irradiation and the solubility of the grass was shown to be fairly high (*ca* 30 %) which may affect the outcome of short fermentations and should be explored further.

6.3 Experiment 2

The aim of this experiment was to determine the effect of the epiphytic bacterial community over an extended *in vitro* batch culture fermentation (six days; 144 hours).

6.3.1 Methods

The four treatments used were as follows: sterilised grass plus buffer (SGB), grass plus buffer (GB), rumen fluid, sterilised grass plus buffer (RFSGB) and rumen fluid, grass plus buffer (RGB). All glassware and equipment was sterilised prior to use. A total of 96 bottles were prepared with 3 bottles removed for each treatment at 0, 12, 24, 36, 48, 72, 96, 120 and 144 hours of fermentation. Samples for VFA, MCP and NH_3-N were removed from each bottle prior to IVDMD analysis. Due to a limited amount of sterilised grass, bottles were not included at 144 hours for SGB and 120 and 144 hours for RSGB. The experiment was run for 144 hours (six days) to match the longest fermentation within this thesis (Chapter 7).

All statistical analysis was performed in IBM SPSS Statistics 21. Data was tested for normality (Kolmogorov-Smirnoff test) and homogeneity of variance (Levene's test) prior to further statistical analysis. A general linear model with Treatment and Time as fixed factors was performed. If non-significant, interactions were removed from the model and main effects were analysed separately. Tukey's *post-hoc* test was used to analyse significant differences within a treatment or time point. A two-way repeated measures ANOVA was used to analyse the gas volume data. Differences were considered significant if $p < 0.05$. Data are expressed as means with pooled SEM.

6.3.2 Results and Discussion

Experiment 1 revealed that the epiphytic bacterial community was capable of digesting the dried grass substrate following 24 hours of fermentation. As experiments contained within this thesis were performed for a range of time periods (24 -144 hours), it was necessary to determine the contribution of the epiphytic microbial community to fermentation across this time frame. The results from Experiment 1 also highlighted that

the solubility of the substrate may mask the actual dry matter digestion performed by the microbial population and therefore a shorter fermentation (12 hours) was also included.

There was found to be a significant interaction between Treatment and Time for IVDMD ($F_{18, 85}$ = 70.575, p < 0.001; Figure 6-3) where bottles containing rumen fluid (RSGB, RGB) showed increased DM digestibility with time. The GB treatment showed increased IVDMD after 72 hours. Between zero and 12 hours, more of the sterilised grass substrate leeched into the buffer, increasing the IVDMD of the SGB by *ca* 5% (not significant; p = 0.244), thereafter IVDMD for this treatment remained stable for the remainder of the experiment. No further leeching or fermentation took place after 12 hours.

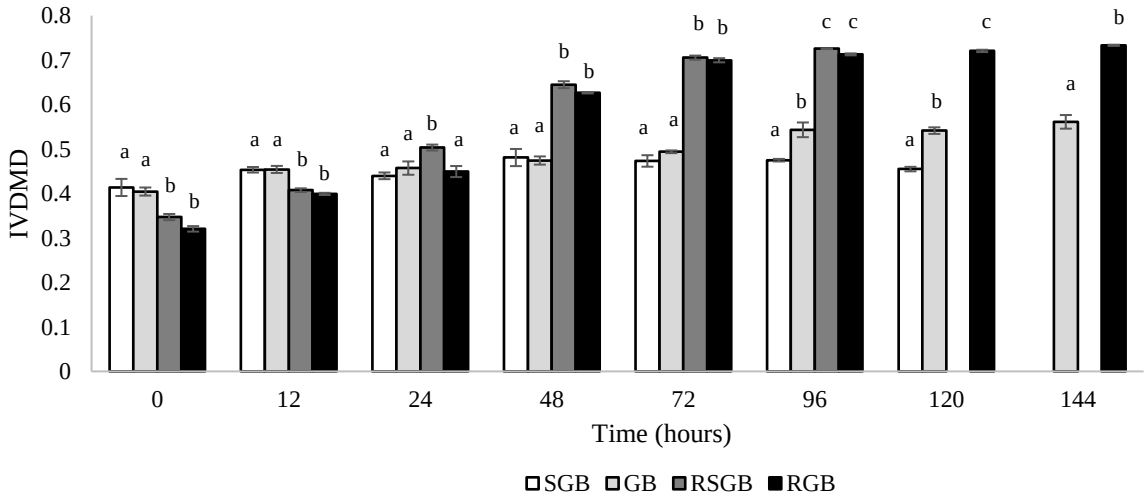

Figure 6-3 *In vitro* dry matter digestibility of samples with and without rumen inoculum with either sterilised or non-sterilised grass as a substrate over 144 hours of incubation. SGB = sterilised grass plus buffer, GB = grass plus buffer, RSGB = rumen fluid, sterilised grass plus buffer, RGB = rumen fluid, grass plus buffer. Error bars show SE. Different superscript letters within a time point show *post-hoc* differences between the four groups p < 0.05.

It is clear from Figure 6-3 that 24 hours of fermentation or less is not sufficient to determine a statistical difference between bottles either containing rumen inoculum or not. Sampling should therefore take place after 24 hours. When using dried grass as a substrate, the epiphytic community was able to ferment the substrate after 72 hours of fermentation. For the dried grass substrate used in this thesis, maximum digestibility was reached after 96 hours at *ca* 72 g/ 100g DM.

As with Experiment 1 above, due to the soluble nutrient fraction, there was a relatively high digestibility value of *ca* 30-40 g/100g DM at zero hours despite the fact that no fermentation, and therefore digestion, had taken place. The DM value at zero hours

represents the soluble fraction of the dried grass substrate. In a study by Chaudry and Mohammed (2012) grass nuts were shown to have an initial digestibility (i.e. solubility) of 280g/kg supporting the findings shown here. The IVDMD value was also shown to be higher at zero hours in bottles that did not contain rumen fluid (0.42 vs 0.33; $t = 6.31$, $df = 10$, $p < 0.001$). This difference is likely an artefact of the DM contained within the inoculum.

There was also a significant interaction between Treatment and Time for gas production ($F= 660.94$, $df = 1$, $p < 0.001$ – 2 way repeated measures ANOVA). After 96 hours of fermentation, the gas production plateaus (Figure 6-4) indicating that no more fermentation is occurring beyond this point. There was found to be an increased lag time for the GB treatment when compared with bottles containing rumen inoculum (RGB, RSGB), which is probably a result of a smaller starting microbial inoculum. As gas production is indicative of fermentation, and therefore digestibility, it would be expected that an increase in gas production would result in an increase in IVDMD. For the GB treatment, gas production began at 12 hours, but it was not until 72 hours that an increase in IVDMD was observed. This fermentation may therefore be indicative of the microbial population digesting the soluble fraction that had leeched from the substrate. The SGB treatment showed negligible gas production as was expected.

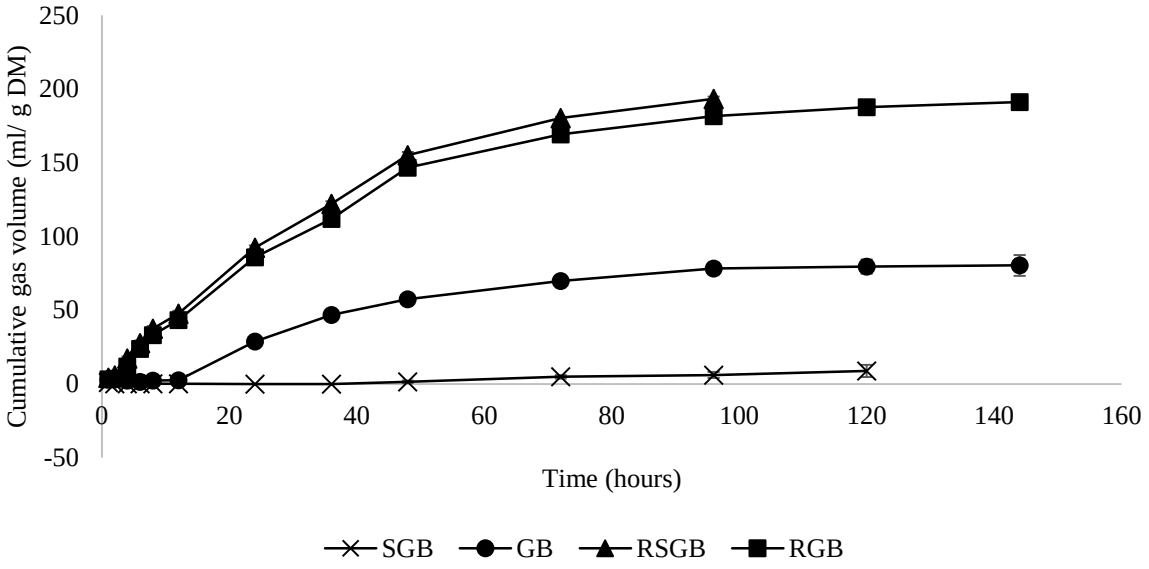

Figure 6-4 Gas production profile for bottles containing either sterilised or non-sterilised grass with or without rumen inoculum fermented over 6 days SGB; cross = sterilised grass plus buffer, GB; circles = grass plus buffer, RSGB; triangle = rumen fluid, sterilised grass plus buffer, RGB; square = rumen fluid, grass plus buffer. Error bars show SE. All values are corrected per g DM added to the bottles.

Analysis of fermentation parameters was undertaken to compare the bottles containing rumen fluid (RSGB, RGB) to those without rumen inoculum (SGB, GB). Ammonia-nitrogen, microbial crude protein and total volatile fatty acid analysis can be seen in Table 6-1. The sterilised grass plus buffer treatment showed a consistent level of ammonia nitrogen (*ca* 800 µg/ml) representing the ammonia present in the buffer, no microbial protein and no volatile fatty acid production confirming, along with the gas production above, that no fermentation was taking place and therefore the substrate was suitably sterilised. The consistent concentration of ammonia was as expected as there was no microbial community present to utilise the ammonia to synthesise microbial amino acids (Hackmann and Firkins, 2015). When un-sterilised grass was used (GB) there was found to be an increase in both NH_3-N concentration and VFA production after 12 hours of fermentation which coincides with the gas production profiles (Figure 6-4). As IVDMD showed no increase until 72 hours, this may be due to fermentation of the soluble fraction of the grass substrate. However, no MCP was detected in the GB treatment throughout the fermentation (Table 6-1). This may be because the technique used to measure MCP was not sensitive enough at the lower end of microbial concentrations. The author noted

that with early time point samples, where the microbial concentration was low, either no microbial pellet or only a small pellet was formed after centrifugation, and the small pellet was easily re-suspended and removed with the supernatant. As the first step of the assay was to remove feed particles, the bacteria present may have been attached to the substrate. The Lowry assay, on which the MCP protocol was based, is sensitive down to 0.01 mg/ml of protein (Walker, 1996).

The process of sterilising the grass had little to no effect on the fermentation parameters measured when rumen fluid was also added to the fermentation bottles.

Figure 6-5 shows the molar proportions of the three VFAs that were measured (acetate, propionate and butyrate). Interestingly, the GB treatment produced a different VFA profile to the bottles containing rumen fluid, with no propionate production throughout the entire fermentation. No butyrate was produced at the first time point where VFA was detected (12 hours) for any of the samples.

Table 6-1 Fermentation parameters following a single batch *in vitro* fermentation over a 144 hour period for bottles containing either a standard or sterilised dried grass substrate, with or without rumen inoculum All results are corrected for dry matter added to each of the bottles

	Time (hours)									p value		
	0	**12**	**24**	**48**	**72**	**96**	**120[1]**	**144[1]**	**SEM**	**Time**	**Treatment**	**Time*Treatment**
Ammonia - N (µg/ml)												
SGB	835.1	787.3	778.1	781.7	816.8	798.1	850.1					
GB	775.3	775.1	904.5	910.6	1035.6	980.0	1007.5	1238.8	35.75	**<0.001**	**<0.001**	**<0.001**
RSGB	945.1	979.1	813.6	920.9	969.1	1078.4						
RGB	912.7	894.2	801.2	824.4	974.1	1087.1	1116.8	1122.4				
Microbial Crude Protein (µg/ml)												
SGB	0.0	0.0	0.0	0.0	0.0	0.0	0.0					
GB	0.0	0.0	0.0	12.8	0.0	0.0	0.0	0.0	32.27	**<0.001**	**< 0.001**	**< 0.001**
RSGB	459.4	217.4	305.3	706.1	607.0	579.5						
RGB	322.5	187.1	363.0	401.1	487.6	495.9	536.7	334.6				
Total VFA (mM)												
SGB	0.00	0.00	0.00	0.00	0.00	0.00	0.00					
GB	0.00	0.00	13.47	21.88	29.87	31.64	33.28	41.71	2.12	**< 0.001**	**< 0.001**	**< 0.001**
RSGB	0.00	15.85	57.27	92.87	102.55	110.41						
RGB	0.00	22.16	53.44	83.58	98.12	110.25	113.23	115.07				

Where SEM = standard error of the mean, SGB = sterilised grass plus buffer, GB = grass plus buffer, RSGB = rumen fluid, sterilised grass plus buffer, RGB = rumen fluid, grass plus buffer

[1]Missing values at 120 hours (RSGB) and 144 hours (SGB, RSGB) were due to a lack of sterilised grass substrate, bottles were removed prior to fermentation

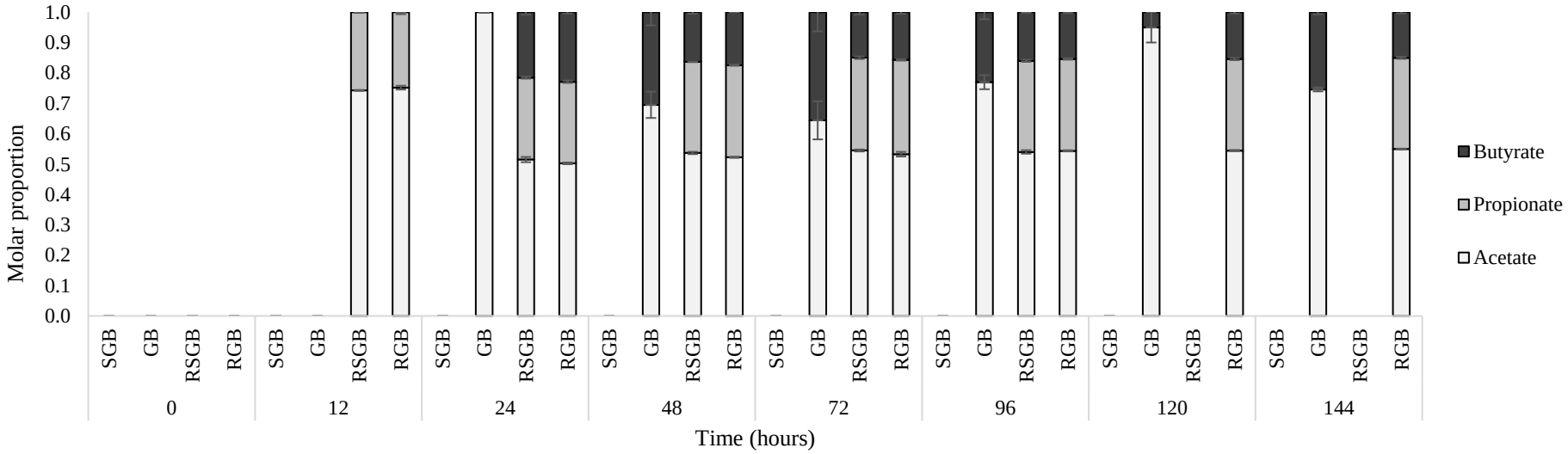

Figure 6-5 The molar proportion of acetate, propionate and butyrate produced across a single 144 hour batch *in vitro* fermentation Error bars show SE. No VFAs were produced at 0 hours for all samples, at 12 hours for GB, and throughout the experiment for SGB. Missing bars at 120 hours (RSGB) and 144 hours (SGB, RSGB) were due to there being no samples for these time points.

Over the course of the experimental period, bottles containing only grass and buffer did not produce any propionate. Acetate was the major VFA produced across all groups (except for sterilised grass plus buffer for which no VFAs were produced). The lack of propionate production in the treatments that did not contain rumen fluid (SGB, GB) could be due to the microbial community present. As the community on the surface of the grass was much less diverse than that found in the rumen (see Appendix B) it may simply be that the species present were unable to convert the dried grass substrate to propionate. Alternatively, any propionate produced may have been used as a substrate by another bacterial species. *Xanthomonas* species for example, can use propionate to synthesise even-numbered volatiles (Weise et al., 2012), in fact all Proteobacteria (which comprised 60% of the sequencing reads associated with the dried grass; Figure 6-6) utilise propionate as a single carbon source for metabolism (Suvorova et al., 2012). Bacteroidetes was the second most abundant phyla present on the surface of the dried grass (24%), which is thought to be the main phyla that produces both acetate and propionate (Chakraborti, 2015). Therefore, the microbial species present on the surface of the dried grass theoretically were capable of propionate production.

Overall, this experiment confirmed that when dried grass was used as a substrate, fermentations must be performed for longer than 24 hours in order to allow differences to be observed between fermentation bottles containing rumen fluid (RSGB, RGB) and those where the substrate was incubated in buffer only (SGB, GB). It took 72 hours of *in vitro* fermentation for the epiphytic community to digest the in-soluble fraction of the substrate and no propionate was detected in fermentations resulting from bottles containing grass and buffer only. Sterilisation of the substrate was shown to have no significant effect on digestibility when rumen fluid was included in the fermentation bottle.

6.4 Experiment 3

The aim of this experiment was to identify the epiphytic bacterial community associated with the dried grass substrate.

6.4.1 Methods

To extract DNA from the grass substrate, a subsample of the dried grass was added into a volume of water and swirled. Then, a 1.5 ml aliquot was removed and the method of bacterial isolation was performed as previously described (Chapter 2.3.1). Sequencing

was performed as previously described (Chapter 2.3.2) alongside the samples from Chapter 4.

6.4.2 Results and Discussion

As the microbial community associated with the feedstuff is capable of digesting the insoluble fraction of the substrate given a long enough time (*ca* 72 hours), 16S rRNA sequencing was used to identify the bacterial community on the surface of the dried grass substrate. A total of 405,862 reads were initially obtained for the epiphytic bacterial community and a total of 8,654 unique, high-quality sequences remained after all processing steps. The grass associated bacterial community identified were all associated with soil and/or plants. The predominant phyla were identified as Proteobacteria (60.8 %), Bacteroidetes (24.2 %), Actinobacteria (7.5 %) and Firmicutes (5.8 %). Other phyla identified were at a relative abundance of < 1%. When considering the bacterial community at the genus level, *Xanthamonas* had the greatest relative abundance (33.6 %) and dominated the grass bacterial community (Figure 6-6).

The grass associated bacterial community was also compared to profiles at the end of an *in vitro* fermentation (samples presented in Chapter 5.3.4). This revealed that the grass associated bacteria did not persist in the *in vitro* model of rumen fermentation and did not appear to be responsible for fermentation seen in the model. The bacterial community associated with the substrate, despite being able to digest the substrate, were not identified in the bacterial 16S rRNA profiles from fermentation bottles containing rumen fluid. It is likely that the rumen bacterial community quickly colonised the substrate and outcompeted the native bacterial community on the surface of the dried grass. This is in agreement with Belanche et al. (2017) who found a rapid colonisation of rumen microorganisms on both grass and hay substrates (< 2 hours) replacing the OTUs identified at zero hours from the grass associated community.

In the absence of rumen fluid, the epiphytic community were able to ferment the grass substrate, but at a slower rate than when the rumen fluid was included which may be due to a combination of lower microbial number and the need for the bacterial cells to re-hydrate before fermentation could begin.

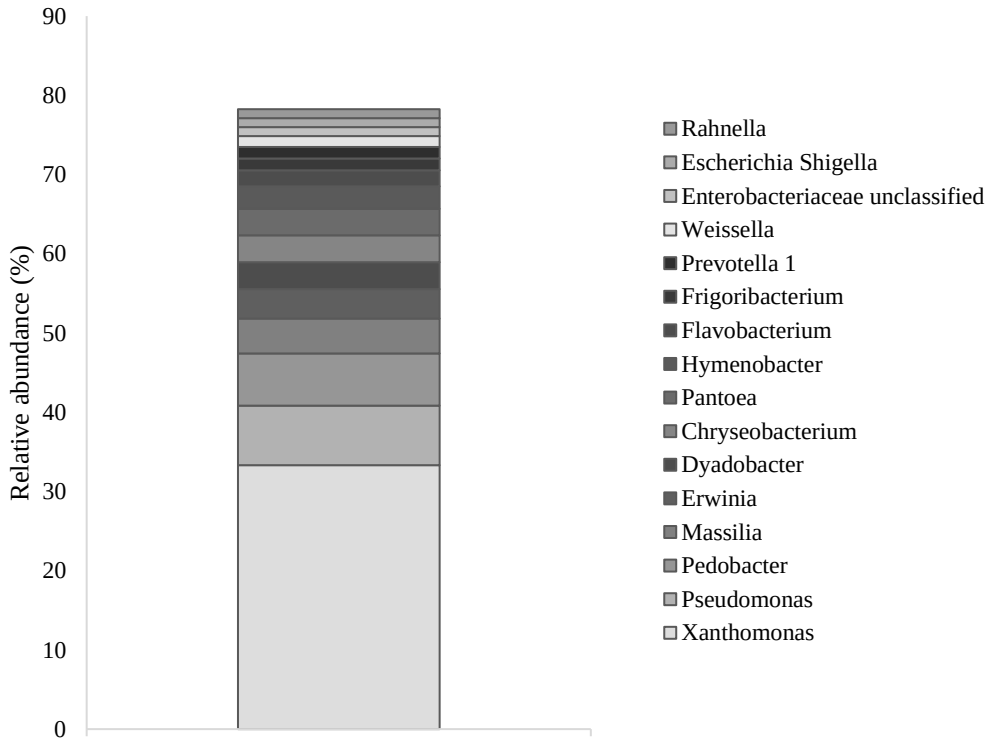

Figure 6-6 The relative abundance (> 1%) of the genera associated with the grass substrate The different shades represent different genera

The grass associated bacteria identified were all found to be associated with plant/soil communities. *Xanthomonas*, for example, which was the most common genera associated with the grass substrate, is a common plant pathogen (Ryan et al., 2011) and is used in the production of Xantham gum (García-Ochoa et al., 2000). The grass associated bacterial community that were identified are capable of digesting the feedstuff. Flavobacterium, for example, has been shown to have genes encoding the glycoside hydrolase families GH78 and GH106, which are involved in the degradation of hemicellulose (Kolton et al., 2013). The relative abundance of all genera and phyla of the grass associated bacteria can be seen in Appendix C alongside the bacterial communities from rumen fermentation samples.

Some of the bacterial genera isolated from the grass substrate have been identified as common DNA/PCR kit contaminants that are usually identified in samples with low DNA quantity (Salter et al., 2014). Bacterial contaminants are introduced through buffers

in both DNA extraction (Mohammadi et al., 2005) and PCR master mixes (Grahn et al., 2003). The concentration of DNA extracted from the grass sample was similar to DNA concentration from samples containing rumen fluid (32.1 ng/ml *vs* 39.3 ± 6.4 ng/µl for dried grass and rumen samples respectively; from Chapter 5). After PCR amplification and purification, the concentration of purified PCR amplicons was lower for the epiphytic community than the experimental rumen samples although the bacterial community was amplified with the same conditions as the rumen inoculum (55.8 *vs* 153.2 ± 22.6 ng/µl) suggesting that after selecting for bacterial DNA through universal bacterial primers, a large proportion of the DNA originally extracted was plant in origin, resulting in a smaller bacterial target for PCR. Negative controls from DNA extraction were PCR amplified and no band was present on the gel, however, it will be imperative in future sequencing work to include a negative control at the sequencing stage to remove any sequences that are associated with contaminants from the analysis when low DNA quantity is analysed. Contaminating amplicons do not appear to be an issue in DNA rich environments such as faecal samples (Salter et al., 2014), therefore, contamination should not be an issue in the experimental samples presented elsewhere in this thesis.

6.5 Conclusion

This chapter has shown that the epiphytic microbial community associated with the dried grass was capable of digesting the substrate within the *in vitro* batch model of rumen fermentation. However, it took more than 72 hours for this community to start digesting the insoluble fraction of the substrate. Digestion by the grass associated community was not to the same extent as when rumen inoculum was included in the fermentation bottles. The bacterial population associated with the substrate was not identified in fermentation bottles containing rumen fluid, suggesting that the substrate is rapidly colonised by rumen species which outcompete the epiphytic community.

Gamma irradiation of the dried grass substrate prevented fermentation, however, it increased the solubility of dried grass, presumably through modification to the plant cell wall. When using dried grass as a substrate, this chapter has shown that 24 hours of fermentation is not long enough to allow differentiation between bottles either containing rumen fluid or not due to a combination of the solubility of the feedstuff and, carry-over of dry matter in the rumen inoculum.

The chapter has also shown that the technique used to sample from the *in vitro* fermentation bottles can affect the sensitivity of the IVDMD assay and therefore the methodology used should be clearly stated in *in vitro* batch culture studies.

6.5.1 Recommendations:

Based on the experimental work presented in this chapter the following recommendations were compiled for future work using the *in vitro* batch culture model of rumen fermentation.

- The substrate used should be examined for its solubility
- In the case of the dried grass used in this thesis, fermentations should be performed for a minimum of 36 – 48 hours to ensure that the solubility of the substrate does not mask potential differences in performance
- Where possible, samples collected from fermentation bottles should be taken from a separate set of bottles to those used for IVDMD analysis to avoid a reduction in the sensitivity of the digestibility assay
- The dry matter of each rumen fluid used as an inoculum should be recorded to allow the dry matter associated with the rumen fluid to be added to the dry matter content of the fermentation bottles
- The bacterial community associated with the substrate does not appear to contribute to fermentation when rumen inoculum is also included, however this should be taken into consideration when different substrates are used.

Chapter 7 The effect of rumen fluid to buffer ratio on fermentation parameters and the stability of the bacterial community

7.1 Introduction

Alongside their role in determining the fermentative digestion of feed, *in vitro* models of rumen fermentation have also been used to examine the effect of dietary manipulations and treatments on the residing microbial community (Vargas et al., 2017; Oh et al., 2017; Kim et al., 2018). Rumen fluid used to inoculate the *in vitro* model contains a rich microbial ecosystem consisting of bacteria, fungi, protozoa, archaea and viruses which enable the host animal to ferment cellulose rich feedstuff. Despite being used for decades, knowledge is limited on how the microbial community establishes and changes over the course of the incubation process especially within the batch culture model.

Mateos et al. (2015) compared the bacterial diversity in the rumen of sheep and in a batch culture model of rumen fermentation using automated ribosomal intergenic space analysis (ARISA). The sheep were fed four different diets with forage to concentrate ratios of either 70:30 or 30:70 with either alfalfa hay or grass hay as forage. The similarity index as shown by ARISA ranged from 67.2 to 74.7 % between the bacterial community in the rumen inocula and the community in the batch culture model and showed that diversity was lower in the model. Using real time PCR (qPCR), Weimer et al. (2011) indicated that the *in vitro* conditions within the batch model could substantially change the bacterial population present.

Soto et al. (2013) evaluated the capability of different *in vitro* models to maintain a microbial population like that of the inoculated rumen fluid. Using three different *in vitro* systems (Daisy II ANKOM incubator (DAI), Wheaton bottles (WB), and single flow continuous culture fermenters (CC)), the authors found that total bacterial population, measured by qPCR, declined in both the DAI and WB system after 48 and 72 hours respectively and after four days in CC. The CC system is continuously receiving feed and the buffer is replenished, therefore it is perhaps not surprising that the population size is more stable than in the batch model systems. Alpha diversity (Shannon diversity and Pielou evenness) decreased in DAI (48 hours) and WB (24 hours) when compared with the original rumen inoculum, but no change was seen in CC. Similarly, the bacterial community structure was similar to the original inoculum in CC, but was found to be

different for both DAI (48 hours) and WB (24 hours) when measured using terminal restriction fragment length polymorphisms. With the development of next generation sequencing technologies, there is scope to confirm these findings and to identify which members of the community are changing during an *in vitro* fermentation on a much finer scale (e.g. at the Genus level).

A more recent study by Mateos et al. (2017) examined the establishment of microorganisms associated with both the solid and liquid phase content of a continuous culture fermenter (RUSITEC) when fed either a medium or high concentrate diet. The study showed that the fermentation parameters measured within the model remained fairly stable over the commonly used sampling period of 8 - 14 days, however, microbial populations differed markedly from those in the initial rumen inoculum when measured using qPCR and ARISA. The authors concluded it was difficult to directly compare treatment effects on the microbial population *in vitro* with that *in vivo*. Also in the RUSITEC, Lengowski et al. (2016) determined that the model provided a stable system after an initial 48 hour adaptation period, however, the microbial community continued to adapt across the experimental period as measured by qPCR. Further studies, again in the RUSITEC, determined that there were differences in the microbial community and fermentation parameters between the model and the animal itself, however, the RUSITEC more closely resembled *in vivo* fermentation when high-forage diets were used compared to diets rich in concentrates (Martínez et al.; Martínez et al., 2010b).

In the literature, there is little recommendation for a standard rumen fluid inclusion rate. Pell and Schofield (1993) suggested that a minimum of 20 ml of inoculum should be used per 100 ml of buffered medium to reduce the chances of inoculum limiting gas production. More recently, Yáñez-Ruiz et al. (2016) suggested that a 1:2 ratio of rumen fluid to buffer gave the most reliable results over a 24-hour fermentation, and that the ratio of rumen fluid to buffer should be decreased with increased incubation length and the inclusion of rapidly fermentable substrate. When comparing results between studies, it would be beneficial to have a standardised protocol for batch *in vitro* fermentations. Increasing the proportion of rumen fluid within the fermenters has been shown to increase the rate of gas production (Pell and Schofield, 1993; Rymer et al., 1999) and reduce the lag time prior to gas production (Pell and Schofield, 1993; Rymer et al., 1999). Despite the importance of the microbial community in digestion, little is known about how the concentration of rumen fluid within a fermenter affects the stability of this population.

There is currently no published work describing the effect of rumen fluid to buffer ratio on the microbial community composition and its stability within a batch culture *in vitro* model using next generation sequencing techniques. The three most common rumen fluid to buffer ratios reported for use in batch culture models are 1:2, 1:4 and 1:9, but a wide range have been used (Rymer et al., 2005). Previous work presented in this thesis used a rumen fluid to buffer ratio of 1:9. With this inclusion rate, results presented in Chapter 4 and 5 revealed a sharp decline in alpha diversity and a divergence in the microbial community present within the fermenters over time, suggesting that the bacterial community is not stable and diverges from that of the initial inoculum. Therefore, it is important to determine whether increased concentrations of rumen inoculum result in a more representative microbial community.

As well as understanding the changes in the microbial community within the *in vitro* batch culture model, it is important to understand how the concentration of the microbial population present affects *in vitro* fermentation. If too much rumen fluid is added to the model, the capacity of the buffer may be exceeded resulting in a pH lower than required for fibre digestion (6.0- 7.0) which may limit further fermentation. On the other hand, if too little rumen fluid is added, there may be limited contact between the rumen microorganisms and the substrate again affecting the rate of fermentation. It is unclear at what point dilution of rumen fluid becomes detrimental to fermentation. It could be assumed that an animal that has a lower concentration of microorganisms within the rumen may be less efficient at digesting feedstuff, as there is less contact between the microorganisms and the substrate.

The aim of this chapter was to determine the effect of rumen fluid to buffer ratio on dry matter digestibility, fermentation parameters and the composition and stability of the bacterial community. By examining the effects on the microbial community through next generation sequencing the chapter sought to identify the rumen fluid to buffer ratio that ensured microbial stability within a batch culture model of rumen fermentation. Gas pressure was frequently vented and bottles removed at different fermentation times for sample collection. By diluting rumen inoculum below a 1:9 dilution ratio, this chapter also aimed to determine whether the concentration of the bacterial population was a possible explanation for the poor fibre digesting ability of some ruminant animals as an alternative to differences in the bacterial population. It was hypothesised that a more diluted rumen inoculum would show the same rate of digestion but an increased lag time.

7.2 Materials and Methods

7.2.1 Experiment 1: Increasing the concentration of rumen inoculum above a 1:9 dilution ratio

Rumen fluid was collected at time of slaughter (11 October 2017, Dawn Meats, Hatherleigh) from a Charolais cross steer raised on permanent pasture on the North Wyke Farm Platform (NWFP, Okehampton, Devon, UK). On the day of slaughter a sample of grass (1 kg) was collected from the field that the animal had been grazing prior to slaughter for use as a substrate in the *in vitro* model and immediately frozen at -20°C. Dry matter (DM) was calculated for both the fresh grass and the rumen fluid used as an inoculum. To measure the DM content of the rumen fluid it was freeze dried to constant weight and then transferred to a drying oven (95°C) for *ca* 16 hours. Rumen fluid samples were then transferred to a desiccator to cool to room temperature before weighing.

Within a week of freezing (-80°C), the rumen fluid was used as an inoculum in the *in vitro* model. The experiment was set up as described in the General Methods (Chapter 2.1.5) with the exception that a time course experiment was designed to incorporate the three most commonly used rumen fluid to buffer ratios as described in the literature (1 in 2, 1 in 4 and 1 in 9). Fresh grass was prepared immediately prior to starting the experiment. Whilst frozen, grass was chopped to 2-5 mm lengths and approximately 0.5 g DM was accurately weighed into each fermentation bottle (fresh grass had a DM content of 16.8%). Control bottles containing no inoculum, and only grass (substrate) and buffer were also included to calculate the solubility of the substrate and estimate the contribution of the epiphytic microbial community to fermentation. A no substrate blank (inoculum and buffer) was also included for each treatment at each time point to allow correction for fermentation of organic matter contained within the inoculum. A total of 105 bottles were used with three bottles per treatment per time point.

Digestibility of the substrate was measured at 0, 6, 12, 18, 24, 36 and 48 hours after samples had been collected from the fermentation bottles for MCP, NH_3-N and VFA analysis. A microbial pellet was also collected from each bottle for sequencing of the bacterial community. Gas pressure was recorded at 1, 2, 4, 6, 12, 18, 24, 36 and 48 hours and pH was measured immediately after the bottles were uncapped. Samples for MCP, NH_3-N, VFA analysis and bacterial community collection were also collected from the rumen fluid used as the inoculum. Microbial pellets were collected from the neat inoculum at the time of collection (before freezing) and at the beginning of the

experiment (after thawing) to determine any changes in the microbial community due to processing and storage.

All measures were standardised to per g of DM and blank corrected (inoculum and buffer, no substrate) where appropriate. For digestibility analysis, rumen fluid DM was added to the substrate DM within each bottle.

Molecular biology and sequence analysis was performed as described in the General Methods (Section 2.3). Samples were analysed from each of the three experimental treatments (1 in 2, 1 in 4, 1 in 9 rumen fluid to buffer ratio) and the no inoculum control bottles (grass and buffer) at each time point (0, 6, 12, 18, 24, 36 and 48 hours) alongside the neat rumen fluid used as an inoculum for the *in vitro* model at both the time of setting up the experimental bottles as well as a microbial pellet that was collected from the rumen fluid before freezing. The latest SILVA alignment was used during OTU assignment (v132).

7.2.1.1 Statistical analysis:

Data was tested for normality with a Kolmogorov-Smirnoff test and homogeneity of variance. If data met these assumptions, they were analysed using a general linear model in SPSS with Ratio and Time as fixed factors. Gas pressure (kPa) was converted to volume (ml) as described in the General Methods (Section 2.2.1) and blank corrected. Microbial crude protein was square root transformed prior to analysis. To correct for the VFAs present in the inoculum, total VFA values were blank corrected prior to analysis. All values were normalised for substrate dry matter added to the model.

7.2.2 Experiment 2: Dilution of the rumen inoculum below a 1:9 dilution ratio

Rumen fluid was collected at time of slaughter from a range of beef cattle at a local commercial abattoir (Penny and Sons, Leeds, UK). Rumen fluid from each animal was pooled into pre-heated thermos flasks. Dried grass was used as a substrate for the *in vitro* fermentations as described in the General Methods (Section 2.1.3).

Rumen fluid was serially diluted in Mould's Buffer to produce inoculum that when added (5 ml) to Mould's Buffer (45 ml) in preparation of the batch cultures the following rumen fluid to buffer dilution ratios (treatments) were obtained; 1 in 10, 1 in 100, 1 in 1,000, 1in 10,000 and 1 in 100,000. A time course experiment was performed, with samples taken and the digestibility of the substrate measured at 0, 12, 24, 36, 48, 72, 96, 120 and

144 hours. Gas pressure was manually recorded at 1, 2, 4, 6, 8, 12, 18, 24, 30, 36, 48, 72, 96, 120 and 144 hours. A total of 135 bottles were used with three bottles per dilution per time point. Due to the number of bottles, blanks were not included in this experiment. Three bottles were removed at each time point and samples for MCP, VFA and NH_3-N were collected from each bottle prior to IVDMD analysis.

7.2.2.1 Statistical analysis:

Data was checked for normality and homogeneity of variance and then analysed using a general linear model with Dilution and Time as fixed factors in IBM Statistics SPSS 21. The data for pH was non-normal and could not be transformed, therefore a generalised linear model was performed. IVDMD was non-normal and the distribution was U shaped, therefore a beta regression was performed in R (package betareg). All values were normalised for substrate DM added to the model.

Right-handed Gompertz curves (see Chapter 5.2.2) were fitted to the digestibility data in GenStat (12th Edition) using the standard curve function to determine whether the rate (the slope) of the curve differed between the 5 dilution rates. Using the web-based software as described in Assaad et al. (2014), one way ANOVA and Tukey *post hoc* tests were performed on summary data for the slopes, inflection point, and upper and lower asymptotes.

7.3 Results

7.3.1 Experiment 1 - Rumen fluid to buffer ratio affects *in vitro* fermentation

To determine the effect of rumen fluid to buffer ratio on fermentation, 1 in 2, 1 in 4 and 1 in 9 ratios were prepared alongside bottles containing no inoculum (grass and buffer). *In vitro* dry matter digestibility showed a significant interaction with Ratio and Time ($F_{18, 83} = 13.570$, $p < 0.001$; Figure 7-1) with both main effects also significant (Ratio $F_{3, 83} = 506.323$, $p < 0.001$; Time $F_{6, 83} = 222.884$, $p < 0.001$). The highest concentration of rumen fluid to buffer (1 in 2) showed significantly higher IVDMD than each of the other concentrations until 18 hours of fermentation. There was no significant difference in IVDMD between 1 in 2 and 1 in 4 ratio at 18 hours (64 *vs* 63 g/ 100g DM respectively) and thereafter. After 36 hours of fermentation, 1 in 2, 1 in 4 and 1 in 9 ratios of rumen fluid to buffer produced the same amount of IVDMD (68, 69 and 64 g/100g respectively). After 48 hours of fermentation, there was no differences in IVDMD between the

experimental bottles containing rumen fluid and the no-inoculum control bottles (grass and buffer only). Bottles containing only grass and buffer showed stable IVDMD at 35 g/100 g until around 36 hours whereupon IVDMD increased to 47 g/100 g and then up to 62 g /100 g at 48 hours.

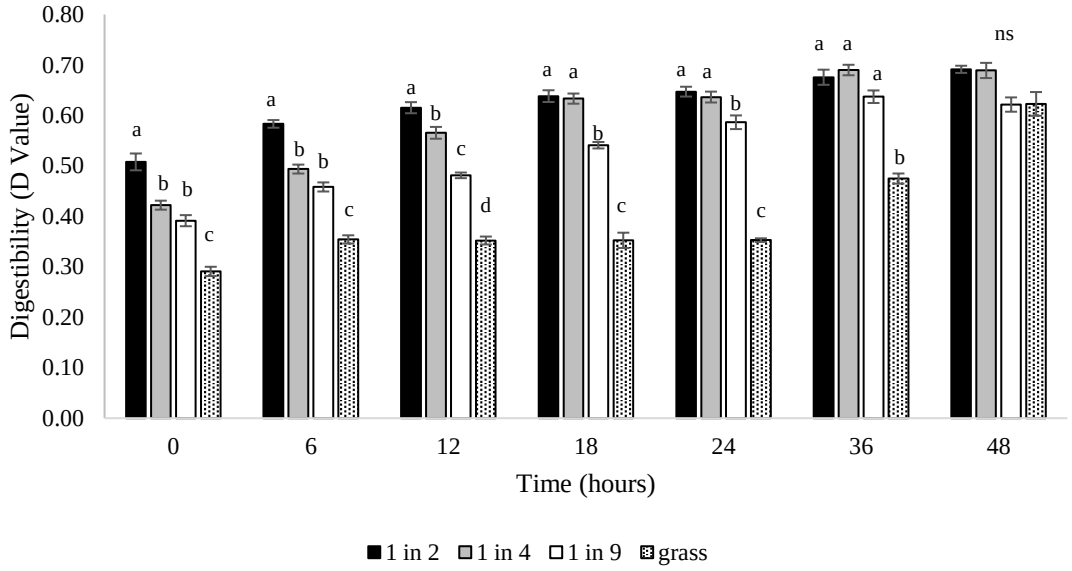

Figure 7-1 *In vitro* dry matter digestibility for a range of rumen fluid to buffer ratios across a 48 hour fermentation Error bars show SE. Different superscript letters show significant *post-hoc* differences between treatments within a time point, ns = no significant difference, $p < 0.05$

Increasing the concentration of rumen fluid within the fermenters was also shown to affect fermentation parameters measured (Table 7-1). A significant interaction between ratio of rumen fluid to buffer and time was observed for each of the measured parameters (gas volume $F_{16, 350} = 65.206$, $p < 0.001$; pH $F_{15, 43} = 30.362$, $p < 0.001$; ammonia-nitrogen $F_{18,83} = 27.70$, $p < 0.001$; microbial crude protein $F_{18, 83} = 2.24$, $p = 0.008$; total volatile fatty acid $F_{12, 62} = 5.348$, $p < 0.001$). The volume of gas produced was shown to be highest initially for the 1 in 2 ratio, but from 36 hours of fermentation onward there was shown to be no difference between the 1 in 2 and 1 in 4 ratios of rumen fluid to buffer. The 1 in 9 ratio had significantly lower gas volume production than both the 1 in 2 and 1 in 4 ratio until 36 hours of fermentation, after which there was no differences between the 1 in 4 and 1 in 9 treatments.

The pH of the fermentation fluid decreased significantly with amount of rumen fluid added to the model. From 18 to 36 hours the pH of the three rumen fluid concentrations were all different from each other (Table 7-1). The pH of bottles containing no inoculum was shown to be stable, as for gas volume, from 12-24 hours (7.19, 7.15 and 7.14 for 12,

18 and 24 hours respectively) and then decreased gradually with time as fermentation progressed.

Ammonia-nitrogen concentration increased with time across the course of the fermentation with a significantly higher concentration in bottles containing the largest amount of rumen inoculum throughout the fermentation (average concentrations of 1.23 ± 0.30, 1.01 ± 0.24 and 0.82 ± 0.17 mg/ml for 1 in 2, 1 in 4 and 1 in 9 respectively).

As expected, the highest concentration of microbial crude protein was observed in fermentation bottles containing the greatest proportion of rumen inoculum (622.1 ± 180.9, 347.9 ± 74.6 and 191.6 ± 80.3 µg/ml for 1 in 2, 1 in 4 and 1 in 9 respectively). After 18 hours of fermentation, bottles containing 1 in 4 or 1 in 9 ratios of rumen fluid to buffer were found to contain similar concentrations of microbial protein. It is interesting to note that the MCP of the three treatments were very different at the start of the experimental period (508.2, 380.6 and 135.6 µg / ml for 1 in 2, 1 in 4 and 1 in 9 respectively) and by 48 hours, no statistical difference was observed between the fluids (597.3, 469.5 and 358.1 for 1 in 2, 1 in 4 and 1 in 9 respectively). The concentration of microbial crude protein was negligible for bottles that did not contain rumen fluid until 48 hours of fermentation where concentration increased from 2.7 µg/ ml at 36 hours to 33.4 µg/ml at 48 hours.

Although there was a significant interaction between Ratio and Time for volatile fatty acid concentration (VFA; mM), no *post-hoc* differences were observed between the three rumen fluid to buffer ratios across the fermentation suggesting that the VFA concentrations were similar across all three ratios.

Table 7-1 Fermentation parameters for different ratios of rumen fluid to buffer over a 48 hour fermentation.

Parameter and rumen fluid to buffer ratio	Time (hours)								p value		
	0	**6**	**12**	**18**	**24**	**36**	**48**	**SEM[1]**	**Time**	**Ratio**	**Time*Ratio**
Gas volume (ml)[3]											
1 in 2	-	18.63[a]	72.94[a]	104.45[a]	128.45[a]	135.48[a]	137.84[a]				
1 in 4	-	6.47[b]	37.41[b]	66.89[b]	102.41[b]	123.62[a]	127.71[ab]	0.709	**< 0.001**	**< 0.001**	**< 0.001**
1 in 9	-	3.37[c]	20.89[c]	45.54[c]	79.26[c]	102.92[b]	109.83[b]				
pH											
1 in 2	-	6.47[a]	6.35[a]	6.19[a]	6.40[a]	6.44[a]	6.48[a]				
1 in 4	-	6.61[ab]	6.58[ab]	6.70[b]	6.66[b]	6.69[b]	6.64[ab]	0.021	**< 0.001**	**< 0.001**	**< 0.001**
1 in 9	-	6.96[bc]	6.96[bc]	6.85[c]	6.90[c]	6.81[c]	6.81[b]				
No inoculum[2]	-	7.28[c]	7.19[c]	7.15[d]	7.14[d]	6.93[d]	6.83[b]				
Ammonia nitrogen (mg/ml)											
1 in 2	0.89[a]	0.92[a]	1.07[a]	1.23[a]	1.28[a]	1.53[a]	1.69[a]				
1 in 4	0.77[b]	0.77[b]	0.87[b]	0.96[b]	1.08[b]	1.24[b]	1.39[b]	0.004	**< 0.001**	**< 0.001**	**< 0.001**
1 in 9	0.63[c]	0.63[c]	0.75[c]	0.80[c]	0.89[c]	1.02[c]	1.04[c]				
No inoculum	0.52[d]	0.52[d]	0.62[d]	0.63[d]	0.65[d]	0.65[d]	0.70[d]				
Microbial crude protein (µg/ml)											
1 in 2	508.2[a]	486.1[a]	588.5[a]	453.1[a]	962.3[a]	759.2[a]	597.3[a]				
1 in 4	380.6[b]	374.2[a]	298.6[b]	371.1[a]	239.3[b]	301.9[b]	469.5[a]	14.88	**0.021**	**< 0.001**	**0.008**
1 in 9	135.6[c]	200.4[b]	118.7[c]	200.2[b]	143.7[b]	184.2[b]	358.1[a]				
No inoculum	0.0[d]	3.6[c]	10.6[d]	11.0[c]	0.0[c]	2.7[c]	33.4[b]				
Total volatile fatty acids (mM)[3]											
1 in 2	0.04	44.71	20.50	21.58	29.89	37.59	33.31				
1 in 4	0.76	0.28	15.56	22.16	22.54	36.61	48.25	1.18	**< 0.001**	0.118	**<0.001**
1 in 9	0.00	0.21	10.52	19.24	36.48	50.79	61.99				

[1] SEM = standard error of the mean, [2] No inoculum = bottles containing only grass and buffer

[a-c] Means within a row that do not share a common superscript are significantly different $p < 0.05$

[3] No inoculum values are not included for gas volume and total volatile fatty acid concentration as there was no suitable blank correction

The individual VFA breakdown can be seen in Table 7-2. To include the no inoculum samples (grass + buffer) values shown are not blank corrected. There was found to be an interaction between Time and Ratio for acetate ($F_{18, 80} = 11.846$, $p < 0.001$) with main effects of both Time ($F_{6, 80} = 129.198$, $p < 0.001$) and Ratio ($F_{3, 80} = 342.544$, $p < 0.001$). In general, difference between the ratios at the start of the experimental period were lost with time. After 24 hours of fermentation, the three treatments including rumen fluid were very similar at each time point (34.5 ± 3.74, 37.0 ± 3.28 and 41.4 ± 2.36 mM for 24, 36 and 48 hours respectively). After 36 hours, there was no difference between the 1 in 4 and grass + buffer samples (33.2 *vs* 26.3 and 41.0 *vs* 33.8 for 36 and 48 hours of fermentation respectively, $p > 0.05$).

For propionate, there was main effects of both Time ($F_{6, 80} = 31.187$, $p < 0.001$) and Ratio ($F_{3, 80} = 308.683$, $p < 0.001$), but no interactive term ($F_{18, 80} = 1.548$, $p = 0.110$). Propionate concentrations at both 36 and 48 hours were significantly higher than all other time points. All ratios of rumen fluid to buffer were significantly different to each other ($p < 0.001$) with concentration generally highest in the most concentrated samples.

Butyrate concentration again showed an interaction between Time and Ratio ($F_{18, 80} = 4.480$, $p < 0.001$) with main effects of Time ($F_{6, 80} = 179.061$, $p < 0.001$) and Ratio ($F_{3, 80} = 300.849$, $p < 0.001$). Although concentrations of butyrate were generally different between the ratios across the fermentation, profiles had converged by 48 hours to a similar point (20.5 ± 3.97 mM). Only the 1 in 2 ratio was significantly higher than the other samples.

Finally, the acetate to propionate ratio also showed an interaction between Time and Ration ($F_{18, 80} = 40.925$, $p < 0.001$) and main effects of both Time ($F_{6, 80} = 42.899$, $p < 0.001$) and Ratio ($F_{3, 80} = 287.151$, $p < 0.001$). Due to the lack of propionate production in the grass + buffer samples until 48 hours, this resulted in a division by zero. Of the samples that contained rumen inoculum, each ratio showed the same A:P initially (3.2 at 0 hours and 3.63 at 6 hours), diverged at 12 and 18 hours and then became more similar again in the second half of the fermentation (4.5 ± 0.53, 3.6 ± 0.89 and 3.6 ± 0.67 at 24, 36 and 48 hours respectively).

Table 7-2 Volatile fatty acid analysis for different rumen fluid to buffer ratios over 48 hours. Mean values are presented and corrected per g DM. All concentrations shown are in mM. Significant values are shown in bold

		Time (hours)								P value		
Rumen fluid to buffer ratio and individual VFAs		0	6	12	18	24	36	48	SEM[1]	Time	Ratio	Time*Ratio
Acetate	1 in 2	31.6^{a}	31.6^{a}	38.5^{a}	41.0^{a}	38.7^{a}	38.7^{a}	40.3^{ab}	1.31	**< 0.001**	**< 0.001**	**< 0.001**
	1 in 4	25.3^{b}	25.9^{b}	32.3^{a}	32.1^{b}	33.0^{ab}	33.2^{ab}	41.0^{ab}				
	1 in 9	16.3^{c}	17.3^{c}	24.8^{b}	27.0^{c}	31.7^{b}	39.0^{a}	42.9^{a}				
	Grass + buffer	0.0^{d}	0.0^{d}	14.6^{c}	11.6^{d}	14.8^{c}	26.3^{b}	33.8^{b}				
Propionate	1 in 2[a]	9.8	8.8	9.6	10.4	9.7	13.2	13.3	0.403	**< 0.001**	**< 0.001**	0.110
	1 in 4[b]	7.8	7.1	7.6	7.4	7.4	10.4	11.9				
	1 in 9[c]	5.0	4.7	5.6	5.2	6.3	8.5	10.8				
	Grass + buffer[d]	0.0	0.0	0.0	0.0	0.0	0.0	4.4				
Butyrate	1 in 2	11.0^{a}	10.2^{a}	13.1^{a}	16.8^{a}	21.0^{a}	24.6^{a}	25.4^{a}	1.01	**< 0.001**	**< 0.001**	**< 0.001**
	1 in 4	8.9^{a}	8.4^{b}	10.7^{b}	12.3^{b}	17.9^{ab}	18.4^{b}	20.2^{b}				
	1 in 9	6.0^{b}	5.6^{c}	7.1^{c}	9.1^{c}	15.4^{b}	19.2^{ab}	20.5^{b}				
	Grass + buffer	0.0^{b}	0.0^{d}	0.0^{d}	0.8^{d}	2.2^{c}	8.3^{d}	15.7^{b}				
A:P	1 in 2	3.2^{a}	3.6^{a}	4.0^{a}	4.0^{a}	4.0^{a}	2.9^{a}	3.0^{a}	0.211	**< 0.001**	**< 0.001**	**< 0.001**
	1 in 4	3.2^{a}	3.6^{a}	4.3^{b}	4.3^{b}	4.4^{b}	3.2^{a}	3.4^{a}				
	1 in 9	3.2^{a}	3.7^{a}	4.4^{c}	5.2^{c}	5.1^{b}	4.6^{b}	4.3^{a}				
	Grass + buffer	0.0^{b*}	0.0^{b*}	0.0^{d*}	0.0^{d*}	0.0^{c*}	0.0^{c*}	7.6^{b}				

[1] SEM standard error of the mean, A:P acetate to propionate ratio

[a-d] Means within a column that do not share a common superscript are significantly different ($p < 0.05$). When a fluid effect was observed, fluid type names that do not share a common superscript are significantly different ($p < 0.05$).

[*] Divided by zero

7.3.1.1 The bacterial population present in the fermenters

To determine whether the ratio of rumen fluid to buffer could affect the stability of the bacterial community, 16S rRNA sequencing was performed on DNA extracts from fermentation fluid sampled at each time point. A total of 14,234,874 sequences were obtained with an average of 474,495 ± 88,001 for each group. After all filtering and clustering steps, a total of 1,258,303 unique sequences remained with an average of 41,943 ± 15,736 for each group. A total of 12 phyla had a minimum relative abundance of 1% in at least one of the experimental samples; Firmicutes, Bacteroidetes, Proteobacteria, Kirimatiellaeota, Tenericutes, Unclassified bacteria, Spirochaetes, Planctomycetes, Actinobacteria, Patescibacteria, Verrucomicrobia and Fibrobacteres.

In the neat rumen inoculum that was used to inoculate the fermentation bottles, the most abundant Phylum was found to be Bacteroidetes (51.1 ± 1.31 %), followed by Firmicutes (36.5 ± 3.15 %). At the last sampling time in the experimental samples (48 hours), the most abundant phylum was Firmicutes (42.1 ± 2.76 %), followed by Bacteroidetes (27.0 ± 1.26 %). A large increase in the relative abundance of the phylum Proteobacteria was seen in experimental bottles containing rumen fluid when compared to the neat inoculum (0.9 ± 0.12 % *vs* 16.1 ± 6.99 % for neat rumen fluid and the mean relative abundance of bottles containing rumen fluid at 48 hours respectively). Interestingly, the relative abundance of Proteobacteria increased inversely to the proportion of rumen fluid within the model; bottles containing less rumen fluid showed higher relative abundance of Proteobacteria (Figure 7-2). Fibrobacteres was identified at 2.37% abundance in the neat rumen fluid collected at time of processing. However, at the start of the experiment after freeze-thaw, Fibrobacteres was present at a much lower abundance (0.06%) and did not recover across the experimental period (Appendix D-1).

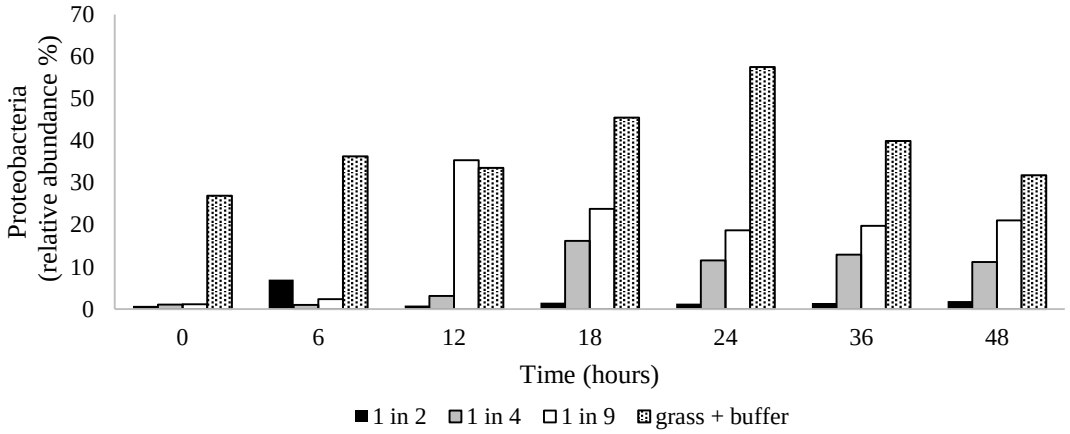

Figure 7-2 The relative abundance of Proteobacteria across the three rumen fluid to buffer ratios and the no inoculum control (grass and buffer) fermentation bottles

From the 12 Phyla identified with a minimum relative abundance of 1%, the most abundant genera at zero hours in bottles containing 1 in 2, 1 in 4 and 1 in 9 rumen fluid to buffer ratios were *Prevotella 1* (23.2 ± 0.66 %), *F082 ge* (10.6 ± 0.42), *Ruminococcaceae NK4A214 group* (9.1 ± 0.11 %), *Christensenellacae R-7 group* (7.3 ± 0.21 %) and *Rikenellaceae RC9 gut group* (4.9 ± 0.43 %). At the end of the experiment at 48 hours, the most abundant genera were *Oribacterium* (14.4 ± 1.57 %), *Streptococcus* (11.5 ± 3.57 %), *Rikenellaceae RC9 gut group* (10.8 ± 2.60 %), *Prevotella 1* (9.0 ± 3.37 %) and *Ruminococcaceae NK4A214 group* (5.2 ± 3.14 %).

In bottles that did not contain rumen inoculum (grass and buffer) the most abundant phyla were Bacteroidetes (54.0 %), Proteobacteria (27.0 %) and Actinobacteria (9.3%) at the first sampling time (0 hours). By the end of the experiment at 48 hours, Firmicutes was the most abundant phyla (40.1 %) followed by Proteobacteria (31.8 %) and Bacteroidetes (27.9 %). The most abundant genera in the grass samples were largely not seen in the experimental bottles (< 0.1 %). The most abundant genera at the beginning of the experiment were *Pedobacter* (8.9 %), *Chryseobacterium* (8.4 %), *Flavobacterium* (7.5 %) and *Pseudomonas* (6.0 %). At the end of the experiment, the genera associated with the grass and buffer only bottles were dominated by *Escherichia-Shigella* (24.4 %), *Bacteroides* (15.2 %) and *Cellulosilyticum* (14.0 %). All relative abundances can be seen in Appendix D 1-4.

7.3.1.2 An increased concentration of rumen fluid improved the stability of the rumen bacterial community

Alpha and beta diversity were analysed to establish the effect of rumen fluid to buffer ratio on the bacterial community composition. It was found that alpha diversity declined as early as 6 hours into the experiment for all experimental treatments. Alpha diversity plateaued at *ca* 24 hours for 1 in 2 and 1 in 4 dilution and 36 hours for the 1 in 9 dilution (Table 7-3). The more concentrated the rumen fluid (i.e. 1 in 2), the smaller the loss of alpha diversity. Using the Chao1 measure of alpha diversity, there was found to be a significant effect of both Time ($F_{1, 20} = 66.516$, $p < 0.001$) and Ratio ($F_{1, 20} = 16.407$, $p < 0.001$) on alpha diversity with no interaction between the two ($F_{1, 20} = 0.5447$, $p = 0.4695$). The same was shown for Shannon (Time $F_{1, 20} = 38.469$, $p < 0.001$; Ratio $F_{1, 20} = 7.5271$, $p = 0.013$; Time*Ratio $F_{1, 20} = 0.7342$, $p = 0.402$) and Simpson's diversity index (Time $F_{1, 20} = 9.0347$, $p = 0.007$, Ratio $F_{1, 20} = 4.8887$, $p = 0.039$, Time*Ratio $F_{1, 20} = 0.2859$, $p = 0.599$).

Table 7-3 Chao 1, Shannon and Simpson's indices of alpha diversity obtained from neat rumen inoculum at time of collection (Pre) and prior to starting the experiment (0 h) along with experimental samples across 48 hours Significant values are shown in bold

		Time (hours)								p value		
		Pre[1]	**0**	**6**	**12**	**18**	**24**	**36**	**48**	**Time**	**Ratio**	**Time*Ratio**
Chao1	**Neat**	11124.6	11748.6							**< 0.001**	**< 0.001**	0.4695
	1 in 2		12051.5	8684.1	9021.7	7820.4	7109.9	6671.4	7434.4			
	1 in 4		9896.1	10237.2	8103.3	7014.8	6673.1	6678.0	6781.1			
	1 in 9		9978.0	8762.6	6505.4	6103.6	5574.5	5008.5	5340.6			
Shannon	**Neat**	7.63	7.65							**< 0.001**	**0.0125**	0.4022
	1 in 2		7.52	6.69	6.24	5.59	5.07	5.15	5.91			
	1 in 4		7.52	6.93	5.61	4.83	4.66	4.84	5.13			
	1 in 9		7.40	6.50	4.55	4.41	4.34	4.38	4.67			
Simpson's	**Neat**	0.998	0.998							**0.0070**	**0.0388**	0.5991
	1 in 2		0.998	0.991	0.977	0.955	0.933	0.956	0.984			
	1 in 4		0.998	0.992	0.967	0.946	0.942	0.952	0.960			
	1 in 9		0.998	0.978	0.900	0.927	0.935	0.944	0.943			

[1] Pre samples were collected at time of processing, prior to freezing (-80°C), * Cells filled in grey denote that samples were not collected at these time points

PERMANOVA analysis, a measure of beta diversity, of bacterial community composition revealed a significant effect of both Time ($F_{7, 22} = 5.967$, $p < 0.001$) and Ratio ($F_{3, 22} = 2.527$, $p = 0.009$). The NMDS plot in Figure 7-3 shows the data points close together to begin with, but over the course of the fermentation the distance between the points increased indicating that community composition was changing. A clear effect of rumen fluid to buffer concentration can be seen with the points from the most concentrated rumen fluid remaining closer to the initial inoculum than the other concentrations (1:4, 1:9) . After 18 hours, the 1 in 2 dilution begins to more rapidly diverge, but divergence is seen much earlier for 1 in 4 (12 hours) and 1 in 9 ratios (6 hours). Although the alpha diversity shown in Figure 7-3 appeared to stabilise around 24 hours, the beta diversity plot indicates the community continues to adapt to the environment within the model after this.

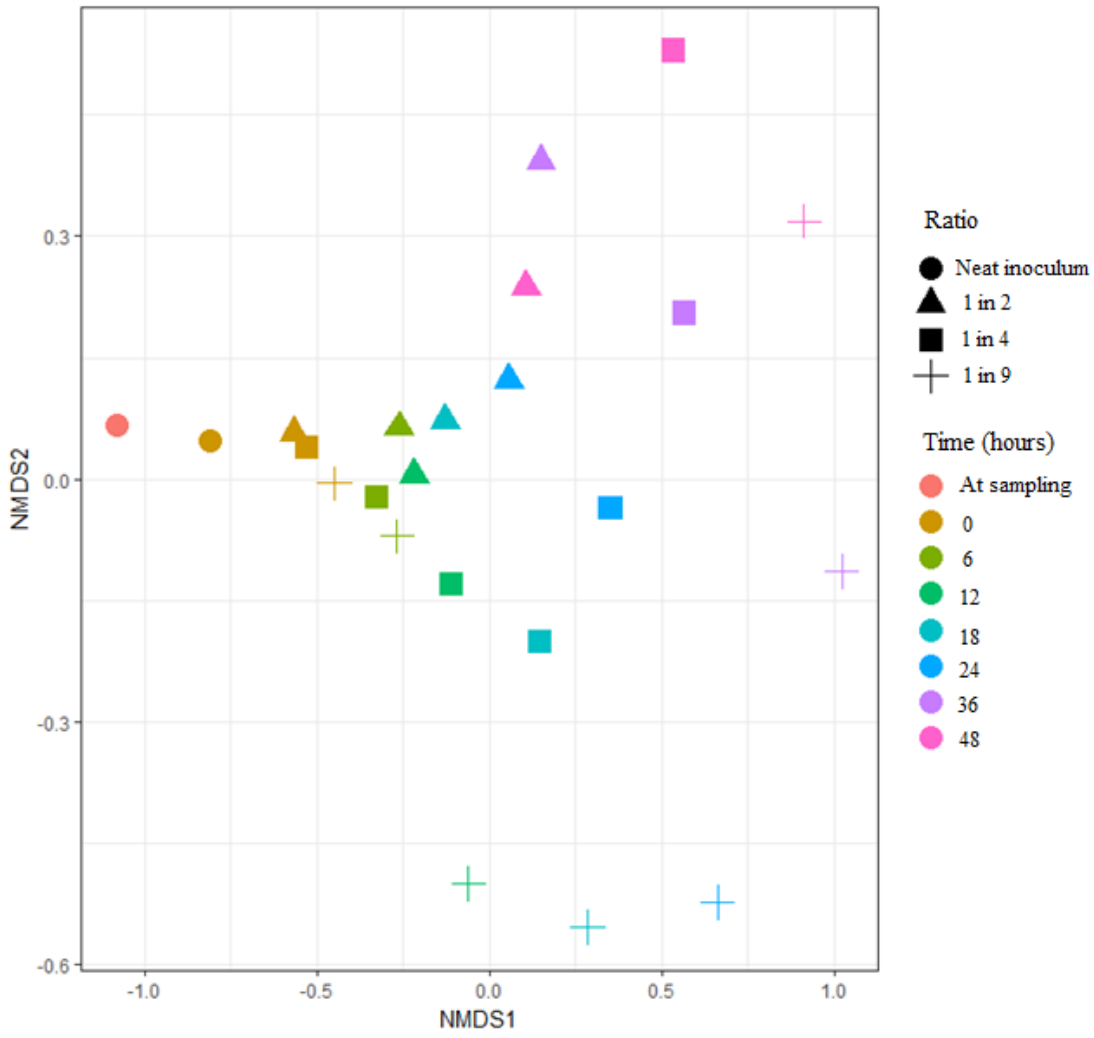

Figure 7-3 Non-metric multidimensional scaling (NMDS) plot using Bray-Curtis distances for different rumen fluid to buffer ratios across a 48 hour fermentation

When the epiphytic community (grass-associated bacteria) was included in the beta diversity analysis, both Time ($F_{7, 29} = 3.096$, $p < 0.001$) and Ratio ($F_{4, 29} = 5.446$, $p < 0.001$) had a significant effect on community composition, but the experimental data points clustered much closer to each other than to the grass samples (Figure 7-4).

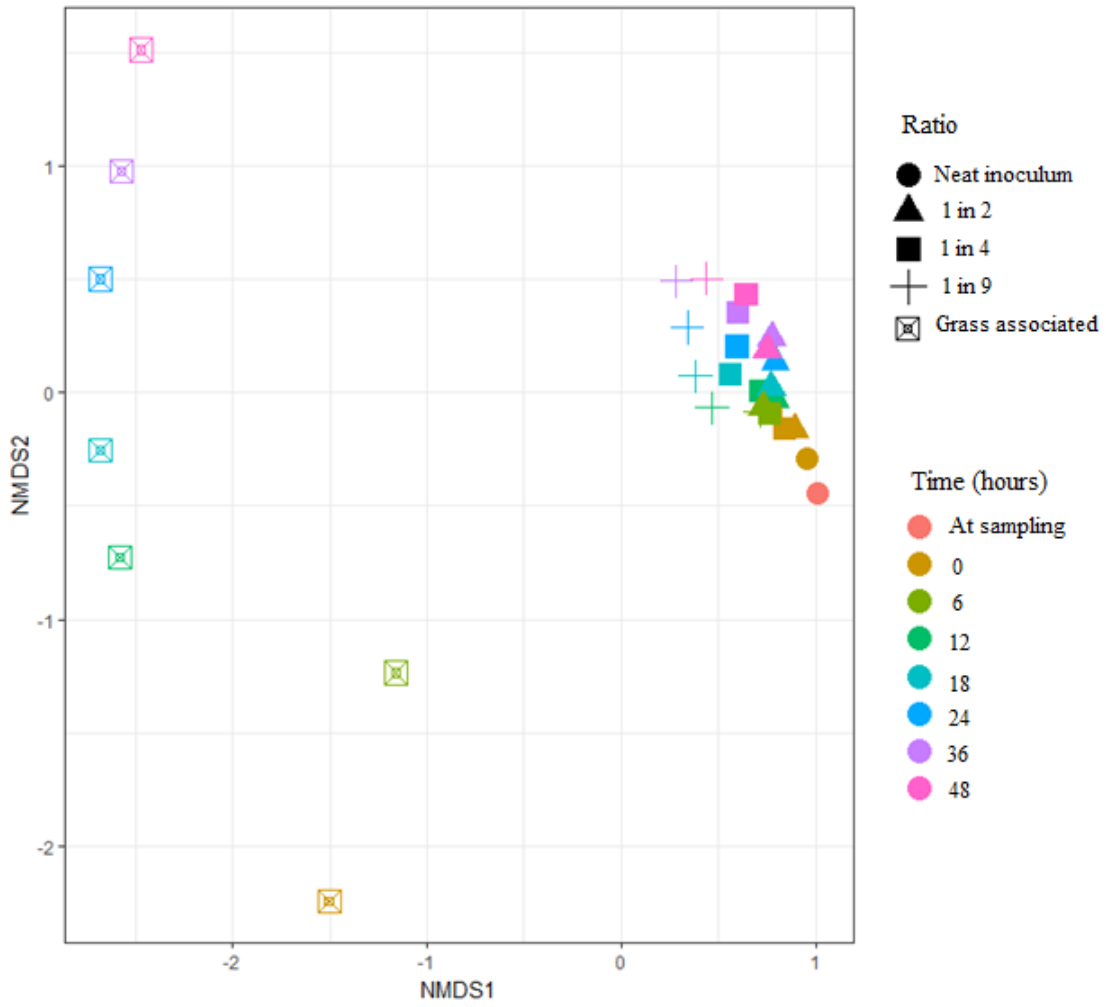

Figure 7-4 Non-metric multidimensional scaling plot using Bray Curtis distances for the three rumen inclusion ratios (1:2, 1:4 and 1:9) along with the epiphytic bacterial community associated with the grass substrate

7.3.2 Experiment 2 -The effect of dilution of rumen inoculum on *in vitro* dry matter digestibility and fermentation parameters

To determine the effect of a smaller starting bacterial community on rumen fermentation within a batch *in vitro* model, a serial dilution of rumen fluid was performed with resulting dilutions ranging from 10 to 100,000 times. Throughout this thesis, rumen fluid was added to the model in a 1 in 9 ratio with buffer, resulting in a 10 x dilution. Serial dilution of rumen inoculum into the *in vitro* model revealed that there was a significant interaction between ratio and time for IVDMD ($z = -2.435$, $p = 0.0149$) with a significant main effect of Time ($z = 13.244$, $p < 0.001$) and no main effect of ratio ($z = 1.253$, $p = 0.210$).

The parameters from the fitted curves in Figure 7-5 revealed a significant difference in both the slope ($F_{4, 134} = 38.723$, $p < 0.001$) and inflection point ($F_{4, 134} = 7.310$, $p < 0.001$) of the dilutions. No significant difference was observed for either the upper ($F_{4, 134} = 0.495$, $p = 0.739$) or lower asymptote ($F_{4, 134} = 0.573$, $p = 0.6826$). Predicted values and *post-hoc* tests can be seen in Table 7-4. There was found to be no difference between the 1 in 10, standard preparation of rumen fluid and a further 10x dilution (1 in 100) in terms of both slope and inflection point.

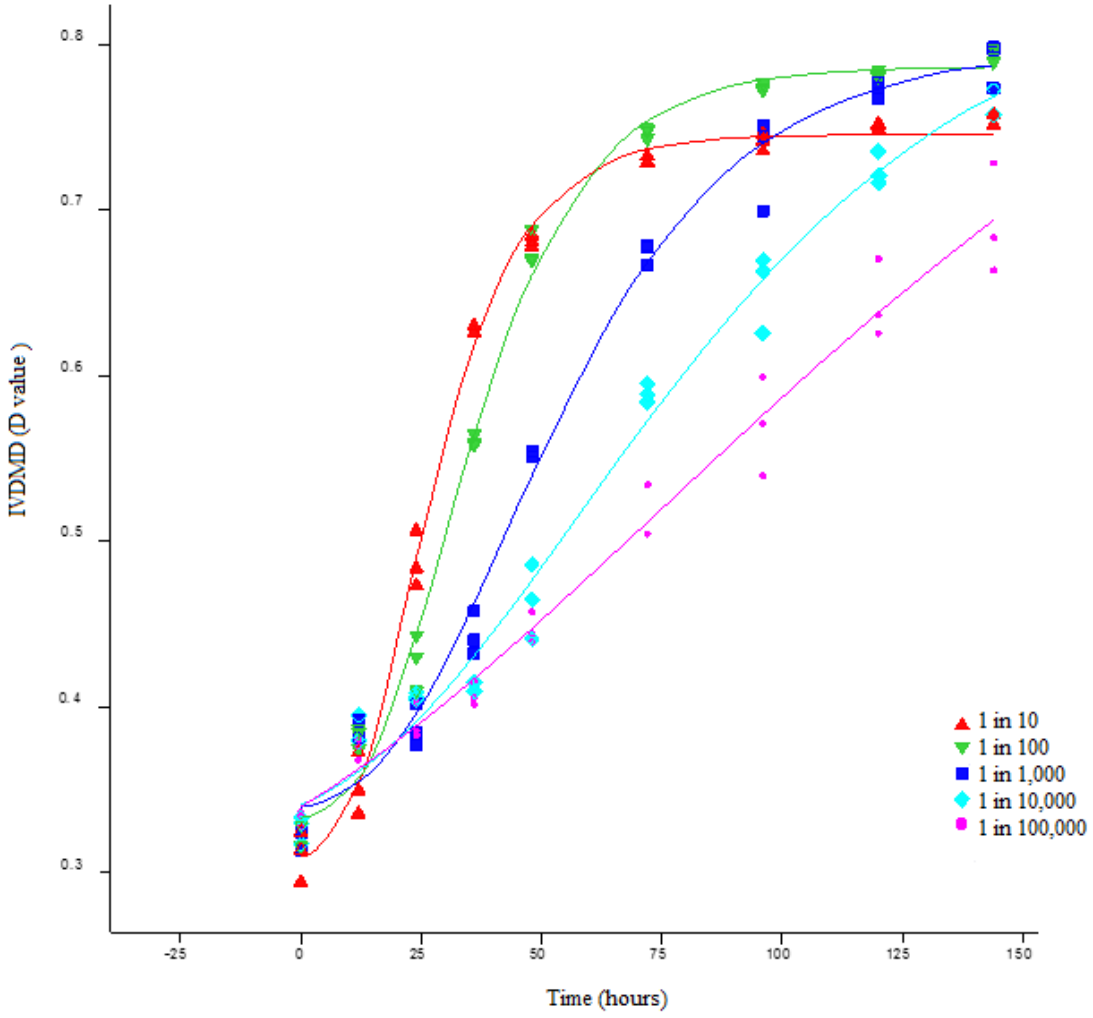

Figure 7-5 Observed (points) and fitted (lines) values for the five dilutions of rumen inoculum across a 144 hour fermentation. Lines were fitted using the Gompertz standard curve function in GenStat (12th Edition). Parameters can be seen in Table 7-4.

Table 7-4 Predicted parameters of the fitted curves (Fig. 7.6) for the five rumen inoculum dilutions

	Rumen fluid to buffer dilution factor				
	1 in 10	**1 in 100**	**1 in 1,000**	**1 in 10,000**	**1 in 100,000**
Slope	0.08 ± 0.01^{a}	0.06 ± 0^{a}	0.04 ± 0^{b}	0.02 ± 0^{bc}	0.01 ± 0.01^{c}
Inflection	22.36 ± 0.92^{c}	29.53 ± 1.03^{bc}	42.77 ± 1.56^{bc}	57.8 ± 3.6^{ab}	75.2 ± 17.2^{a}
Lower Asymptote[1]	0.44 ± 0.01	0.45 ± 0.01	0.46 ± 0.02	0.52 ± 0.05	0.69 ± 0.3
Upper Asymptote[1]	0.31 ± 0.01	0.33 ± 0.01	0.34 ± 0.01	0.32 ± 0.02	0.27 ± 0.09

[1] The sum of these denotes overall upper asymptote value

[a-c] Means within a row that do not share a common superscript are significantly different $p < 0.05$

The effect of rumen fluid dilution on fermentation parameters are presented in Table 7-5. Similar to Experiment 1, there was a significant interaction between Dilution and Time for each of the measured parameters (gas volume $F_{28, 119} = 888.23$, $p < 0.001$; pH Wald $X^2 = 435.18$, df = 28 $p < 0.001$; ammonia-nitrogen $F_{32, 133} = 5.27$, $p < 0.001$; microbial crude protein $F_{32, 115} = 6.67$, $p < 0.001$; total volatile fatty acids $F_{32, 130} = 16.87$, $p < 0.001$).

The highest gas production was produced for the most concentrated rumen fluid (1 in 10), as also seen in Experiment 1, and the difference between the five dilutions was most pronounced at the 12 hour sampling time with the 1 in 10 dilution producing almost double the amount of gas when compared with the 1 in 100 dilution (72.0 *vs* 44.2 ml respectively). From 48 to 144 hours of fermentation, incremental dilutions resulted in a significant reduction in gas volume, with the exception of 120 hours where the only significant difference was between the 1 in 10 and 1 in 100,000 dilution (259.9 *vs* 161.7 ml respectively).

The pH was shown to generally decrease with time across the fermentation with the lowest pH recorded for the most concentrated sample (1 in 10). Generally, little difference was seen between the 1 in 10 and 1 in 100 dilution across the fermentation (average pH across the fermentation of 6.53 for both). In general, there was no difference in pH values between the 1 in 10,000 and 1 in 100,000 samples.

Ammonia-nitrogen concentration increased with time across the fermentation. There was no significant difference in concentration between 1 in 10, 1 in 100 and 1 in 1,000 dilutions at any time during the fermentation, and no differences were observed between all five dilution rates at 12, 24, 48 and 72 hours.

As expected, the higher concentrations of microbial protein were observed for the most concentrated samples (1 in 10). After 36 hours of fermentation, no difference in crude protein concentration was observed between 1 in 10 and 1 in 100 dilutions. There was no difference in MCP by the end of the experiment between all five dilution rates (926.1 ± 47.0 µg/ml; $p = 0.714$). Across the first 48 hours of fermentation, the concentration of microbial protein was similar for the three least concentrated treatments (655.3 ± 294.2, 572.7 ± 273.7 and 529.1 ± 228.8; $F_{2, 26} = 0.487$, $p = 0.60$ for 1 in 1000, 1 in 10,000 and 1 in 100,000 respectively).

Finally, for total VFA concentration, after 24 hours of fermentation the 1 in 10 dilution had a significantly higher total VFA concentration than each of the other dilutions (56.2, 23.45 11.2, 10.7 and 9.9 mM for 1 in 10, 1 in 100, 1 in 1,000, 1 in 10,000 and 1 in 100,000

respectively). After 96 hours of fermentation no significant difference was observed between the 1 in 10, 1 in 100 and 1 in 1,000 dilutions (104.4, 90.4 and 90.1 mM respectively). At the end of the experimental period, with the exception of the most dilute samples (1 in 100,000; 79.6 mM) there was no significant difference in volatile fatty acid concentration between the samples (100.7, 98.7, 96.5 and 95.8 mM for 1 in 10, 1 in 100, 1 in 1,000 and 1 in 10,000 respectively).

Table 7-5 Fermentation parameters for the five rumen fluid dilutions across a 144 hour fermentation

	Time (hours)										p value		
	0	**12**	**24**	**36**	**48**	**72**	**96**	**120**	**144**	**SEM[1]**	**Time**	**Dilution**	**Time*Dilution**
Gas volume (ml)													
1 in 10	-	72.03^{a}	136.3^{a}	189.05^{a}	207.99^{a}	238.27^{a}	250.78^{a}	259.88^{a}	261.1^{a}				
1 in 100	-	44.24^{b}	82.07^{ab}	138.32^{ab}	171.76^{b}	197.03^{b}	218.73^{b}	234.31^{ab}	241.47^{b}				
1 in 1,000	-	38.76^{c}	68.74^{ab}	91.83^{ab}	121.92^{c}	158.13^{c}	183.24^{c}	196.03^{ab}	210.8^{c}	0.057	**< 0.001**	**< 0.001**	**< 0.001**
1 in 10,000	-	39.22^{c}	65.24^{b}	77.61^{ab}	92.45^{d}	134.08^{d}	173.64^{d}	192.45^{ab}	203.61^{d}				
1 in 100,000	-	29.16^{d}	66.71^{ab}	75.73^{b}	81.44^{e}	111.7^{e}	139.62^{e}	161.7^{b}	177.44^{e}				
pH													
1 in 10	-	6.66^{a}	6.60^{a}	6.54^{a}	6.49^{a}	6.48^{ab}	6.48^{ab}	6.48^{a}	6.51^{ac}				
1 in 100	-	6.62^{b}	6.60^{a}	6.57^{b}	6.51^{ab}	6.48^{ab}	6.46^{a}	6.45^{a}	6.48^{abc}				
1 in 1,000	-	6.64^{c}	6.63^{b}	6.63^{c}	6.52^{ab}	6.45^{a}	6.43^{a}	6.40^{b}	6.44^{bc}	0.009	**< 0.001**	**< 0.001**	**< 0.001**
1 in 10,000	-	6.65^{ac}	6.64^{bc}	6.65^{d}	6.63^{ab}	6.50^{ab}	6.48^{ab}	6.44^{a}	6.42^{b}				
1 in 100,000	-	6.66^{a}	6.66^{d}	6.66^{d}	6.66^{b}	6.55^{b}	6.56^{b}	6.48^{a}	6.50^{c}				
Ammonia-nitrogen (mg/ml)													
1 in 10	1.23^{ac}	1.31	1.13	1.11^{a}	1.31	1.60	1.51^{a}	1.76^{a}	1.76^{a}				
1 in 100	1.24^{ac}	1.39	1.21	1.17^{ab}	1.15	1.33	1.48^{ab}	1.60^{ab}	1.64^{ab}				
1 in 1,000	1.28^{ac}	1.24	1.31	1.25^{ab}	1.31	1.13	1.45^{ab}	1.50^{ab}	1.40^{bc}	0.009	**< 0.001**	**< 0.001**	**< 0.001**
1 in 10,000	1.38^{b}	1.21	1.31	1.37^{b}	1.25	1.19	1.24^{bc}	1.34^{bc}	1.42^{bc}				
1 in 100,000	1.26^{c}	1.32	1.31	1.34^{b}	1.36	1.12	1.17^{c}	1.12^{c}	1.23^{c}				
Microbial crude protein (ug/ml)													
1 in 10	551.8^{a}	598.3^{a}	735.8	915.3^{a}	1257.2^{a}	1186.7^{a}	911.1^{ab}	1157.1^{a}	929.5				
1 in 100	395.0^{b}	462.9^{b}	445.4	632.4^{b}	912.4^{a}	940.1^{ab}	1063.5^{a}	955.6^{ab}	927.5				
1 in 1,000	375.1^{bc}	345.0^{c}	439.0	495.3^{c}	449.6^{b}	909.5^{ab}	878.3^{b}	1155.9^{a}	849.9	7.230	**< 0.001**	**<0.001**	**<0.001**
1 in 10,000	270.4^{c}	287.8^{c}	372.5	396.1^{c}	462.0^{b}	745.3^{bc}	707.9^{c}	934.8^{ab}	977.5				
1 in 100,000	250.7^{c}	349.0^{c}	442.2	349.2^{c}	440.8^{b}	526.0^{c}	667.9^{c}	789.8^{b}	945.9				
Total volatile fatty acids (mM)													
1 in 10	0.00	17.96	56.22^{a}	74.56^{a}	87.11^{a}	100.02^{a}	104.44^{a}	103.42^{a}	100.67^{a}				
1 in 100	0.00	0.00	23.48	55.13^{b}	71.34^{b}	85.03^{b}	90.39^{ab}	92.21^{ab}	98.73^{a}				
1 in 1,000	0.00	0.00	11.20	30.77^{c}	59.18^{c}	79.35^{bc}	90.12^{ab}	93.60^{ab}	96.53^{a}	0.369	**< 0.001**	**< 0.001**	**< 0.001**
1 in 10,000	0.00	0.00	10.74	12.79^{d}	41.17^{d}	71.64^{c}	76.98^{bc}	88.53^{ab}	95.80^{a}				
1 in 100,000	0.00	0.00	9.92^{b}	11.79^{d}	24.39^{e}	54.16^{d}	62.10^{c}	78.69^{b}	79.57^{b}				

[1] SEM = standard error of the mean, [a-e] Means within a column that do not share a common superscript are significantly different $p < 0.05$

A breakdown of the individual VFAs can be seen in Table 7-6. For acetate, there was a significant interaction between Time and Dilution ($F_{32, 130} = 6.926$, $p < 0.001$) with significant main effects of Time ($F_{8, 130} = 591.557$, $p < 0.001$) and Dilution ($F_{4, 130} = 169.321$, $p < 0.001$). The least dilute sample (1 in 10) showed the highest concentration of acetate compared to other dilutions across all time points. With time, the concentration of acetate between the other dilutions became more similar (72 hours +). Overall, there was no significant difference between dilutions of 1 in 1,000 and 1 in 10,000, but all other dilutions showed significantly different acetate concentrations ($p < 0.001$). Looking only at Time, there was no difference in acetate concentration at 0 and 6 hours ($p = 0.157$) or at 120 and 144 hour ($p = 0.724$).

Propionate concentration also showed an interaction between Time and Dilution ($F_{32, 129} = 18.376$, $p < 0.001$) with main effects of Time ($F_{8, 129} = 640.241$, $p < 0.001$) and Dilution ($F_{4, 129} = 271.799$, $p < 0.001$). Over the course of the experimental period, the concentration of propionate became more similar between the different dilutions with only the largest dilution (1 in 100,000) significantly different to the others at the last time point. Looking at the main effect of Dilution, all five dilutions were significantly different to one another. In terms of time points, 0 and 6 hours ($p = 1.00$), 72 and 96 hours ($p = 1.00$) and 120 and 144 hours ($p = 1.00$) showed no significant difference in propionate concentration.

A similar pattern was seen for butyrate with again, an interaction between Time and Dilution ($F_{32, 127} = 12.974$, $p < 0.001$) and main effects of Time ($F_{8, 127} = 456.869$, $p < 0.001$) and Dilution ($F_{4, 127} = 18.898$, $p < 0.001$). Butyrate production began for the least dilute (1 in 10) samples at 12 hours and differences between the samples existed until 72 hours of fermentation. After this, no difference was seen between the different dilutions until 144 hours, where the most dilute sample (1 in 100, 000) showed significantly higher butyrate concentration compared to 1 in 10 and 1 in 100 ($p < 0.05$). Examining the main effects, there was no difference between 0 and 6 hours nor 96, 120 and 144 hours. For dilution, the 1 in 100 and 1 in 1,000 were not different in terms of butyrate production nor were the 1 in 10,000 and 1 in 100,000 dilutions.

Finally for A:P, there was again an interaction between Dilution and Time ($F_{32, 129} = 11.468$, $p < 0.001$) and both main effects were significant in the model ($F_{4, 129} = 13.001$, $p < 0.001$ for Dilution and $F_{8, 129} = 59.982$, $p < 0.001$ for Dilution). Initial differences between the dilutions at earlier time pointes were lost between 72 and 120 hours. Interestingly, for Dilution, only the 1 in 10 was significantly different to the other dilutions ($p < 0.001$).

Table 7-6 Volatile fatty acid analysis for each dilution over 144 hours. Mean values are presented and corrected per g DM. All concentrations shown are in mM. Significant values are shown in bold

		Time (hours)										P value		
		0	12	24	36	48	72	96	120	144	SEM	Time	Dilution	Time*Dilution
Acetate	1 in 10	0.0	15.4^{a}	29.0^{a}	38.4^{a}	46.8^{a}	54.3^{a}	56.1^{a}	56.1^{a}	53.2^{a}	2.00	**< 0.001**	**< 0.001**	**< 0.001**
	1 in 100	0.0	3.1^{b}	15.9^{b}	24.2^{b}	32.0^{b}	39.3^{b}	41.2^{b}	42.7^{b}	48.3^{ab}				
	1 in 1000	0.0	0.0^{b}	11.2^{c}	16.4^{c}	27.2^{b}	38.2^{b}	42.7^{bc}	44.5^{b}	46.4^{ab}				
	1 in 10,000	0.0	0.0^{b}	10.7^{c}	12.8^{c}	19.6^{c}	36.9^{b}	38.9^{bc}	44.4^{b}	49.0^{ab}				
	1 in 100,000	0.0	0.0^{b}	9.9^{c}	11.8^{c}	14.8^{c}	25.7^{c}	30.5^{c}	40.9^{b}	40.6^{b}				
Propionate	1 in 10	0.0	2.6	16.0^{a}	23.0^{a}	26.2^{a}	30.2^{a}	31.7^{ab}	31.4^{a}	31.6^{a}	1.26	**< 0.001**	**< 0.001**	**< 0.001**
	1 in 100	0.0	0.0	6.0^{b}	19.3^{a}	25.5^{a}	30.8^{a}	33.0^{a}	32.7^{a}	34.0^{a}				
	1 in 1000	0.0	0.0	0.0^{c}	10.3^{b}	19.8^{b}	25.5^{ab}	28.4^{b}	30.5^{a}	31.5^{a}				
	1 in 10,000	0.0	0.0	0.0^{c}	0.0^{c}	11.1^{c}	20.3^{bc}	21.5^{c}	26.5^{ab}	27.9^{a}				
	1 in 100,000	0.0	0.0	0.0^{c}	0.0^{c}	0.0^{d}	15.2^{c}	11.2^{d}	20.5^{b}	16.7^{b}				
Butyrate	1 in 10	0.0	0.0	11.2^{a}	13.2^{a}	14.1^{a}	15.5	16.7	16.0	15.9^{a}	0.958	**< 0.001**	**< 0.001**	**< 0.001**
	1 in 100	0.0	0.0	0.0^{b}	11.7^{b}	13.9^{a}	14.9	16.2	16.8	16.4^{a}				
	1 in 1000	0.0	0.0	0.0^{b}	6.1^{c}	12.2^{a}	15.6	18.9	18.6	18.6^{ab}				
	1 in 10,000	0.0	0.0	0.0^{b}	0.0^{d}	10.5^{ab}	14.4	16.6	17.6	18.8^{ab}				
	1 in 100,000	0.0	0.0	0.0^{b}	0.0^{d}	7.1^{b}	13.3	16.6	17.3	22.3^{b}				
A:P	1 in 10	0.0	6.0	1.8^{a}	1.7^{a}	1.8^{a}	1.8	1.8	1.8	1.7^{a}	0.231	**< 0.001**	**< 0.001**	**< 0.001**
	1 in 100	0.0	0.0	2.7^{b}	1.3^{a}	1.3^{ab}	1.3	1.3	1.3	1.4^{a}				
	1 in 1000	0.0	0.0	0.0^{b}	1.6^{b}	1.4^{ab}	1.5	1.5	1.5	1.5^{ab}				
	1 in 10,000	0.0	0.0	0.0^{b}	0.0^{c}	1.8^{a}	1.8	1.8	1.7	1.8^{ab}				
	1 in 100,000	0.0	0.0	0.0^{b}	0.0^{c}	0.0^{b}	1.7	2.7	2.0	2.4^{b}				

[1] SEM standard error of the mean, A:P acetate to propionate ratio

[a-c] Means within a column that do not share a common superscript are significantly different ($p < 0.05$).

7.4 Discussion

7.4.1 A higher concentration of rumen fluid results in a more stable bacterial profile but the community continued to diverge from that of the initial inoculum with time

An overarching aim within this thesis was to determine the feasibility of using a batch culture *in vitro* model of the rumen to compare the ability of rumen fluid obtained from different animals within a herd to digest fibre. To be able to do this, it was important to understand how fermentation within a batch culture *in vitro* model affected the complex microbial community within the rumen inoculum. Therefore, a key objective of this chapter was to determine how the concentration of rumen fluid within a batch culture *in vitro* model of rumen fermentation affected the stability of the bacterial community.

Previous results presented in Chapters 4 and 5 showed a rapid decline in alpha diversity across fermentations when using a 1 in 9 ratio of rumen fluid to buffer, with community composition shifting away from that of the rumen fluid inoculum. The underlying assumption for the use of an *in vitro* model is that the microbial community remains functionally similar to that of the rumen (Weimer et al., 2011). To ensure that results seen *in vitro* closely resemble the animal, it is imperative that the microbial community remains as stable as possible through maintenance of microbial diversity and community composition. Within the rumen itself, the microbiome has been shown to converge to an 'adult' profile with age (Rey et al., 2013) and is thought to be relatively stable over time similar to the gastrointestinal tract of humans (Costello et al., 2009; Faith et al., 2013). There are reports of dynamic changes in the rumen bacterial population over the course of two consecutive lactations, however, the bacterial population on the whole were largely similar (Jewell et al., 2015).

As highlighted in the introduction to this chapter, there is little published work within the literature describing the stability of the microbial community within the *in vitro* model, especially the batch culture model and there is no published work examining the effect of the concentration of rumen inoculum on the microbial population using next generation sequencing techniques. The most stable bacterial community, demonstrated through diversity indices in Experiment 1, was achieved with a 1 in 2 ratio of rumen fluid to buffer. Over time, however, all communities diverged from that of the initial rumen fluid irrespective of concentration. This was to be expected somewhat within the batch

model due to the exhaustion of the substrate and build-up of waste products, for example. Therefore, the length of fermentation as well as the rumen fluid to buffer ratio is critical to the stability of the bacterial profile.

Although the 1 in 2 rumen fluid to buffer ratio resulted in a more stable bacterial community profile compared to the higher dilutions, even at this dilution alpha diversity decreased after 6 hours of incubation (first sampling point) suggesting that the immediate environment within the model caused rapid changes to community composition with the loss of alpha diversity highlighting, potentially, the poor survivability of some rumen bacteria, either within the model or following the freeze-thaw process (Prates et al., 2010). Alternatively, the decline in diversity may be an artefact of the feeding process. Shaani et al. (2018) showed a decline in alpha diversity (Chao1) following feeding *in vivo*, which they stated was due to niche modification (e.g. a decrease in pH) selecting for a different microbial population.

The increased concentration of rumen fluid (1 in 2) was found to mitigate the loss of diversity somewhat compared to other concentrations (1 in 4, 1 in 9), but, compared to the neat inoculum, a decline of *ca* 36.7, 42.3 and 54.5 % of the Chao1 measure of alpha diversity was observed when comparing values at the beginning (0 hours) and end of the incubation (48 hours) for 1 in 2, 1 in 4 and 1 in 9 respectively. All three concentrations of rumen fluid to buffer ratio stabilised at around 24 to 48 hours shown by a plateau in alpha diversity indices reaching final Chao1 values of 7071.9 ± 312.7, 6710 ± 49.8 and 5307.8 ± 232.2 for 1 in 2, 1 in 4 and 1 in 9 respectively across the 24-48 hour period. The stabilisation of the microbial diversity may have been due to the exhaustion of substrate. However as the IVDMD continued to increase with time, it may be that the soluble fraction became exhausted leaving a more 'stable' fibre fraction for the microbiota to digest.

Lengowski et al. (2016) found a decline in bacterial number within the first few hours of sampling in a RUSITEC system measured using qPCR and found that bacterial number stabilised around 24 hours into the experimental period. As discussed in Chapter 5, Soto et al. (2013) also saw a decrease in alpha diversity (Shannon index and Pielou evenness) after 24 hours of fermentation in a batch model system. It would appear, therefore, that when using an *in vitro* batch culture model of rumen fermentation, a decline in alpha diversity is inevitable and this may be an artefact of the model, a conclusion also arrived at by Fraga et al. (2015).

The observed decline in alpha diversity occurred despite providing the same substrate to the fermenters as the animal was fed immediately prior to slaughter. The same diet was provided to prevent any changes in microbial composition due to substrate as diet has been shown to be the greatest cause of variation in the rumen microbial population (Henderson et al., 2015). In previous chapters (Chapters 4 & 5), where a decline in alpha diversity was observed, a dried grass substrate was provided to the fermenters which differed from the diet provided to the animal prior to slaughter. This suggests that the loss of diversity seen across this thesis was not due solely to substrate and was also due in part to the different environmental conditions imposed by the *in vitro* model when compared with the rumen.

Beta diversity, the difference in community structure between samples, was also found to be significantly affected by both rumen fluid to buffer ratio and time of fermentation. This change in beta diversity indicated that the bacterial community was diverging with time from that of the inoculating rumen fluid across all rumen fluid to buffer ratios and the effect was more pronounced with increased dilution of rumen fluid shown by the increased distance between points on the NMDS plots. Batch cultures, in a study by Machado et al. (2018), showed a consistent decline in Bacteroidetes:Firmicutes ratio across fermentations when compared to the neat inoculum as was also shown in this study, with ratios decreasing from 1.52 in the neat inoculum to 0.54, 0.52 and 0.59 for 1:2, 1:4 and 1:9 respectively. The authors also showed a clear difference in community structure from 48 to 72 hours of fermentation through principle coordinate analysis (PCoA). Soto et al. (2013) further showed that the community composition present after 24 hours of fermentation in a batch culture model was rather different to that of the neat inoculum with a large increase in fibrolytic species, likely due to the removal of the solid attached bacteria during inoculum processing.

Benincà et al. (2008) discovered that in a controlled laboratory environment over 2,000 days, a microbial community cultured in a mesocosm showed striking fluctuations in species abundance over orders of magnitude despite constant environmental conditions and it has been suggested that the bacteria themselves host a circadian clock (Lenz and Søgaard-Andersen, 2011). Indeed, Paulose et al. (2016) showed that some members of the human gut microbiome (e.g. *Enterobacter aerogenes*) are reactive to melatonin, resulting in periods of swarming (a period of swimming and division) and motility. Natural fluctuations in the bacterial community may have been responsible for some of the observed changes in bacterial community composition.

The findings presented in this chapter showed that the community diverged away from that of the neat inoculum, but the difference was greater in less concentrated rumen fluid:buffer ratios. In order for the bacterial community to remain as stable as possible, when using fresh grass as a substrate, a 1:2 ratio of rumen fluid to buffer should be used.

7.4.1.1 Key differences in bacterial composition between the rumen fluid to buffer ratios

A key difference in the bacterial composition of fermentation fluid between the different concentrations of rumen fluid used to inoculate the fermentation bottles was the relative abundance of the phylum Proteobacteria. Only the 1 in 2 ratio was found to maintain Proteobacteria at a similar relative abundance as recorded for the neat inoculum (0.98 vs 2.10 ± 2.19, 8.14 ± 6.25 and 17.46 ± 12.08 % for neat vs 1 in 2, 1 in 4 and 1 in 9 respectively). With the larger dilutions (1 in 4, 1 in 9) the level of Proteobacteria generally increased with time and Proteobacteria had the highest relative abundance in the 1 in 9 ratio samples. Proteobacteria is a commonly identified phylum present in the rumen and has been shown to be a phylum with high metabolic activity despite relatively low abundance (Kang et al., 2013). The ratio of Proteobacteria to Firmicutes plus Bacteroidetes has been suggested by Auffret et al. (2017) to be an indicator of dysbiosis in the rumen. In the current study, the Proteobacteria to Firmicutes plus Bacteroidetes ratio was found to be similar in the 1 in 2 ratio as in the neat inoculum (0.01 ± 0.002 *vs* 0.02 ± 0.03 for neat and 1 in 2 respectively), and values increased with a reduction in the concentration of rumen fluid (0.10 ± 0.08 for 1 in 4 and 0.25 ± 0.19 for 1in 9). This would suggest the increase in Proteobacteria, especially in the 1 in 9 ratio of rumen fluid to buffer, is indicative of dysbiosis in the *in vitro* model. As mentioned previously, an element of dysbiosis is inevitable when using a batch culture model, however, it would appear that by using an increased concentration of rumen fluid to buffer (1 in 2) the effects can be mitigated somewhat.

In addition to changes in the phylum Proteobacteria, the genus *Oribacterium* (Phylum: Firmicutes, Order*: clostridia)* was found to increase over the course of the fermentation. In Chapter 4 it was discussed that *Oribacterium* is an opportunistic genus that can thrive in conditions of dysbyosis (Shen et al., 2017). *Oribacterium* was at its most abundant at the last sampling time point (1.52 ± 0.06 *vs* 14.40 ± 1.57 % at 0 hours and 48 hours respectively) and increased in all three ratios of rumen fluid to buffer across the fermentation (average abundance of 8.87 ± 4.11, 11.28 ± 6.23 and 10.34 ± 6.23 % for

1:2, 1:4 and 1:9 respectively). The batch culture *in vitro* model provides feed and buffer to the system only at the beginning of the experiment and therefore substrate may become limiting towards the end of a fermentation. For example, the water-soluble carbohydrates, which are readily available at the beginning of the fermentation, will be rapidly used and will become limiting with more highly fibrous plant particles remaining. Indeed, IVDMD and fermentation parameters such as gas volume produced indicated that fermentation plateaued by *ca* 36 hours, with IVDMD reaching a maximum of 69 g/100g DM for the 1:2 and 1:4 ratio of rumen fluid to buffer.

For this chapter, the most up to date version of the SILVA database was used for sequence alignment resulting in the identification of phyla not previously identified in this thesis or indeed previously published literature on the rumen microbiome. In the latest update, the new Phyla added that were identified in samples were Kirimatiellaeota and Patescibacteria. Kirimatiellaeota is part of the PVC superphylum, named after the three main phyla that make up this group, namely Planctomycetes, Verrucomicrobia and Chlamydiae (Rivas-Marín and Devos, 2017), and has been shown to be widespread in anoxic environments from hypersaline sediments to the gastrointestinal tract of animals (Spring et al., 2016). Patescibacteria is another super phylum, first described by Rinke et al. (2013), which have limited metabolic capabilities. The presence of these phyla within the rumen microbiome are not unexpected.

The findings of this section suggest that for the microbial community to more closely resemble that of the host animal, the highest rumen fluid to buffer ratio should be used. After *ca* 24 hours of fermentation, alpha diversity profiles appear to stabilise, but community composition (beta diversity) continues to diverge from the initial rumen inoculum with time. When different substrates are used, the stability of the bacterial population should be further explored as the community dynamics may be different.

Further work should explore the effect of rumen fluid to buffer ratio on metabolic function and activity. As described by de la Fuente et al. (2017), functional resilience in the microbial community of the rumen dictates that changes in microbial composition may not necessarily be indicative of changes in function. It would be of interest to study the microbiota and fermentation parameters of the batch culture model inoculuated with rumen fluids sourced from fistulated animals that had been performance tested *in vivo,* the same as in the batch culture model at the time of withdrawing the rumen fluid to inoculate the batch culture model.

7.4.2 Rumen fluid to buffer ratio affects both IVDMD and fermentation parameters

Throughout this thesis a 1 in 9 ratio of rumen fluid to buffer was used. When fistulated animals are not freely available to allow rumen fluid collection, it is important to maximise the use of the rumen fluid collected from the abattoir. A 1 in 9 ratio maximised the number of fermentation bottles that could be used in each experiment whilst maintaining a good rate of gas production. In this chapter, as well as establishing the effect of rumen fluid to buffer ratio on the rumen bacterial community, fermentation parameters were also compared to examine how different ratios affect *in vitro* performance in a batch culture model of rumen fermentation.

Each of the three rumen fluid to buffer ratios showed no significant difference in IVDMD after 36 hours of fermentation. Maximum digestibility of the fresh grass over a 48 hour fermentation was 69 g/100 g DM. With fresh grass as a substrate, fermentations should not be performed for longer than 36 hours, as there was found to be no significant difference between the fermentation bottles containing rumen inoculum and blank bottles containing only grass and buffer after this (48 hours). It is important to know how the solubility of the substrate and the microbial community associated with the substrate behave within the fermentation to ensure that any differences in digestibility observed early in the fermentation between treatments are not masked, especially by the solubility of the substrate.

Rymer et al. (1999) showed that increasing the concentration of rumen fluid in a batch culture *in vitro* model advanced the digestion of the insoluble fraction, but the rate of fermentation remained the same. This suggests that an increased proportion of rumen fluid improved the ability of the microorganisms to bind to the substrate, or the time taken for the microorganisms to bind, as a function of microbial number, but did not affect the rate at which the substrate was digested. This is supported by data presented in Experiment 2, where a 10 fold difference in rumen fluid concentration (1 in 10 *vs* 1 in 100) showed no significant difference in the slope of the fitted curve.

Interestingly, for total volatile fatty acid production, there was little difference between the three ratios (Experiment 1) in terms of concentration within a time point. This is supported by an experiment performed by Navarro-Villa et al. (2011) who showed that across three substrates (barley straw, grass silage and barley grain) at three different

amounts of substrate added (0.3, 0.5 or 0.7 g) there was no effect of rumen fluid to buffer ratio (1 in 2, 1 in 4, 1 in 6) on total VFA production.

In previous experiments within this thesis, as well as Experiment 2 within this chapter, dried grass was used as a substrate in comparison to fresh grass in Experiment 1. When comparing performance of the 1 in 9 rumen fluid to buffer ratio across the two substrates within this Chapter, acknowledging the grass substrates were not related in anyway, after 48 hours of incubation gas production (207.9 *vs* 109.8 ml per g DM) and VFA concentration (87.1 *vs* 62.0 mM per g DM) were lower when fresh grass was used. The pH (6.49 *vs* 6.81) was lower and NH_3-N (1.31 *vs* 1.04 mg/ml) and MCP (1257.2 *vs* 358.1 µg/ml) were higher after 48 hours of fermentation when dried grass was provided. Mohammed et al. (2014) also showed that when heifers were transferred from orchard grass pasture, to orchard grass hay and then back to pasture, total VFA production was also highest when the dried forage was provided (182.2 *vs* 132.7 mM for orchard grass hay and pasture respectively). Conversely, higher VFA production with grazing animals compared to grass hay and silage has also been observed (Holden et al., 1994). Lowman et al. (2002) suggested that the process of drying grass increased the surface area available to microorganisms, which therefore increased microbial attachment and resulted in both higher gas production and volatile fatty acid production in dried versus fresh grass despite similar digestibility values.

7.4.3 The epiphytic community fermented the substrate to the same extent as rumen inoculum after 48 hours of fermentation

No inoculum blanks were included within Experiment 1 to determine both the solubility of the fresh grass and the ability of the grass associated (or "epiphytic") community to digest the substrate. The epiphytic community was shown to digest the fresh grass to the same extent as bottles containing rumen inoculum at 48 hours of *in vitro* fermentation (69 *vs* 69 *vs* 62 *vs* 62 g/100g DM for 1 in 2, 1 in 4, 1in 9 and grass associated bacteria respectively; $p > 0.05$). In previous chapters, in which dried grass was used as a substrate, digestion by the epiphytic community was found to take *ca* 72 hours in comparison to 48 hours within this chapter. The difference between the two substrates is likely due to the difference in processing however it must be reiterated that the two grass samples were not related in any way. The fresh grass used in this chapter was cut from the field and immediately frozen at -20°C until use in the model. The dried grass was cut and then flash-dried. It is probable that the process of flash drying may have reduced the surviving

microbial load on the surface prior to fermentation or may have reduced microbial growth through dehydration of cells (Monteiro et al., 2016).

The microbial community associated with the fresh grass substrate was dominated initially by Bacteroidetes (54%), Proteobacteria (26.9%) and Actinobacteria (9.3%) in agreement with Belanche et al. (2017). The epiphytic community was shown to clearly digest the grass substrate as evidenced by the increased IVDMD alongside production of both gas and VFAs across the experimental period in bottles that did not contain rumen inoculum. The concentration of MCP was negligible relative to samples containing rumen fluid across the experimental period, but appeared to increase over the fermentation reaching a final concentration of 33.4 µg/ml at 48 hours compared to an average of 467.5 µg/ml for samples containing rumen fluid. Despite an MCP concentration an order of magnitude lower than that of bottles containing rumen fluid, IVDMD was the same after 48 hours of fermentation. As discussed previously (Section 6.3.2), the MCP assay determines the 'free' MCP in the rumen liquor as feed particles are removed via centrifugation prior to the assay. The negligible MCP concentrations measured prior to the 48 hour sample may be due to the majority of the microorganisms being attached to the substrate and therefore not being included in the assay and/or simply that the concentration of microorganisms present was below the lower limit of quantification of the assay (0.01 mg/ml of protein (Walker, 1996)). Similar to Chapter 6, no propionate was produced from the no inoculum blank bottles until 48 hours of fermentation had occurred (data not shown). Despite their ability to digest the substrate, the epiphytic bacterial community were not observed (< 0.1% relative abundance) in bottles that also contained rumen fluid. The bacterial genera associated with the substrate would thus appear to be rapidly outcompeted by the rumen inoculum, again in agreement with Belanche et al. (2017). To further highlight this, the NMDS plot in Figure 5 shows a clear separation of all samples containing rumen inoculum from those containing only grass and buffer.

7.4.4 Incredibly large dilution rates have little effect on digestibility of dried grass within an *in vitro* model of rumen fermentation

Cattle likely vary in the concentration of microorganisms that reside within their rumen due to many factors, such as flow rate which can be partitioned into the rumen fluid dilution rate and the rumen particle dilution rate. It is a reasonable assumption that an animal with fewer bacterial cells present will have a lower digestive capability due to the

simple fact that there are fewer microorganisms present to digest the substrate as well as lower microbial activity. The batch *in vitro* model used throughout this thesis was performed with a 1 in 9 ratio of rumen fluid to buffer resulting in a 10 x dilution of the original rumen inoculum. In Experiment 2, a serial dilution of rumen inoculum was performed resulting in a range of dilutions from 10 to 100,000 x.

The extremely large dilution rates had little effect on the ability of the microbial community to digest the substrate. Over a 144 hour period, all of the ratios of rumen fluid to buffer with the exception of 1 in 100,000 had digested the substrate to the same extent reaching final digestibility values of 75.3, 79.3, 79.0, 76.8 and 69.2 g/kg DM for 1 in 10, 1 in 100, 1 in 1000, 1 in 10,000 and 1 in 100,000 ratios of rumen fluid to buffer respectively, although progressive dilutions affected the rate of digestion and thus the time taken to achieve the stated digestibility values. Rymer et al. (1999) showed that with increasing concentration of rumen inoculum in an *in vitro* model, organic matter apparently degraded tended to be reduced (e.g. 70.3, 68.3 and 65.9 % for 5, 15 and 30 % rumen fluid concentration). The authors suggested that when the concentration of rumen fluid was low, more of the degraded substrate was used for microbial growth and diverted away from gas production. Although lower in number, the microbial community present had the potential to grow rapidly as there was more substrate available to support growth.

Indeed, there was little difference in microbial concentration (MCP) between the 1 in 1,000, 1 in 10,000 and 1 in 100,000 ratios across the course of the experiment (Table 7-4) and after 144 hours, there was no difference in concentration between any of the dilutions, reaching a final concentration of 926.1 ± 47.01 µg/ml. Gas production, however, was lower in the more diluted samples as suggested by Rymer et al. (1999). Due to the initial lower microbial concentration in the larger dilutions, there was likely less competition for binding sites on the substrate, therefore the microorganisms that were best adapted to the substrate and environment within the model may have been able to divide and dominate. Due to the short time needed for bacterial replication, the population was able to recover from dilution. As the rumen is estimated to contain *ca* 10^{10} bacterial cells per ml (Hungate, 1966; Rey et al., 2013), even after a 1 in 100,000 dilution an estimated 10^5 cells per ml of rumen fluid were still transferred into the model. Large dilution of rumen inoculum may provide different selection pressures in comparison to more concentrated rumen fluid.

It is unlikely that large differences in microbial population were responsible for the differences between the ‘Good’ and ‘Bad’ animals identified in Chapters 4 and 5. Indeed,

the OD600 of the rumen fluids used as the 'Good' in Chapter 4 was higher than that of the 'Bad' (0.391 ± 0.008 *vs* 0.337 ± 0.006; t = -3.30, df =4, p = 0.030), but this was reversed in Chapter 5 where the OD600 was significantly higher in the 'Bad' than the 'Good' (0.358 ± 0.022 *vs* 0.307 ± 0.015; t = 9.13, df =4, p < 0.001). OD600 is a measure of absorbency with the optical density frequently used to measure bacterial population size (Rehse et al., 2010; Zhou et al., 2012b). For Chapter 5, the ODs of 0.307 for the Good and 0.358 for the Bad corresponded to MCP values of 970 and 1078.3 µg/ml respectively. Due to a lack of 'Bad' rumen fluid, MCP was not recorded for the inoculum from Chapter 4.

Where the ratio of rumen fluid to buffer was low (1 in 10,000, 1 in 100,000) the rumen microbial community was shown to recover over the course of the extended fermentation. However, retention time of feed in the rumen is typically 36-48 hours depending upon the diet fed (Krämer et al., 2013; Ribeiro et al., 2017), therefore it is likely that extremely low microbial concentrations would results in lower IVDMD and VFA provided to the host animal. In this case, it is likely that some groups of microbes may be washed out of the rumen before they are able to attach to the substrate and begin digestion, or, may be subject to washout if the rate of dilution is greater than their rate of growth (Allen and Mertens, 1988). However, despite this, Experiment 2 showed that the rumen microbial population is extremely resilient to huge perturbations to their numbers.

7.4.5 Conclusion

As the microbial community play such a pivotal role in digestion in ruminant animals, it is imperative that we understand the dynamics of this community in order to allow us to manipulate fermentation in such a way that can improve the efficiency, productivity and health of the animal whilst reducing greenhouse gasses and environmental pollutants. The *in vitro* model provides a platform to study the rumen microbiota in a controlled laboratory setting, however, little is known about how the concentration of rumen fluid affects the microbial population in an *in vitro* batch model of rumen fermentation.

This study is the first where NGS has been used to explore the stability of the bacterial community in a batch model under different rumen fluid to buffer ratios. The microbial community within the *in vitro* batch model of rumen fermentation was shown to change over the course of a fermentation, regardless of the concentration of rumen fluid added. However, increasing the concentration of rumen fluid maintained more bacterial diversity. The microbial community within control fermentation bottles and samples

from the initial inoculum must be included in NGS studies. Large increases in the amount of buffer relative to rumen fluid were found to have only short term effects on fermentation over 144 hours.

In order to directly relate *in vitro* studies to the animal there is a need for a greater understanding of microbial dynamics within the model. To aid with this there is call for a unified approach to *in vitro* batch studies between institutions. This chapter has highlighted how rapidly the bacterial community changes when the effect of the host is removed, despite providing the same diet to the fermenters as the animal was fed. Future work should examine whether microbial activity is perturbed by different inclusion rates of rumen fluid.

Chapter 8 General Discussion

Individuals within a herd differ in their ability to digest fibre despite the same diet, breed and management (Jami and Mizrahi, 2012; Shabat et al., 2016), suggesting that animal factors play a large role in an animal's fermentative digestion. With the expected increase in the human population towards 2050 and the increased demand for meat and milk (FAO, 2017), there is much interest in understanding differences in digestive performance between individuals. By manipulating rumen fermentation, efficiency could be increased and the negative environmental impacts associated with ruminant production reduced (Díaz et al., 2017). Previous attempts to manipulate the mature rumen community through introduction of bacterial species (see Table 1-1) and cross-inoculation of entire rumen content (Weimer et al., 2010; Ribeiro et al., 2017; Zhou et al., 2018) have proven unsuccessful and it is thought that this is due to 'host-effects' on the residing microbial population (Section 1.4.9).

The aims of this thesis were to determine whether a batch *in vitro* model of rumen fermentation could be used to study the fermentative digestion of high fibre feeds by different rumen fluids and their associated microbiota, specifically the bacterial population and to identify whether the *in vitro* model could be used to determine if it is possible to manipulate the rumen microbiota in favour of fibre digestion *in vitro* where attempts to do so *in vivo* have failed, due to the absence of control by host regulatory mechanisms. The extent to which the thesis presents evidence to answer these aims are discussed below.

8.1 The use of an *in vitro* model to study fermentative digestion of different rumen fluids

In vitro models of the rumen provide a potential means to study the rumen microbiota and their fermentative digestion of feed in the absence of host regulatory mechanisms. Although *in vitro* models have been in use for decades, as described in Section 1.5.1.1, the usual application of the batch culture model involves the use of a pooled rumen fluid and multiple different test substrates or feed additives. There has been little consideration for whether the *in vitro* model can be used to identify and explain possible differences in fermentation, digestibility and effects on microbial populations between individual donor animals.

Findings in this thesis have shown that the batch culture *in vitro* model of the rumen can be used to identify differences in fermentative digestion of high fibre diets between rumen fluids sourced from different animals (Chapters 4 and 5). In Chapter 4, there was a clear difference in fermentative digestion of a high fibre substrate between 10 rumen fluids collected from a group of Holstein-Friesian cattle at time of slaughter. These animals were from the same herd. All fermentation parameters (gas production, pH, total VFA production, ammonia-nitrogen, microbial crude protein and the acetate to propionate ratio), as well as digestibility, were shown to differ between the individual rumen fluids when the same substrate and environmental conditions were provided. *In vitro* dry matter digestibility (IVDMD) ranged from 22 to 37 g DM/ 100g DM over a 24 hour period. In Chapter 5, rumen fluid was collected from 11 genetically similar (sired by the same bull) Charolais-cross steers that had been raised on the same farm from birth on a high fibre diet. In this chapter, although the rumen fluids were more similar in their ability to digest DM (ranging from 36 to 40 g DM/ 100 g DM), there was still shown to be a significant difference in IVDMD between the individual rumen fluids.

To ensure that the microbial concentration of the rumen fluid was not responsible for the differences in performance observed, a rumen fluid dilution series was performed (Chapter 7, Experiment 2). Even with incredibly large dilutions to the rumen fluid, fermentative digestion was able to recover. There was shown to be no difference in the rate of fermentation between a 1 in 10 and 1 in 100 dilution of rumen fluid. In Chapter 4, while the OD600 of the Good fluid was higher than that of the Bad, this was reversed in Chapter 5. Therefore, it is very unlikely that differences in bacterial concentration were responsible for the differences in observed performance.

Overall, the batch *in vitro* model was found to be a useful tool to explore the differences in fermentative performance between rumen fluids sourced from different animals. This opens up avenues beyond the scope of this thesis to allow thorough investigation of individual responses to different substrates and additives in a controlled environment. It would be useful to compare *in vivo* and *in vitro* digestibility across a range of animals when provided with the same substrate to confirm that the differences seen in the model are reflective of the true animal performance. Although comparisons between *in vitro* and *in vivo* performance have been performed (Section 1.5.1), there is little available data across a range of individual animals. The model allows larger sample sizes and allows for experimentation in isolation from the animal, therefore reducing the costs associated with animal trials and adhering to the 3Rs (Animals (Scientific Procedures) Act (1986)).

8.2 The use of an *in vitro* model to study the bacterial population of rumen fluids that differ in their fermentative digestion of high fibre feeds

Due to the nature of the *in vitro* model, the differences in performance as described above were assumed to be microbial in origin. Therefore, it was of interest to determine whether a pipeline could be established to study the microbial population within the batch *in vitro* model across a fermentation. The bacterial population was the focus of this thesis as this community is the most numerous in the rumen and has received by far the most research attention (Hungate, 1966; Zhou et al., 2015). A pipeline was successfully established and examined for its accuracy (Chapter 3).

The bacterial rumen community has been shown to differ under different dietary conditions such a diet rich in fibre compared with a diet rich in concentrate (McDermott, 2014; Henderson et al., 2015) and when exposed to different experimental treatments such as the addition of essential oils and vitamin E supplementation (Khorrami et al., 2015; Belanche et al., 2016). As diet is considered to be the largest determinant of microbial composition in the gut of the animal (Henderson et al., 2015), it is logical to expect animals managed in the same way have a similar community composition. However, there are numerous studies reporting differences in the rumen microbiota between animals within a herd (Guan et al., 2008; Hernandez-Sanabria et al., 2012; Jami and Mizrahi, 2012; McCann et al., 2014), suggesting variation in animal performance is likely, to some extent, to be determined by the rumen microbiota present and their metabolism. Therefore, it was hypothesised that the difference in performance between a 'Good' and 'Bad' rumen fluid, in terms of its ability to digest DM, would be reflected in the bacterial community. However, no difference in bacterial composition was observed across both alpha and beta diversity measures.

Bacterial composition was very similar between the Good and Bad rumen fluids throughout fermentations (Chapters 4 and 5) and in the neat inoculum (Chapter 5). Therefore, it may be that the bacterial community is not an accurate representation of performance and it may be more important to explore what the community is doing through the use of 'omics technologies. Prediction of gene function can be performed from 16S rRNA sequencing reads through programs such as PICRUSt (Langille et al., 2013) and Piphillin (Iwai et al., 2016), however, due to a lack of information on a large number of the rumen species present, this has not been done on the data presented in this

thesis. Upon completion of projects such as the Hungate 1000 (http://genome.jgi.doe.gov/TheHunmicrobiome/TheHunmicrobiome.info.html), which aims to sequence the genome of un-cultured rumen species, this may be something that can be routinely performed to supplement rumen microbial studies. Furthermore, as the rumen contains much more than just the bacterial community, it follows that other elements within the rumen fluid may be responsible for the differences in digestive performance observed. Or indeed, it may be a combination of the two.

8.2.1 Limitations of the use of a batch *in vitro* model to study the bacterial population

Whilst it was possible to use the *in vitro* model to study the bacterial population associated with different rumen fluids, the population structure was largely affected by the time at which the samples were taken and differences between the fluids were minimal. The bacterial population within the *in vitro* model was shown to change over the course of a fermentation (Chapters 4, 5 and 7) away from that of the initial inoculum. Despite this, the changes in bacterial population structure were found to be repeatable between fermentation bottles (Chapter 3) and could be mitigated somewhat by increasing the concentration of rumen fluid used as an inoculum (Chapter 7). For future studies that wish to observe the microbial population within an *in vitro* model when using a high fibre diet, a high concentration of rumen fluid to buffer (such as 1:2) is recommended to ensure that changes in the bacterial population due to the model are limited.

There are three possible reasons for the observed change in bacterial population with time. The first is that the environment within the *in vitro* model exerted a different selection pressure than the rumen itself. This is especially likely over longer fermentations where the substrate becomes limiting and products of fermentation are not removed. Indeed, changes over time across the fermentation were to be expected as the water soluble carbohydrate fraction of the diet became limiting and fermentation shifted to the lignocellulose fraction of the substrate. The second factor to consider is the removal of the selection pressure exerted by the host animal within the model. Although not fully elucidated, the host is thought to select for a particular microbial community through a range of factors as described in the General Introduction (Section 1.4.9) including the immune system, rumen environment (e.g. pH and VFA absorption rate) and the retention time of feed. Through the use of an *in vitro* model, the direct influence of the animal is removed, therefore, these constraints on the bacterial population have

been removed allowing the community to adapt to the substrate and environment. The final possible reason was the influence of the epiphytic community associated with the substrate. In the mature animal, this community is likely to be transient, however, as the batch model is a closed system these organisms may have been able to establish.

Evidence from this thesis and the literature suggests that the observed change in the bacterial population with time is due to a combination of both the environment and the removal of host constraint. As these two factors are confounded, it is not possible to distinguish one from the other. The bacterial epiphytic community is not thought to have any effect as shown from evidence accrued in Chapter 6. Here, the role of the epiphytic community was examined. Although able to ferment the substrate given enough time, the epiphytic community were not responsible for driving microbial composition change. The epiphytic bacterial community associated with the substrate was not identified in fermentation bottles that also contained rumen fluid suggesting that the community was rapidly outcompeted by ruminal bacteria as also shown by Belanche et al. (2017).

The change of bacterial population over time within *in vitro* models of rumen fermentation has been observed previously (Weimer et al., 2011; Soto et al., 2013; Lengowski et al., 2016; Mateos et al., 2017). It is likely that the model is selecting for a sub-community of the bacterial community for the reasons described above (change in environment and loss of host control), indicated by the loss of alpha diversity over the first 24 hours of fermentation (Chapters 4, 5 and 7). The loss of diversity was initially thought to be due to the use of a different substrate within the model compared to that which the animal was fed. Diet has been shown to be the leading factor in determining microbial community composition (Henderson et al., 2015). The decline in alpha diversity, however, was still observed when the same substrate was provided to the model as the donor animal was grazing immediately prior to slaughter. The change in bacterial composition over time was shown with both a dried grass substrate (Chapters 4 and 5) and the same substrate as the animals were grazing prior to slaughter (fresh grass; Chapter 7). There is evidence to suggest that the observed decline in alpha diversity may in fact be a result of the addition of substrate and not a detrimental effect of the model (Shaani et al., 2018). After a meal, the environment within the rumen, and indeed the model undergoes transitory modification due to an increase in carbohydrate fermentation, resulting in an increase in VFA concentration and a reduced pH. Shaani et al. (2018) showed that as a function of time relative to the delivery of feed, alpha diversity decreased quadratically.

As mentioned at the beginning of this section, the change in the bacterial population invoked by the environment within the model and/or the removal of host constraint was shown to be consistent across multiple fermentation bottles (Chapter 3). Therefore, for any studies that examine the bacterial community composition within a batch *in vitro* model, confidence can be placed in the fact that the individual fermentation bottles are providing the same selection pressures, resulting in the same bacterial communities within a treatment group. However, when using a batch *in vitro* model of rumen fermentation, it is important to consider the effect of the model environment on the bacterial structure and document the initial starting bacterial composition to allow determination of treatment *vs* environment effects.

A further possible explanation for the change in population structure over time was that the protozoal population (although not measured in this thesis) was expected to be minimal within the batch *in vitro* model of rumen fermentation. This is due to the fact that the rumen fluid used was frozen (-80°C) for storage prior to use in the model and protozoa are lost after freezing (Yáñez-Ruiz et al., 2016). Protozoa predate on bacteria within the rumen and therefore have an important role to play in the establishment and maintenance of the bacterial population. Protozoa are known to not persist well in both batch (Soto et al., 2013) and continuous culture (Cabeza-Luna et al., 2018) models of rumen fermentation and due to this, an element of control of the bacterial community is also lost. The bacterial community is no longer under the same constraints and therefore species that may have been previously kept at low levels are potentially able to flourish. In defaunated animals, it has been shown that in the absence of protozoa the bacterial community composition is simplified and less diverse (Belanche et al., 2012b; Belanche et al., 2015).

8.3 The use of an *in vitro* model to manipulate the rumen bacterial population to favour improved digestibility in the absence of host effects

Some animals are more efficient than others at digesting fibre. Some of this difference is believed to be due to the rumen microbial community. Therefore, there is interest in improving the performance of less efficient animals by manipulating their microbiota, e.g. by inoculating them with rumen fluid from efficient animals, the assumption being the microbiota of the more efficient animal will have a competitive advantage over that of the less efficient animals due to its better energy harvesting ability. However, attempts

to manipulate the rumen microbiota *in vivo* have failed (Weimer et al., 2010; Ribeiro et al., 2017; Zhou et al., 2018). The microbiota reverts back to 'type', and it is hypothesised this is due to the influence of the host on its microbial population (Section 1.4.9). Using an *in vitro* model of the rumen, the influence of the host can be removed and therefore it was hypothesised that attempts to manipulate the microbiota should be successful, success being measured as a conversion of the initial mixed microbiota to that of the microbiota from the efficient animal. However, this was not the observed outcome. Instead the bacterial composition of all three inocula (Good, Bad and Mix) changed with time converging on a similar bacterial composition and level of performance, a performance that was markedly better than that of the Good after the initial 24 or 48 hours of incubation.

In Chapter 4, there was an initial improvement in IVDMD after 24 hours of fermentation, with the cross inoculated fluid (1:1 Mix) of intermediate performance between the Good and Bad. However, this difference was lost with time as the three rumen inoculums reached similar performance across a range of fermentation parameters. In Chapter 5, there was no improvement in the 1:1 Mix compared with the Bad and the two fluids performed similarly. As with Chapter 4, all three fluids improved in terms of their IVDMD across the course of the experimental period, reaching the same end point.

The diet of the cattle used in Chapter 4 was unknown, but it was likely that there had been at least some concentrate in the diet prior to slaughter. As only a high fibre diet with no concentrate (dried grass) was provided to the *in vitro* model, it is possible that the dietary change caused perturbation to the community and this allowed the microbial community from the 'Good' fluid to establish somewhat. As described in the General Introduction (Section 1.4.8.1), some promising results were observed for inoculation of steers with *Megasphaera elsdenii* and *Butyrivibrio fibrisolvens* when these species were inoculated into the rumen at the time of dietary change (Klieve et al., 2003), which supports the hypothesis that a disturbance to the community may be needed to improve the success of inoculation studies. In Chapter 5, although the substrate was not the same as the cattle had been fed prior to slaughter, the composition was more similar such that each was high in fibre (dried grass *vs* fresh grass respectively). The microbial community associated with these samples was likely more suited to the substrate, resulting in less perturbation upon inoculation of the model and this may explain the lack of improvement observed and the differences between the two chapters.

The gut microbiota has been described as an ‘ecosystem on a leash’ due to the controlling effect of the host on the composition of the microbial community (Foster et al., 2017). It is possible that even in the absence of this controlling host effect, conferred by the *in vitro* model, the bacterial community itself is resilient to manipulation through many of the principals associated with community ecology. Principals such as priority effect, niche defence and competitive exclusion may all play a role in the establishment and malleability of the rumen community. Network analysis of microbial communities is likely to play a key role moving forwards in understanding the complexity of the rumen ecosystem.

8.3.1 Other members of the microbial ecosystem may be responsible for unsuccessful attempts to manipulate the bacterial population

As well as bacteria, there are many different microorganisms that inhabit the rumen, all of which may play a role in the success of cross inoculation experiments and may explain the differences in performance observed in this thesis.

The rumen virome has been largely overlooked when compared with bacteria. From the late 1960’s until the 1990’s there were many studies documenting morphology of rumen phages (see Gilbert et al. (2017)), however, there are only a few studies using new sequencing technologies (Anderson et al., 2017; Gilbert et al., 2017; Parmar et al., 2016; Ross et al., 2013; Berg Miller et al., 2012). Large individual variation in phage populations has been identified when using animals of the same breed, age and from animals fed the same diet housed together (Ross et al., 2013). Bacteriophages have been shown to play a role in structuring, activity, dynamics and diversity of microbial communities and are a part of the normal gut virome (Letarov and Kulikov, 2009).

The individuality of the rumen viral community and its close association with bacteria suggests this may be a key factor in successful manipulation of the rumen bacterial community. The results observed may also have been influenced by the viral community composition. It may be that when bacteria that are not equipped to deal with the phages present are introduced into a non-native rumen, they are quickly lost through infection or may become less competitive through disruption of genes during lysogeny of viral DNA resulting in unsuccessful cross inoculation. Indeed, the virome has been shown to be very important in the success of human faecal transplants. When treating *Clostridium difficile* infection (CDI), human faecal transplant (FT) has been a successful treatment, but in 10-15% of cases the transplant does not work. In human transplants, the large

intestine is first cleansed of its microbial community through the use of antibiotics and enema. The transplant from a healthy donor is then used to re-seed the large intestine and out-compete *C. difficile*. Recently, it has been identified that unsuccessful transplants may be due to the viral community present both in the recipient and in the donor samples (Zuo et al., 2017). Patients with CDI show an increased number of Caudovirales bacteriophages, but with low richness and evenness compared to healthy controls. Successful transplants were associated with donor samples that showed a higher Caudovirales richness than the recipient. Alterations in both the viral and bacterial community were necessary for successful transplants. With improvements in sequencing technologies and bioinformatics tools, it is becoming easier and cheaper to study the viral community. To understand the interactions of the different microbial populations within the rumen is an area of research that requires greater efforts in future endeavours.

As mentioned above, protozoa predate on bacteria within the rumen and therefore have an important role to play in the establishment and maintenance of the bacterial population. In experiments where defaunated animals have been used, fibre digestibility was shown to decrease (Newbold et al., 2015). Protozoal groups are able to degrade soluble carbohydrates, pectins, cellulose and hemicellulose (Jouany and Ushida, 1990), as well as bacterial cells.

Anaerobic fungi are thought to be the most effective fibre digesting microorganisms within the mammalian gut (Edwards et al., 2017). Due to their large contribution to fibre digestion, it is possible that the differences in *in vitro* performance between the 'Good' and 'Bad' animals used in the work presented here was in part due to the fungal community. Therefore, it would be of interest in future work to determine if there is a difference in fungal community between rumen fluids that differ in their ability to digest dry matter *in vitro*. As the fungal community are largely associated with the solid phase of the rumen content (Carberry et al., 2012), it would also be of interest to isolate the fungal community and transplant it between *in vitro* fermentation bottles, or indeed *in vivo*, to identify if transferring the fungal community improved fibre digestibility of a poorer performing rumen fluid.

Bacteria (and archaea) can also produce bacteriocins. This may be an alternative mechanism by which the microbial population is controlled within a mixed ruminal community. Bacteriocins are a diverse group of ribosomally synthesized antimicrobial peptides that act against similar or closely related strains to inhibit their growth (Dobson et al., 2012). Bacteriocins have been suggested as an alternative to antibiotics to improve

feed efficiency in the rumen, as they act in a similar way to monensin (Shen et al., 2017). It may be that the 'Bad' fluids used in Chapters 4 and 5 contained more bacteriocins and therefore similar bacterial species (that may have differed at the strain level) may have been prevented from establishing in this environment. This is especially likely in the case of Chapter 5 where animals were raised from birth on the same farm, within the same herd.

8.3.2 Manipulating a developing community

There may be scope to target the developing, naïve rumen community prior to its establishment. It has been established that the three major microbial groups (bacteria, protozoa and archaea) are present in the rumen from one day of age and that early life management can affect community composition (Abecia et al., 2014). Studies are providing evidence that manipulations to the rumen community pre-weaning can have lasting effects on community composition (Yáñez-Ruiz et al., 2010; Abecia et al., 2013) and therefore provide evidence for the possibility of programming the community of the naïve rumen. However, as reviewed by Yáñez-Ruiz et al. (2015)there is still a lack of understanding of many of the mechanisms associated with the development of the rumen community and the interactions between the microbes and the host. *In vitro* studies as presented in this thesis provide a platform with which to explore these interactions in a controlled environment alongside animal studies.

8.4 Conclusion

Exploring the dynamics of the microbial community is an exciting area of ruminant science that warrants further investigation. Seeding the rumen holds possibility to improve ruminant production and reduce environmental impact, however, due to the complex interactions between the different microbial populations within the rumen, it follows that future experiments should consider the whole microbial community and not just the bacteria.

The pipeline developed throughout this thesis provided a repeatable platform to perform bacterial community analysis and this was the first study to apply next generation sequencing techniques to explore the dynamics of the bacterial community across a fermentation within a batch culture model of rumen fermentation. The community composition was found to diverge from that of the host animal within the *in vitro* model with time, which was not unexpected due to the exhaustion of substrate and build-up of

waste products over time. Alongside this change in bacterial composition with time came an improvement in IVDMD with consecutive batch culturing. Despite the fact the community diverged from the initial inoculum, as long as suitable controls are included in experimental design, the batch *in vitro* model provides an opportunity to explore microbial dynamics in a controlled laboratory setting in the absence of confounding animal factors. The community within the rumen fluid used to inoculate the *in vitro* model should be known.

The *in vitro* model has been successfully used to identify rumen fluids that differ in their ability to digest dry matter and the pipeline used to determine the bacterial community has been shown to be repeatable, accurate and suitable for use on different environmental samples. The *in vitro* batch model may not be a direct reflection of what would be seen *in vivo*, however, it provides a useful platform to explore complex microbial dynamics and the mechanisms through which these can be manipulated in a controlled environment.

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Appendix A Sequencing data for the experiments presented in Chapter 3

Appendix A-1 The relative abundance (%) at the Phyla and Genera level for each of the repeated DNA extractions for both the QIAamp DNA Stool Mini Kit (QK) and the ZymoBIOMICS DNA Mini Kit (ZK) along with the theoretical values as given by the manufacturer

	Relative abundance (%)						
	QK			ZK			
	1	2	3	1	2	3	Theoretical
Phyla[a]							
Firmicutes	53.69	50.93	52.91	57.67	53.98	56.08	
Proteobacteria	46.17	48.89	46.97	42.21	45.90	43.83	
Bacteroidetes	0.02	0.04	0.02	0.02	0.03	0.01	
Bacteria unclassified	0.12	0.13	0.09	0.06	0.09	0.07	
Actinobacteria	0.00	0.01	0.00	0.03	0.00	0.00	
Genera[a]							
Lactobacillus	18.13	21.55	21.51	17.66	19.41	20.58	18.40
Salmonella	11.31	15.48	13.74	13.14	17.82	17.64	10.40
Bacillus	14.16	13.49	14.12	13.54	17.29	19.08	17.40
Escherichia-Shigella	16.20	17.40	15.73	14.52	15.63	13.78	10.10
Pseudomonas	17.71	15.08	16.67	13.86	11.39	11.41	4.20
Staphylococcus	15.46	11.49	12.57	18.21	12.44	11.89	15.50
Enterococcus	4.96	3.43	3.72	4.57	2.26	2.32	9.90
Listeria	0.50	0.48	0.47	3.37	1.95	1.49	14.10
Enterobacteriaceae unclassified	0.85	0.84	0.75	0.63	0.91	0.90	
Bacillales unclassified	0.14	0.20	0.07	0.06	0.30	0.25	
Bacilli unclassified	0.06	0.05	0.12	0.05	0.04	0.12	
Bacillaceae unclassified	0.19	0.12	0.22	0.14	0.23	0.25	
Gammaproteobacteria unclassified	0.05	0.04	0.03	0.03	0.10	0.06	
Bacteria unclassified	0.12	0.13	0.09	0.06	0.09	0.07	

[a] Phyla and genera with > 0.1% relative abundance in at least one sample are shown

Appendix A-2 The relative abundance (%) of the phyla and genera (> 1%) for a sample of neat rumen inoculum extracted with two different DNA extraction kits Where QK = QIAamp DNA Stool Mini Kit and ZK = ZymoBIOMICS DNA Mini Kit

	Relative abundance (%)	
	QK	**ZK**
Phyla[a]		
Bacteroidetes	52.13	48.94
Firmicutes	34.21	31.64
Patescibacteria	2.58	2.67
Fibrobacteres	2.33	4.33
Kiritimatiellaeota	2.23	4.03
Spirochaetes	1.69	2.12
Tenericutes	1.31	1.65
Bacteria unclassified	0.83	1.24
Genera[a]		
Prevotella 1	31.55	27.09
F082 ge	7.65	9.38
Rikenellaceae RC9 gut group	3.62	4.72
Ruminococcaceae NK4A214 group	3.59	4.13
Lachnospiraceae unclassified	3.16	3.29
Ruminococcus 1	3.05	3.26
Christensenellaceae R-7 group	2.38	3.27
Fibrobacter	2.32	4.32
Prevotellaceae UCG-001	2.24	1.1
WCHB1-41 ge	2.23	4.03
Muribaculaceae ge	2.23	0.29
Absconditabacteriales (SR1) ge	2.08	2.53
Succiniclasticum	1.89	0.18
Selenomonas 1	1.6	1.13
Lachnospiraceae AC2044 group	1.53	0.67
Treponema 2	1.51	1.77
Ruminococcaceae UCG-014	1.37	1.07
Oribacterium	1.19	0.54
Ruminococcaceae UCG-002	1.15	1.38
Mollicutes RF39 ge	1.12	1.12
Butyrivibrio 2	1.08	0.53
Ruminococcaceae unclassified	1.04	1.05
Saccharofermentans	1.02	0.72
Prevotellaceae UCG-003	0.93	1.19
Bacteroidales RF16 group ge	0.9	1.52
Bacteria unclassified	0.83	1.24
Ruminococcaceae UCG-010	0.8	1.04
Bacteroidales UCG-001 ge	0.64	1.14
Erysipelotrichaceae UCG-004	0.47	1.17

[a] Phyla and genera with >1% relative abundance in a minimum of one sample are shown

Appendix A-3 The relative abundance (%) of the phyla and genera from the same rumen fluid DNA extract amplified and sequenced three times

	Relative abundance[a] (%)			
	PCR 1	**PCR 2**	**PCR 3**	**CV (%)**
Phyla				
Firmicutes	44.99	45.34	46.46	1.69
Bacteroidetes	39.41	40.86	38.93	2.53
Spirochaetae	4.01	3.7	3.6	5.67
Fibrobacteres	3.64	3.3	3.34	5.5
Tenericutes	2.65	2.54	2.63	2.13
Bacteria_unclassified	2.21	1.7	1.84	13.79
Lentisphaerae	1.33	0.81	1.13	24.27
Genera				
Rikenellaceae RC9 gut group	14.09	14.07	13.86	0.91
Bacteroidales UCG-001 unclassified	8.55	8.84	8.83	1.83
Bacteroidales BS11 gut group unclassified	6.51	6.98	6.01	7.49
Oribacterium	5.59	5.96	5.51	4.21
Ruminococcus 1	5.08	5.37	5.91	7.79
Pseudobutyrivibrio	4.84	4.82	5.04	2.48
Prevotella 1	4.67	5.13	4.76	5.01
Fibrobacter	3.64	3.3	3.32	5.66
[Eubacterium] oxidoreducens group	3.57	3.69	3.5	2.65
Treponema 2	3.25	3.06	2.97	4.51
Lachnospiraceae unclassified	3.11	3.18	3.5	6.31
Anaerovibrio	2.52	2.45	2.78	6.81
Bacteria unclassified	2.21	1.7	1.84	13.79
Ruminococcaceae UCG-010	1.94	2.09	2.25	7.5
Lachnospiraceae AC2044 group	1.78	1.86	1.65	5.97
Butyrivibrio 2	1.78	1.52	1.4	12.33
Lachnospiraceae NK4A136 group	1.75	1.66	1.66	2.86
Prevotellaceae UCG-003	1.55	1.47	1.55	3.18
Bacteroidales S24-7 group unclassified	1.16	1.21	1.13	3.62
Mollicutes RF9 unclassified	1.09	0.94	1.17	10.92
Erysipelotrichaceae UCG-004	1.06	0.79	1.06	16.26
Christensenellaceae R-7 group	1.06	1.11	1.1	2.54
Anaeroplasma	0.98	1.09	0.82	14.26

[a] Samples with >1% relative abundance in at least one sample

Appendix B Sequencing data for the fermentation fluid samples from Chapter 4

Appendix B -1 The relative abundance (> 1%) of bacterial phyla and genera associated with samples taken at Day 1 and Day 16 along with the grass associated bacterial community for Good, Bad and 1:1 Mix rumen inoculums

		End of Day 1			End of Day 16		
	Grass[1]	**Good**	**Bad**	**Mix**	**Good**	**Bad**	**Mix**
Phyla							
Bacteroidetes	24.23	37.81	37.23	38.89	36.05	34.63	36.25
Firmicutes	5.79	29.95	30.13	30.65	29.42	26.95	29.42
Fibrobacteres	0.25	20.38	14.96	17.30	15.77	20.86	13.63
Spirochaetae	0.10	7.63	8.74	5.69	8.56	4.51	6.05
Tenericutes	0.05	2.09	5.89	4.63	3.65	9.55	9.29
Bacteria unclassified	0.86	0.83	1.39	1.31	0.76	0.63	0.78
Proteobacteria	60.83	0.35	0.30	0.30	4.33	1.51	3.15
Actinobacteria	7.46	0.10	0.08	0.03	0.05	0.03	0.03
Synergistetes	0.00	0.05	0.10	0.10	1.03	0.65	0.86
Genera							
Prevotella 1	1.46	21.84	21.06	23.35	10.28	14.81	11.41
Fibrobacter	0.23	20.38	14.96	17.28	15.77	20.86	13.63
Treponema 2	0.08	7.53	8.72	5.67	8.16	4.21	5.82
Probable genus 10	0.13	4.66	3.35	7.46	1.01	1.61	2.90
Rikenellaceae RC9 gut group	0.38	3.38	3.53	2.87	3.25	2.64	3.30
Saccharofermentans	0.03	2.29	0.98	1.59	0.73	0.43	0.03
Lachnospiraceae unclassified	0.13	2.22	2.29	1.86	0.86	1.26	1.31
Prevotellaceae unclassified	0.03	2.02	1.61	1.44	0.13	0.23	0.28
Prevotellaceae UCG-001	0.18	1.96	1.06	1.74	4.94	4.79	2.62
Bacteroidales BS11 gut group unclassified	0.13	1.91	1.66	1.81	2.59	3.95	2.64
Ruminococcus 1	0.13	1.86	1.64	2.12	2.97	2.85	4.03
Prevotella 7	0.10	1.64	1.34	1.54	0.00	0.00	0.00

Table continued …

	End of Day 1				End of Day 16		
	Grass	**Good**	**Bad**	**1:1 Mix**	**Good**	**Bad**	**1:1 Mix**
Roseburia	0.08	1.59	1.03	1.44	0.91	0.68	0.88
Bacteroidales S24-7 group unclassified	0.08	1.54	3.17	1.99	2.85	0.98	5.31
Erysipelotrichaceae UCG-004	0.13	1.49	1.21	1.13	1.13	0.93	0.98
Bacteroidales UCG-001 unclassified	0.08	1.16	0.48	0.93	6.85	4.18	6.45
Anaeroplasma	0.05	1.13	4.86	3.50	2.29	1.54	1.96
[Eubacterium] ruminantium group	0.08	1.08	0.38	0.65	0.00	0.13	0.10
Lachnospiraceae NK3A20 group	0.00	0.93	1.71	1.18	0.08	0.00	0.00
Ruminococcaceae NK4A214 group	0.03	0.88	2.32	1.59	0.43	0.45	0.65
Christensenellaceae R-7 group	0.03	0.86	1.49	0.93	0.63	0.35	0.38
Bacteria unclassified	0.86	0.83	1.39	1.31	0.76	0.63	0.78
Ruminococcaceae UCG-010	0.03	0.65	1.08	0.71	0.25	0.23	0.25
Lachnospiraceae NK4A136 group	0.05	0.63	0.05	0.38	0.23	1.18	0.68
Succiniclasticum	0.00	0.63	1.08	0.63	0.23	0.13	0.10
Mollicutes RF9 unclassified	0.00	0.58	0.93	0.81	0.63	0.50	1.28
Butyrivibrio 2	0.05	0.48	0.53	0.93	1.69	1.46	2.14
Lachnospiraceae AC2044 group	0.03	0.43	0.98	0.58	0.76	2.04	0.71
Pseudobutyrivibrio	0.08	0.38	0.28	0.55	6.55	3.90	5.69
Oribacterium	0.05	0.38	0.15	0.25	2.12	2.14	2.14
Ruminococcaceae UCG-014	0.03	0.35	1.59	0.63	0.03	0.00	0.00
Mollicutes unclassified	0.00	0.33	0.00	0.13	0.28	7.10	5.79
Bacteroidetes unclassified	0.18	0.15	0.13	0.05	1.54	0.35	0.28
Prevotellaceae YAB2003 group	0.08	0.13	0.15	0.23	1.13	0.43	1.23
Phocaeicola	0.00	0.08	0.13	0.18	1.08	0.63	1.01
Pyramidobacter	0.00	0.05	0.10	0.10	1.03	0.65	0.86
Streptococcus	0.40	0.00	0.00	0.03	2.12	1.41	1.26
Xanthomonas[a]	33.58	0.00	0.03	0.00	0.00	0.00	0.00
Ruminococcaceae UCG-005	0.03	0.00	0.15	0.10	1.49	0.71	0.08
Escherichia-Shigella	1.13	0.00	0.00	0.00	0.83	0.98	0.50
Basfia	0.00	0.00	0.00	0.00	2.85	0.00	2.17

Table continued…

	End of Day 1				End of Day 16		
	Grass	**Good**	**Bad**	**1:1 Mix**	**Good**	**Bad**	**1:1 Mix**
Massilia[a]	4.41	0.00	0.00	0.00	0.00	0.00	0.00
Pseudomonas[a]	7.56	0.00	0.00	0.00	0.00	0.00	0.00
Pantoea[a]	3.35	0.00	0.00	0.00	0.00	0.00	0.00
Pedobacter[a]	6.60	0.00	0.00	0.00	0.00	0.00	0.00
Frigoribacterium[a]	1.49	0.00	0.00	0.00	0.00	0.00	0.00
Dyadobacter[a]	3.40	0.00	0.00	0.00	0.00	0.00	0.00
Chryseobacterium[a]	3.40	0.00	0.00	0.00	0.00	0.00	0.00
Enterobacteriaceae unclassified	1.79	0.00	0.00	0.00	0.00	0.05	0.03
Weissella[a]	1.31	0.00	0.00	0.00	0.00	0.00	0.00
Hymenobacter[a]	2.82	0.00	0.00	0.00	0.00	0.00	0.00
Flavobacterium[a]	1.96	0.00	0.00	0.00	0.00	0.00	0.00

[1]Where relative abundance is > 1%

[a] Relative abundance is > 1% in the grass associated bacteria, but genus is not seen in the fermented sample

Appendix C Sequencing data for the fermentation fluid samples from Chapter 5

Appendix C-1 The relative abundance (>1%) of bacterial phyla and genera associated with the rumen fluids used as inocula, the fermentation fluids collected at the end of consecutive batch culture 1 (CBC1) and consecutive batch culture 4 (CBC4), and the grass used as the substrate in the experiment reported in Chapter 5

	Neat rumen fluid				End of CBC1 (48h)			End of CBC4 (48h)		
	Grass	**Good**	**Bad**	**Mix**	**Good**	**Bad**	**Mix**	**Good**	**Bad**	**Mix**
Phyla[1]										
Bacteroidetes	24.23	46.68	47.68	45.29	34.58	35.06	34.38	37.18	38.79	37.51
Firmicutes	5.79	30.48	34.84	35.62	38.97	37.83	40.03	48.29	45.06	49.60
Bacteria unclassified	0.86	5.39	4.13	4.01	1.34	1.41	1.11	1.69	2.59	2.07
Lentisphaerae	0.00	4.56	2.82	2.87	0.28	0.33	0.28	1.03	1.44	1.39
Candidate division SR1	0.00	3.88	2.72	3.07	0.18	0.20	0.10	0.00	0.00	0.03
Planctomycetes	0.03	2.59	1.84	2.29	0.40	0.08	0.15	0.13	0.03	0.15
Tenericutes	0.05	2.47	2.77	3.00	6.78	8.29	6.52	2.52	2.37	2.82
Proteobacteria	60.83	0.91	0.71	0.83	0.53	0.40	0.23	1.03	0.88	0.63
Spirochaetae	0.10	0.88	0.45	0.81	2.77	2.75	3.22	4.53	4.26	2.90
Saccharibacteria	0.18	0.68	1.03	0.88	0.13	0.28	0.13	0.18	0.20	0.20
Fibrobacteres	0.25	0.05	0.05	0.03	13.75	13.15	13.55	3.10	3.40	1.91
Actinobacteria	7.46	0.03	0.00	0.08	0.03	0.00	0.03	0.00	0.10	0.13
Genera[1]										
Prevotella 1	1.46	20.23	20.18	18.77	16.42	14.11	16.95	9.57	4.53	7.25
Rikenellaceae RC9 gut group	0.38	8.69	8.92	8.66	5.64	6.15	5.01	14.53	14.31	12.70
Bacteroidales BS11 gut group unclassified	0.13	6.37	6.07	6.65	2.29	3.17	2.70	5.92	6.83	5.52
Bacteria unclassified	0.86	5.39	4.13	4.01	1.34	1.41	1.11	1.69	2.59	2.07
Candidate division SR1 unclassified	0.00	3.88	2.72	3.07	0.18	0.20	0.10	0.00	0.00	0.03
Bacteroidales UCG-001 unclassified	0.08	3.85	4.79	4.41	3.85	5.09	3.68	2.77	7.83	6.42
Prevotellaceae UCG-003	0.00	3.38	2.90	2.90	0.53	0.73	0.20	0.73	1.61	1.13

Table continued…

	Neat rumen fluid				End of CBC1 (48h)			End of CBC4 (48h)		
	Grass	**Good**	**Bad**	**Mix**	**Good**	**Bad**	**Mix**	**Good**	**Bad**	**Mix**
Lentisphaerae RFP12 gut group unclassified	0.00	2.72	1.44	1.99	0.20	0.28	0.13	0.96	0.73	0.93
Ruminococcaceae UCG-010	0.03	2.49	2.85	2.80	0.65	0.53	0.63	1.94	2.19	1.34
Christensenellaceae R-7 group	0.03	2.29	3.35	3.55	0.88	0.86	0.91	1.46	1.21	1.59
Lachnospiraceae unclassified	0.13	2.22	1.81	2.14	2.57	2.80	3.20	3.73	2.87	3.10
Mollicutes RF9 unclassified	0.00	2.14	2.07	2.59	1.06	1.99	0.68	1.76	1.01	1.46
Ruminococcaceae NK4A214 group	0.03	2.04	2.92	2.80	0.28	0.65	0.58	0.76	0.63	1.01
Erysipelotrichaceae UCG-004	0.13	1.89	1.74	2.14	1.08	1.11	0.96	0.91	1.26	0.60
Ruminococcaceae UCG-014	0.03	1.86	1.28	1.79	0.13	0.45	0.15	0.48	0.05	0.13
[Eubacterium] coprostanoligenes group	0.00	1.79	1.99	1.81	0.18	0.35	0.25	0.88	0.63	0.88
Clostridiales unclassified	0.00	1.64	2.04	1.31	1.13	0.76	1.11	0.68	0.73	0.60
Saccharofermentans	0.03	1.51	2.07	1.96	1.56	2.52	2.34	0.88	0.65	0.81
Lachnospiraceae AC2044 group	0.03	1.28	1.59	1.81	3.43	2.64	2.77	2.39	1.64	2.44
Pirellula	0.00	1.28	0.98	1.28	0.20	0.05	0.08	0.03	0.00	0.00
p-1088-a5 gut group	0.00	1.18	0.65	0.86	0.20	0.03	0.08	0.10	0.03	0.13
Probable genus 10	0.13	0.96	1.06	0.86	2.04	3.25	2.92	0.73	0.73	0.73
Prevotellaceae UCG-001	0.18	0.93	0.88	0.96	0.93	0.86	1.01	0.23	0.25	0.53
Ruminococcus 1	0.13	0.91	0.96	1.03	6.85	7.53	7.25	3.85	5.09	3.60
Succiniclasticum	0.00	0.73	1.01	1.13	0.35	0.35	0.38	0.15	0.30	0.20
Candidatus Saccharimonas	0.00	0.68	1.01	0.88	0.13	0.28	0.13	0.18	0.20	0.20
Bacteroidales unclassified	0.08	0.65	0.38	0.43	1.21	1.21	1.01	0.63	0.65	0.73
Lachnospiraceae NK4A136 group	0.05	0.55	0.33	0.35	1.36	0.91	1.69	0.63	1.76	1.06
Ruminococcaceae unclassified	0.03	0.50	0.96	1.16	0.20	0.28	0.43	0.65	0.58	0.50
Bacteroidales S24-7 group unclassified	0.08	0.48	1.18	0.76	2.64	2.54	2.70	0.65	0.98	1.03
Butyrivibrio 2	0.05	0.48	0.50	0.60	0.45	0.81	0.60	3.30	1.64	1.99
Treponema 2	0.08	0.30	0.20	0.35	2.24	2.32	2.67	2.54	3.53	2.02
Pseudobutyrivibrio	0.08	0.28	0.25	0.45	5.34	3.50	3.53	8.39	4.74	4.89
Lachnospiraceae_FCS020_group	0.03	0.25	0.53	0.40	3.73	2.29	3.25	0.33	0.30	0.60

Table continued…

	Grass	Neat rumen fluid Good	Neat rumen fluid Bad	Neat rumen fluid Mix	End of CBC1 (48h) Good	End of CBC1 (48h) Bad	End of CBC1 (48h) Mix	End of CBC4 (48h) Good	End of CBC4 (48h) Bad	End of CBC4 (48h) Mix
Ruminococcaceae_UCG-005	0.03	0.18	0.38	0.33	0.03	0.05	0.03	3.38	0.60	5.77
Oribacterium	0.05	0.08	0.15	0.15	1.13	0.55	0.91	2.22	5.04	4.31
[Eubacterium]_oxidoreducens_group	0.00	0.08	0.08	0.05	0.40	0.10	0.28	1.31	3.90	2.52
Fibrobacter	0.23	0.05	0.05	0.03	13.75	13.15	13.55	3.10	3.40	1.91
Anaeroplasma	0.05	0.05	0.15	0.08	5.42	5.82	5.59	0.28	0.96	1.16
Enterobacteriaceae unclassified	1.79	0.05	0.00	0.05	0.03	0.03	0.03	0.00	0.00	0.00
Pseudomonas	7.56	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Streptococcus	0.40	0.00	0.00	0.00	0.18	0.13	0.15	2.27	0.76	2.44
Xanthomonas[a]	33.58	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Anaerovibrio	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.08	2.27	2.27
Escherichia-Shigella	1.13	0.00	0.00	0.00	0.00	0.00	0.00	0.48	0.25	0.10
Massilia[a]	4.41	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Erwinia[a]	3.70	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Pantoea[a]	3.35	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PL-11B10 unclassified	0.00	0.00	0.00	0.03	0.00	0.00	0.00	1.44	0.00	0.68
Pedobacter[a]	6.60	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Frigoribacterium[a]	1.49	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Dyadobacter[a]	3.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Chryseobacterium[a]	3.40	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Weissella[a]	1.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Hymenobacter[a]	2.82	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Flavobacterium[a]	1.96	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

[1]Where abundance is > 1% in at least one sample

[a] Present at > 1% in the grass sample, but not observed in experimental samples

Appendix D Sequencing data for the fermentation fluid samples from Chapter 7

Appendix D-1 The relative abundance (> 1%) of the phyla and genera observed across a 48 hour fermentation with a 1 in 2 ratio of rumen fluid to buffer and the neat rumen fluid sampled at time of processing (fresh) and at the start of the experiment (start)

			Time (hours)						
	Neat fresh	**Neat start**	**0**	**6**	**12**	**18**	**24**	**36**	**48**
Phyla									
Bacteroidetes	52.07	50.21	49.67	38.29	40.72	42.69	45.57	36.16	31.13
Firmicutes	34.25	38.70	39.34	44.83	48.89	46.36	45.72	54.83	57.93
Patescibacteria	2.57	1.50	1.55	1.08	0.99	0.75	0.58	0.47	0.68
Fibrobacteres	2.37	0.06	0.05	0.01	0.01	0.00	0.00	0.00	0.01
Kiritimatiellaeota	2.25	2.74	3.14	2.42	2.30	1.72	1.35	1.09	1.10
Spirochaetes	1.60	0.90	0.56	1.01	0.50	0.59	0.83	1.13	1.34
Tenericutes	1.38	1.95	2.00	2.48	1.15	1.38	0.82	0.88	0.95
Proteobacteria	0.98	0.81	0.77	6.99	0.85	1.49	1.29	1.45	1.89
Bacteria_unclassified	0.82	0.83	0.52	0.71	2.28	2.93	2.15	2.53	3.22
Planctomycetes	0.52	0.82	1.38	1.15	1.15	0.87	0.86	0.65	0.72
Genera									
Prevotella 1	31.38	26.91	23.98	13.68	8.45	8.23	6.76	6.65	5.38
F082 ge	7.69	9.49	10.84	7.93	7.57	5.50	4.13	3.37	3.76
Rikenellaceae RC9 gut group	3.59	4.41	4.43	5.29	4.73	3.35	4.24	7.04	13.43
Ruminococcaceae NK4A214 group	3.56	9.83	9.57	8.09	10.18	6.87	5.29	4.84	8.72
Lachnospiraceae unclassified	3.10	2.15	1.55	0.94	0.91	0.80	0.58	0.51	0.61
Ruminococcus 1	2.97	1.13	0.81	0.21	0.23	0.10	0.06	0.03	0.03
Christensenellaceae R-7 group	2.37	4.33	7.01	5.94	6.73	4.81	4.22	3.54	4.76
Fibrobacter	2.36	0.06	0.05	0.01	0.00	0.00	0.00	0.00	0.01
Muribaculaceae ge	2.31	2.44	3.27	1.69	1.20	0.38	0.41	0.33	0.44
WCHB1-41 ge	2.25	2.74	3.14	2.42	2.30	1.72	1.35	1.09	1.10
Prevotellaceae UCG-001	2.21	2.56	2.66	1.21	1.25	0.92	0.53	0.42	0.48
Absconditabacteriales (SR1) ge	2.06	0.74	0.54	0.30	0.21	0.14	0.07	0.09	0.03
Succiniclasticum	1.95	2.35	3.09	1.40	1.84	1.11	0.68	0.58	0.79
Selenomonas 1	1.67	1.11	1.16	0.54	0.48	1.16	0.78	1.09	0.64
Lachnospiraceae AC2044 group	1.47	1.18	0.72	0.26	0.20	0.20	0.06	0.03	0.03
Treponema 2	1.43	0.70	0.37	0.89	0.41	0.44	0.80	0.96	0.96
Ruminococcaceae UCG-014	1.37	1.52	1.30	1.06	0.66	0.58	0.41	0.28	0.56
Ruminococcaceae UCG-002	1.24	0.79	0.39	0.21	0.20	0.15	0.09	0.15	0.15
Oribacterium	1.21	0.92	1.46	8.67	9.15	7.07	8.74	13.26	13.73
Mollicutes RF39 ge	1.20	1.69	1.70	1.08	0.84	0.89	0.42	0.31	0.44
Butyrivibrio 2	1.06	0.51	0.58	0.69	0.56	0.51	0.45	0.66	0.66
Saccharofermentans	0.96	1.18	1.36	0.63	0.75	0.53	0.37	0.30	0.25
Ruminococcaceae UCG-005	0.84	1.30	1.05	0.53	0.48	0.34	0.33	0.24	0.30
Bacteria unclassified	0.82	0.83	0.52	0.71	2.28	2.93	2.15	2.53	3.22

Table continued…

	Time (hours)								
	Neat fresh	**Neat start**	**0**	**6**	**12**	**18**	**24**	**36**	**48**
Ruminococcaceae UCG-010	0.81	1.45	1.32	0.92	1.01	0.65	0.47	0.40	0.47
Erysipelotrichaceae UCG-004	0.52	0.77	0.52	0.62	1.67	1.92	1.22	1.35	1.87
Sutterella	0.31	0.30	0.26	0.24	0.42	1.15	1.11	1.29	1.71
Prevotellaceae YAB2003 group	0.14	0.09	0.06	2.26	0.96	1.55	2.20	1.54	0.50
Anaeroplasma	0.09	0.16	0.17	1.27	0.21	0.39	0.33	0.54	0.43
Streptococcus	0.03	0.01	0.03	3.70	1.93	3.99	5.51	13.12	7.62
Prevotella 7	0.02	0.02	0.01	3.18	13.70	20.64	25.47	14.75	3.18
Lachnoclostridium 1	0.02	0.00	0.02	0.45	0.09	1.49	2.06	2.00	2.56
Escherichia-Shigella	0.01	0.01	0.00	4.64	0.05	0.07	0.02	0.01	0.00
Lactobacillus	0.00	0.00	0.00	1.48	5.04	4.28	2.94	1.83	1.71
Megasphaera	0.00	0.00	0.00	0.44	0.07	3.59	6.12	4.43	5.02

Appendix D-2 The relative abundance (> 1%) of the phyla and genera observed across a 48 hour fermentation with a 1 in 4 ratio of rumen fluid to buffer

	Time (hours)						
	0	**6**	**12**	**18**	**24**	**36**	**48**
Phyla							
Bacteroidetes	48.71	36.79	37.69	36.84	37.57	29.24	27.58
Firmicutes	38.88	51.53	51.23	40.42	45.67	51.34	53.05
Patescibacteria	1.44	1.16	0.66	0.43	0.33	0.30	0.29
Kiritimatiellaeota	3.79	3.34	1.71	1.22	0.85	0.73	0.55
Spirochaetes	0.66	0.49	0.30	0.29	0.64	1.64	2.05
Tenericutes	2.09	2.01	0.91	1.15	1.07	1.37	2.38
Proteobacteria	1.04	1.02	3.14	16.17	11.55	12.92	11.15
Bacteria_unclassified	0.65	0.61	2.46	2.29	1.35	1.52	1.65
Planctomycetes	1.43	1.65	0.99	0.52	0.47	0.39	0.80
Genera							
Prevotella 1	22.74	12.72	7.16	9.56	8.41	10.52	9.50
F082 ge	10.79	10.13	6.04	3.03	2.45	1.93	1.69
Rikenellaceae RC9 gut group	5.26	6.18	3.80	2.27	3.07	6.41	10.86
Ruminococcaceae NK4A214 group	9.76	11.48	6.36	3.95	3.37	3.14	4.36
Lachnospiraceae unclassified	1.48	1.20	0.75	0.57	0.46	0.51	0.54
Christensenellaceae R-7 group	7.40	8.74	5.10	3.21	3.25	2.65	3.24
Muribaculaceae ge	2.91	2.12	0.80	0.34	0.27	0.23	0.32
WCHB1-41 ge	3.79	3.34	1.71	1.22	0.85	0.73	0.55
Prevotellaceae UCG-001	2.65	1.64	0.85	0.41	0.30	0.28	0.26
Succiniclasticum	3.17	2.72	1.07	0.42	0.30	0.31	0.33
Treponema 2	0.54	0.38	0.21	0.24	0.58	1.43	1.63
Ruminococcaceae UCG-014	1.26	1.22	0.52	0.33	0.20	0.17	0.21
Oribacterium	1.55	3.41	16.01	11.71	14.13	15.95	16.19
Mollicutes RF39 ge	1.79	1.66	0.60	0.35	0.29	0.21	0.21
Butyrivibrio 2	0.52	0.38	0.83	0.72	1.13	1.26	1.37
Saccharofermentans	1.10	0.89	0.45	0.28	0.16	0.16	0.09
Bacteria unclassified	0.65	0.61	2.46	2.29	1.35	1.52	1.65
Ruminococcaceae UCG-010	1.33	1.22	0.58	0.33	0.29	0.26	0.66
Pirellula	0.95	1.09	0.74	0.36	0.36	0.27	0.36
Prevotellaceae YAB2003 group	0.05	0.05	1.50	3.19	3.26	1.50	1.46
Anaeroplasma	0.10	0.19	0.24	0.76	0.76	1.12	2.05
Streptococcus	0.02	6.90	5.42	6.00	9.18	14.40	14.69
Prevotella 7	0.00	0.11	15.33	16.62	18.65	7.03	1.45
Lachnoclostridium 1	0.01	0.02	0.13	1.59	1.60	1.63	1.59
Escherichia-Shigella	0.05	0.05	2.48	14.31	9.98	10.97	9.41
Lactobacillus	0.02	4.38	4.05	1.17	0.92	0.65	0.45
Megasphaera	0.00	0.00	0.03	0.78	2.77	2.95	1.99
Clostridium sensu stricto 1	0.00	0.00	3.46	3.72	2.23	1.20	0.53

Appendix D-3 The relative abundance (> 1%) of the phyla and genera observed across a 48 hour fermentation with a 1 in 9 ratio of rumen fluid to buffer

	Time (hours)						
	0	**6**	**12**	**18**	**24**	**36**	**48**
Phyla							
Bacteroidetes	47.80	33.24	21.88	36.77	36.83	31.07	26.13
Firmicutes	39.11	53.65	38.74	35.87	40.42	44.10	44.05
Patescibacteria	1.37	1.18	0.46	0.31	0.17	0.13	0.18
Kiritimatiellaeota	4.38	3.97	1.16	0.88	0.64	0.38	0.45
Spirochaetes	0.56	0.40	0.13	0.13	0.33	1.50	2.57
Tenericutes	2.08	1.41	0.44	0.47	1.49	1.92	3.83
Proteobacteria	1.16	2.36	35.39	23.80	18.68	19.77	21.03
Bacteria_unclassified	0.51	0.58	0.61	0.88	0.77	0.51	1.04
Planctomycetes	1.59	1.64	0.54	0.40	0.32	0.28	0.37
Genera							
Prevotella 1	22.95	10.88	3.68	7.35	10.85	12.30	12.05
F082 ge	10.10	8.79	3.47	1.97	1.34	0.89	0.98
Rikenellaceae RC9 gut group	5.02	5.86	2.34	1.42	1.73	3.60	8.22
Ruminococcaceae NK4A214 group	9.74	10.14	3.98	3.09	2.70	2.13	2.63
Lachnospiraceae unclassified	1.54	0.95	0.39	0.36	0.39	0.47	0.65
Christensenellaceae R-7 group	7.37	7.85	3.52	2.81	2.31	1.71	2.26
Muribaculaceae ge	2.70	1.97	0.64	0.25	0.16	0.08	0.26
WCHB1-41 ge	4.37	3.97	1.16	0.88	0.64	0.38	0.45
Prevotellaceae UCG-001	2.71	1.89	0.68	0.33	0.21	0.14	0.15
Succiniclasticum	3.57	2.38	0.53	0.22	0.15	0.11	0.39
Selenomonas 1	1.40	0.59	0.17	0.10	0.22	0.32	0.15
Treponema 2	0.39	0.29	0.10	0.11	0.27	1.37	2.16
Ruminococcaceae UCG-014	1.47	0.94	0.33	0.25	0.16	0.11	0.15
Oribacterium	1.55	1.95	12.06	11.55	14.01	18.00	13.28
Mollicutes RF39 ge	1.73	1.27	0.29	0.22	0.14	0.13	0.08
Butyrivibrio 2	0.51	0.33	0.25	0.43	0.58	0.85	1.71
Saccharofermentans	1.08	0.70	0.28	0.15	0.13	0.07	0.06
Bacteria unclassified	0.51	0.58	0.61	0.88	0.77	0.51	1.04
Ruminococcaceae UCG-010	1.03	0.90	0.42	0.18	0.15	0.17	0.30
Pirellula	0.91	1.10	0.44	0.29	0.25	0.23	0.25
Prevotellaceae YAB2003 group	0.05	0.02	0.56	4.22	4.75	5.27	2.36
Anaeroplasma	0.18	0.11	0.12	0.24	1.33	1.77	3.61
Streptococcus	0.03	12.65	8.40	6.25	6.06	9.32	12.06
Prevotella 7	0.01	0.03	8.82	20.04	16.75	8.04	0.98
Lachnoclostridium 1	0.02	0.01	0.00	0.51	1.06	1.24	0.96
Escherichia-Shigella	0.01	0.85	29.16	16.28	15.92	15.46	18.39
Lactobacillus	0.01	7.34	1.41	0.74	0.37	0.36	0.37
Megasphaera	0.00	0.00	0.00	0.06	0.73	1.50	0.92
Clostridium sensu stricto 1	0.00	0.04	2.08	2.06	3.84	1.62	0.82
Lysinibacillus	0.00	0.00	0.62	2.71	2.79	1.67	0.32
Comamonas	0.00	0.00	0.25	1.82	0.44	1.39	0.31
Enterobacteriaceae unclassified	0.00	0.04	1.30	0.65	0.41	0.58	0.70
Cellulosilyticum	0.00	0.00	0.00	0.02	0.09	0.53	1.10
Acinetobacter	0.01	0.16	3.05	4.12	1.30	1.43	0.80
Clostridium sensu stricto 7	0.00	0.00	0.02	0.28	1.05	0.45	0.19

Appendix D-4 The relative abundance (> 1%) of the phyla and genera observed as the epiphytic community across a 48 hour fermentation

	Time (hours)						
	0	**6**	**12**	**18**	**24**	**36**	**48**
Phyla							
Bacteroidetes	54.00	23.66	1.10	0.45	0.70	0.16	27.91
Proteobacteria	26.95	36.27	33.53	45.50	57.50	39.97	31.80
Actinobacteria	9.27	2.00	0.12	0.12	0.17	0.11	0.04
Verrucomicrobia	3.23	0.86	0.02	0.01	0.02	0.01	0.00
Firmicutes	1.34	36.21	65.13	53.84	41.53	59.65	40.14
Patescibacteria	1.01	0.17	0.00	0.02	0.00	0.00	0.00
Genera							
Hymenobacter	9.32	1.19	0.07	0.02	0.01	0.03	0.00
Pedobacter	8.90	5.17	0.30	0.07	0.04	0.01	0.00
Chryseobacterium	8.37	4.96	0.29	0.14	0.09	0.02	0.01
Flavobacterium	7.52	3.36	0.08	0.08	0.05	0.01	0.01
Pseudomonas	5.98	4.52	0.10	0.15	5.65	1.17	0.07
Mucilaginibacter	4.11	0.88	0.02	0.01	0.00	0.00	0.00
Spirosoma	3.63	1.93	0.09	0.03	0.00	0.02	0.00
Chitinophagaceae unclassified	2.97	0.75	0.02	0.02	0.01	0.01	0.00
Dyadobacter	2.73	1.95	0.07	0.03	0.01	0.01	0.00
FBP ge	1.90	0.26	0.02	0.00	0.00	0.00	0.02
Luteolibacter	1.64	0.64	0.01	0.01	0.01	0.00	0.00
Burkholderiaceae unclassified	1.56	0.26	0.00	0.02	0.02	0.01	0.02
Variovorax	1.43	0.83	0.06	0.02	0.03	0.03	0.00
Erwinia	1.39	2.44	0.14	0.03	0.06	0.01	0.00
uncultured	1.20	0.19	0.00	0.01	0.00	0.00	0.00
Microbacterium	1.04	0.37	0.02	0.02	0.02	0.00	0.00
Escherichia-Shigella	1.00	6.95	22.44	30.61	36.90	27.47	24.40
Acinetobacter	0.99	1.08	0.31	1.15	3.31	2.00	0.62
Pantoea	0.47	6.62	0.29	0.15	0.13	0.08	0.03
Enterobacteriaceae unclassified	0.29	2.57	5.10	1.58	4.89	4.70	2.87
Comamonas	0.25	0.17	0.37	0.91	2.67	1.05	2.18
Bacillus	0.11	1.57	0.17	0.31	0.33	0.24	0.03
Streptococcus	0.08	0.88	18.20	18.59	5.36	3.83	3.43
Lysinibacillus	0.08	0.13	0.30	3.93	10.08	12.62	0.61
Lachnospiraceae unclassified	0.04	0.04	0.06	0.19	1.59	12.04	4.36
Clostridium sensu stricto 1	0.03	26.20	43.11	23.16	5.58	1.57	1.92
Cellulosilyticum	0.01	0.00	0.49	0.68	4.03	12.02	14.03
Prevotellaceae unclassified	0.00	0.04	0.02	0.00	0.00	0.01	6.85
Lachnoclostridium 12	0.00	0.01	0.00	0.08	1.04	2.57	0.68
Lachnoclostridium 10	0.00	0.00	0.00	0.19	1.58	0.66	0.37
Bacteroides	0.00	0.00	0.00	0.00	0.46	0.00	15.24
Hafnia-Obesumbacterium	0.00	2.63	2.95	9.95	1.24	0.72	0.99
Citrobacter	0.00	0.70	1.24	0.62	2.17	2.36	0.49
Lachnoclostridium 5	0.00	0.02	0.23	1.54	2.89	2.14	3.03
Macellibacteroides	0.00	0.00	0.00	0.00	0.00	0.00	5.71
Lachnotalea	0.00	0.00	0.53	1.27	1.85	0.71	0.25
Anaerocolumna	0.00	0.00	0.02	1.34	3.07	2.42	1.57

Table continued…

	Time (hours)						
	0	**6**	**12**	**18**	**24**	**36**	**48**
Crassaminicella	0.00	0.00	0.00	0.01	0.17	1.22	1.02
Clostridiaceae 1 unclassified	0.00	2.01	0.68	0.74	0.72	0.07	0.15
Exiguobacterium	0.00	1.17	0.19	0.29	0.25	0.00	0.00
Lactococcus	0.00	1.06	0.14	0.07	0.06	0.02	0.00
Herbinix	0.00	0.00	0.00	0.02	0.13	1.34	1.44