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APPLICATION OF MOLECULAR TECHNIQUES TO OVERCOME CHALLENGES OF RUMINANT NUTRITION IN THE TROPICS

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SUMMARY OF THESIS

Livestock farmers in many parts of sub-Saharan Africa contend with low production caused by shortage of feed, low quality forages or both. Fibrous feeds and crop residues of low digestibility constitute the major proportion of feeds available to most ruminants under smallholder situations in developing countries. The characteristic feature of tropical forages is their slow rate of microbial breakdown in the rumen with the result that much of the nutrients of the feed are voided in the faeces. The slow rate of breakdown reduces the outflow rate from the rumen and depresses feed intake.

This dissertation is organised into seven chapters aimed at understanding the degradation of low-quality feeds in the rumen with a goal of improving it. Chapter one is comprises of a literature review with background information regarding ruminants, rumen microbiology, molecular techniques, tropical fodder trees and crop residues. The chapter also outlines the aims and objectives of the dissertation.

Chapter two details the materials and methods used for the experiments. Chapter three details the evaluation of the chemical composition, *in vitro* degradation, and effect on composition on rumen bacteria of leaves of seven African fodder tree species to determine their suitability for ruminant nutrition. The results showed that the leaves of the trees could be a source of highly nutritious feed to supplement bulk feeds. However, also contained secondary metabolites which had a significant *in vitro* effect on rumen microbes.

Advances in sequencing technologies have facilitated the study of the rumen microbial community composition with higher accuracy and greater throughput than was previously achievable. In Chapter four, a new entrant into the sequencing platforms, the Oxford Nanopore MinION, was evaluated. The results showed that the platform had potential as a cheaper, faster, and more accurate alternative for taxonomic investigation of the rumen microbial ecosystem.

Crop residues form an important part of ruminant feedstuff in many developing countries. Chapters five and six investigated rice straw as a representative of these residues. The results showed that the effect of the chemical composition *in vitro* degradation was as a result of a complex interaction between the various components of the straw rather than their absolute measurements.

Colonisation of newly ingested plant material by rumen microbes is a prerequisite for ruminal degradation of plant biomass. The *in vitro* colonisation of the rice straw samples was investigated *via* 16S sequencing of attached bacteria. Results in Chapter five showed that the colonisation dynamic with two distinct phases. Further, Chapter six demonstrated that there are interactions between the chemical composition of the substrate and the colonising bacteria. The studies showed that understanding the dynamics of colonisation could enable interventions to improving ruminant fermentation efficiency.

Chapter seven is a summary of the conclusions and recommendations from the studies.

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I would also like to thank my family, for their support and encouragement.

But Mouse, you are not alone,
In proving *foresight* may be vain:
The best-laid schemes of *Mice* and *Men*
Go often askew,
And leave us nothing but grief and pain,
For promised joy!

Still you are blessed, compared with me!
The *present* only touches you:
But oh! I *backward* cast my eye,
On prospects dreary!
And *forward*, though I cannot see,
I *guess* and *fear*!

Robert Burns
To a Mouse
1785

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LIST OF ABBREVIATIONS

ADF	Acid detergent fibre
ADIN	Acid detergent insoluble nitrogen
ADL	Acid detergent lignin
AFEX	Ammonia fibre expansion
ANOSIM	Analysis of similarity
ANOVA	Analysis of variance
ASAL	Arid and semi-arid land
amu	Atomic mass unit
CCA	Canonical Correspondence Analysis
cDNA	Complementary DNA
CF	Crude fat
cm	Centimetre
CO_2	Carbon dioxide
CP	Crude protein
CTAB	Hexadecyltrimethylammonium bromide
DHP	4-hydroxy-4(H)-pyridone
DM	Dry matter
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
e.g.	*exempli gratia* (for example)
EM	Electron microscopy
ESI	Electrospray Ionization
FAO	Food and Agriculture Organization of the United Nations
FID	Flame ionisation detector
g	gram
GDP	Gross domestic product
Gt	Gigaton
ha	Hectare
HI	Harvest index
H_2O	Water
HPLC	High-performance Liquid Chromatography
H_2SO_4	Hydrochloric acid
IBERS	Institute of Biological Environmental and Rural Sciences
i.e.	*id est* (that is)
KNBS	Kenya National Bureau of Statistics
kg	Kilogram
kPa	Kilopascal
kV	Kilovolts
L	Litre
LiCL	Lithium chloride
LSD	Least square difference
M	Molar
mg	Milligram
min	Minutes
MJ	Mega joule
mL	Millilitre
mm	Millimetre
mM	Milimolar
MS	Mass Spectrometry
m/z	Mass-to-charge ratio

NaCl	Sodium chloride
NaOH	Sodium hydroxide
NDF	Neutral detergent fibre
N_2O	Nitrous oxide
OD	Optical density
OM	Organic matter
ONT	Oxford Nanopore Technologies
OTU	Operational taxonomic unit
PCO	Principal coordinate analysis
PCR	Polymerase chain reaction
PDA	Photodiode Array Detection
PEG	Polyethylene glycol
PGM	Personal genome machine
PSM	Plant secondary metabolites
PVP	polyvinylpyrrolidone
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RDP	Ribosomal database project
rRNA	Ribosomal RNA
SMS	Spent Mushroom Substrate
sp.	Species
SSA	Sub-Saharan Africa
SSF	Solid-state fermentation
t	Tonne
TRIS	Trisaminomethane
UK	United Kingdom
µg	Microgram
µL	Microliter
µM	Micromolar
UPGMA	Unweighted pair group method with arithmetic mean
USA	United States of America
UV	Ultraviolet
V	Volts
VFA	Volatile fatty acid
XRF	X-ray fluorescence spectrometry
>	Greater than
<	Less than
x *g*	Times gravity
~	Approximately

CHAPTER 1: LITERATURE REVIEW

1.1 INTRODUCTION

Agricultural scientists are currently faced with the challenge of increasing food production to match the global population, which is set to increase by 50% between 2000 and 2050 to 9 billion people. The challenge is further complicated by the need to accomplish this task without exacerbating the damage to the environment caused by global warming (Tilman et al., 2002). Livestock production already occupies 37% of global ice free land area and at least 570 million farms (Cortner et al., 2019). In order to meet the growing demands, livestock production would have to expand to land occupied by other ventures including land needed for housing, growth of crops for human consumption and more recently, the growth of bioenergy/biomass crops (Kingston-Smith, Marshall, & Moorby, 2013). This limits further expansion of grazing lands (Asner et al., 2004). The alternative is to convert natural ecosystems into pastures and farms for feed production. For instance, in South America, cattle ranching is the major driver for the rapid deforestation of the Amazon currently taking place (Cortner et al., 2019). Livestock production is also a major consumer of water resources, and a major polluter (Steinfeld et al., 2006).

Ruminants are capable of converting plant material not suitable for human consumption into a high-quality protein (Kingston-Smith, Marshall, et al., 2013). Ruminant production is therefore uniquely suited to increase food production without competing with humans for grain foods. Further, ruminant production can be undertaken in marginal land that cannot be used for food crop production (Kingston-Smith et al., 2010). Although ruminants are better at extracting energy from plant material compared to monogastric animals, it has been known for a long time that rumen fermentation is inefficient in the use of feed energy (Kingston-Smith, Marshall, et al., 2013). The major outcomes of this inefficiency are methane and nitrogen emissions. Apart from the ecological concerns of the effect of these emissions on the environment, they also represent the loss of energy (in regards to methane) and nitrogen (that would have otherwise been incorporated in useful products such as meat and milk) from the production system.

The loss of energy *via* methane production due to inefficiency fermentation has been recognised for a long time (Kriss, 1930). More recently, the effect methane and other greenhouse gases on the global climate has placed the contribution of livestock production into sharp focus (Abler, 2004; Shortle et al., 2012). It is estimated that globally ruminal fermentation produces 86 million tonnes of methane a year representing 37% of anthropogenic emissions (Steinfeld et al., 2006). The main source (30.5%) of this methane is directly from inefficient rumen fermentation and the rest is produced from manure decomposition. Methane is a potent greenhouse gas with a warming potential 23 times that of CO_2. Further, livestock production is estimated to be responsible for 9% of the anthropogenic CO_2 emissions (including the effects of deforestation) (Steinfeld et al., 2006).

The inefficiency of nitrogen metabolism in ruminants is particularly high with as much as 70% of ingested nitrogen being lost (Kingston-Smith, Marshall, et al., 2013). This nitrogen is deposited on pastureland in form of manure and urine. The increase of nitrogen in the soil favours proliferation of grasses at the expense of dicotyledonous species, resulting in a reduction the biodiversity of the pasture ecosystems (Kingston-Smith et al., 2010). The excreted nitrogen also acts as a substrate for generation of nitrous oxide (N_2O); although N_2O is present only in low concentrations in the atmosphere, it has 296 times the warming potential of CO_2, and a half-life of 114 years in the atmosphere (Steinfeld et al., 2006). Livestock contribute 65% of anthropogenic N_2O, with 35% of this linked specifically to the decomposition of manure (Steinfeld et al., 2006). The excreted nitrogen is also a major contaminant of surface and ground water; animal wastes contribute about 30% of the nitrogen and phosphorus in watercourses. Apart from manure, the application of organic and inorganic fertilisers to pastures and forage crops is also a significant contributor to nitrogen pollution (Letica et al., 2010). Nitrogen compounds entering the water courses and leaching into ground water cause eutrophication and nitrate poisoning of aquatic life as well as the loss of biodiversity (Oenema et al., 1997; Wachendorf et al., 2008). Apart from the ecological concerns of nitrogen pollution, the excreted nitrogen represents a significant financial loss to the farmer (Kingston-Smith, Marshall, et al., 2013).

There is potential to improve the efficiency of rumen function with the dual goals of increasing production and reducing the environmental impact of the sector.

Considering that there are approximately 3.5 billion cattle, buffalo, sheep and goats globally, even small improvements in efficiency would have a significant impact (Kingston-Smith et al., 2010). The dry matter digestibility of feed is a major determinant of the efficiency of use of feed; therefore, any improvements in fodder digestibility would result in improved efficiency. Since the energy supplied by a feed is a function of its digestibility, increasing the digestibility of feed is a good way of increasing its energy use efficiency (Casler & van Santen, 2010).

This is also applicable for improving the efficiency of nitrogen metabolism in ruminants. The incorporation of dietary proteins into microbial proteins (and ultimately into meat and milk protein) is limited by the availability of energy to drive the process. The imbalance in the timing (or asynchrony) of nitrogen and energy sources in the rumen is attributed slower rates of degradation of the main energy-yielding substrates, the structural carbohydrates, cellulose and hemicellulose (Kingston-Smith et al., 2003). As a result, ammonia accumulates and, if it is not incorporated into microbial proteins, is absorbed from the rumen. It is subsequently converted to urea in the liver and is either recycled back to the rumen or excreted as waste nitrogen in urine (Moorby et al., 2009).

Improving digestibility has traditionally been a major target for plant breeding programmes (Kingston-Smith & Thomas, 2003; Parsons et al., 2011). However, while plant breeding has increased digestibility of pasture grasses used on improved lands (Kingston-Smith, Marshall, et al., 2013), many production systems rely on indigenous forage that is poorly digestible. This is particularly evident in production developing countries, especially in the tropics were nutrition represents one of the most serious limitations to livestock, resulting in significant yield gaps (Henderson et al., 2016). In many tropical countries, feed resources are inadequate in both quality and quantity, particularly during the dry seasons.

Fibrous feeds and crop residues of low digestibility constitute the major proportion of feeds available to most ruminants under smallholder situations in developing countries. Tropical forages show a characteristically slow rate of microbial breakdown in the rumen resulting in much of the nutrients of the feed being voided in the faeces (Minson & McLeod, 1970). The slow rate of breakdown also results in reduced outflow rate of feed residues from the rumen which consequently depresses feed intake. The

slow breakdown is attributed to the lignification of plant cell walls which prevents degradation by cellulase or hemicellulase enzymes. The main treatment methods to improve nutritional value of fibrous forages such as cereal straws are mechanical (*e.g*. grinding), physical (*e.g*. temperature and pressure treatment) or a range of chemical treatments of which sodium hydroxide or ammonia (Greenhalgh, 1984).

The plant cell wall has been studied for many years as it is an important source for textiles, paper and composite materials, and plays a central role in human and animal nutrition. Cell walls are mainly composed of the polysaccharides cellulose, hemicelluloses and pectins in varying proportions and may be impregnated with lignin (Neutelings, 2011). They are complex structures limiting the mobility of enzymes and chemicals necessary for the degradation of carbohydrates in food digestion.

Plant cell walls are composed of up to three layers (Buchanan, Gruissem, & Jones, 2015). The primary cell wall is generally a thin, flexible and extensible layer formed while the cell is growing. The secondary cell wall is not found in all cell types. It is a thick layer formed inside the primary cell wall after the cell is fully grown. It is found in some cells, such as the conducting cells in xylem where it contains lignin, which strengthens and waterproofs the wall (Buchanan et al., 2015). The middle lamella is rich in pectins. This outermost layer forms the interface between adjacent plant cells and glues them together (Buchanan et al., 2015).

The term lignin derives from the Latin name for wood, "*lignum*" (McCarthy, 2000). Lignin synthesis starts at the onset of secondary cell wall formation and, depending on the plant species and cell type, is more or less uniformly distributed in the thick compound wall composed of the middle lamella, primary- and secondary-cell walls of specialized cells (Neutelings, 2011). Lignin is a complex phenolic polymer consisting of three monolignol subunits: *G* (guaiacyl)-, *S* (syringyl)- and *H* (*p*-hydroxyphenyl)-lignin (Loix et al., 2017). The compound is essential for the growth of land plants because it provides mechanical support to the whole plant and is responsible for the hydrophobic proprieties of water-conducting cells in the xylem (Vanholme et al., 2010).

Although beneficial for plants, animal nutritionists view lignin as an undesired or "antinutritive" component in fodder. Lignin is not readily fermented by the rumen bacteria (Krause et al., 2003). Consequently, lignin content limits the feed value of plant materials through two mechanisms:

1. Inaccessible energy content: lignin has about 30% higher gross energy content than cellulose (Novaes et al., 2010). The heat combustion of cellulose is 17kJ/g organic matter (OM) compared to lignin at approximately 25.5 kJ/g (Kienzle et al., 2001). The difference is due to the comparatively higher carbon content of lignin (Demirbas, 2017). The complex structure of lignin contains numerous ether linkages, hydroxyl and methoxy groups and, therefore, a high-oxygen content (Amezcua-Allieri & Aburto, 2018). However, this energy is poorly accessible for ruminants. As a result the lignin content is negatively correlated with digestible energy in ruminant diets (Casler & Jung, 2006).
2. Reduced feed intake due to the association with polysaccharide constituents: lignin forms a physical barrier and thus hinders the access of rumen microbes to fermentable cell wall components. Consequently, the passage rate of feeds through the rumen is slowed down, thus reducing the feed intake capacity (Cannas, Van Soest, & Pell, 2003; Herrero et al., 2001).

Lignin has been identified as a major factor limiting the feed value in perennial grasses (Casler & Jung, 2006), maize stems (Mechin et al., 2000; Riboulet et al., 2008) and tropical forages (Gomes et al., 2011). Lignin content in alfalfa had a more negative effect in long-term than in short-term *in vitro* incubations, indicating that it affected the potential extent of digestion rather than the rate of digestion (Jung & Lamb, 2003). Animal feeding experiments have also confirmed these *in vitro* experiments. For example, it was demonstrated that lignin was the main chemical parameter explaining the *in vivo* organic matter digestibility of 64 different grass silages fed to cattle (De Boever et al., 1996). Another study reported a negative correlation between lignin concentration and *in vitro* digestibility in 36 different forages including legume and grasses and confirmed these results in feeding trials with lambs (Jung, Mertens, & Payne, 1997). Based on these findings, animal nutritionists usually seek to minimize the lignin content of ruminant diets.

One approach to minimising lignin content in fodder is to breed for low lignin content in forage crops. The efforts are comprised of both conventional breeding and biotechnological approaches (Barrière et al., 2003). Selection for high *in vitro* digestibility in four perennial forages was associated with simultaneous selection for

low lignin content (Casler, Buxton, & Vogel, 2002). Naturally occurring or induced brown midrib mutations are known to reduce the lignin content in a number of grass species (Sattler, Funnell-Harris, & Pedersen, 2010) and were also associated with improved digestibility in maize (Mechin et al., 2000), sorghum, and Sudan grass (Ledgerwood et al., 2009; Singh, Prasad, & Katiyar, 2003).

Several studies also tested crop or model species that were genetically modified to contain lower lignin content. The down-regulation of different monolignol biosynthetic genes to engineer transgenic alfalfa plants containing less lignin led to improved digestibility (Baucher et al., 1999; Getachew et al., 2011). Similar results were reported from transgenic maize in which a gene encoding a lignin biosynthetic gene was suppressed (He et al., 2003). This approach may however undermine the health of fodder and food crops (whose residue is used as livestock feed) during cultivation since lignin's function in plants includes defence against environmental and biological stresses, especially pathogens and insects; and conferring stability to xylem vessels for efficient water transport (Voelker et al., 2011).

In addition to lignin, many plant species contain a large diversity of secondary metabolites. These include condensed cyanogenic glycosides, glucosinolates, alkaloids, tannins, phenolics and saponins (Bennett & Wallsgrove, 1994; Kliebenstein, 2004). Two groups of compounds have attracted most attention in regards to ruminant nutrition; the condensed tannins (proanthocyanidins) and phenolics (Kingston-Smith et al., 2010).

Apart from the agricultural and ecological importance of the order *Artiodactyla* (suborder *Ruminantia*), ruminants are also interesting to scientists due to the complexity of the digestive system and the metabolic peculiarity that come with this. (Mackie et al., 2000).

1.2 RUMINANTS

1.2.1 RISE OF THE HERBIVORES

Microbes were the first forms of life to colonise almost every part of the planet and were continuously present for millions of years before multicellular life emerged (Woese, 1994). The consequence of this is that all animals had to occupy ecological environments that were already populated by microbes. The interactions between microbes and the multicellular newcomers ranges from cooperation to competition (Hungate, 1976). Animals could not escape being colonised by microbes on any surface that was open to environment. The digestive system is one such place where the environment favours rapid growth of microbes. The relationship between microbes and the animal in this system could be competition, cooperation or a combination of the two incorporating the advantages and minimising disadvantages to achieve an optimum (rather than a maximum) result (Mackie et al., 2000).

Mammals in the Cretaceous and early Palaeocene periods were most likely to have been eating fruits rather than plant foliage (since the former was presumably easier to digest) (Mackie, White, & Isaacson, 1997, p. 25). Bacterial fermentation is necessary to effectively break down the plant cell wall found in foliage. This requires ingested material to remain longer in the digestive system, which in turn needed to create space to function as a fermentation vat. Mammals therefore had to become physically larger in order to be proper herbivores (Figure 1.1); this finally occurred in the middle Palaeocene (Collinson et al., 1991). Before this, the main herbivores were dinosaurs of the late Cretaceous period (Farlow, 1987). The consensus is that herbivorous dinosaurs were browsers since they existed before the grasslands developed. However, recent evidence from silicified plant tissues (phytoliths) preserved in Late Cretaceous coprolites suggest that dinosaurs in this period consumed and may have evolved together with grasses (Piperno & Sues, 2005; Prasad et al., 2005). The rise of extensive grasslands probably commenced in the Miocene-Pliocene transition (7-5 million years ago) (Axelrod, 1985) and coincided with the appearance of mammalian grazers (Thomasson & Voorhies, 1990). This meant that for nearly 30 million years after the extinction of dinosaurs, plant-eating mammals remained either frugivorous or were browsers feeding mainly on leaves. The expansion in the variety of herbivores started

in the late Eocene when the climate cooled down and the vegetation started to open up (Mackie et al., 2000).

The fossil record does not provide any information on the morphology, physiology, biochemistry or microbiology of the gut of early herbivores. For a long time, any inference made to answer these questions based on the study of present-day animals and plants was mostly speculative (Ley, Hamady, et al., 2008). However, the advent of 16S ribosomal RNA gene sequencing has provided a powerful way to explore the evolutionary history of the vertebrate gut microbiota (Ley, Lozupone, et al., 2008).

Considering the time that microbes lived on the planet before the (relatively) recent arrival of herbivores, it is safe to assume that microbial digestion also existed long before it found a home in the gut. Many observers are quick to attribute the relationship between herbivores and microbes to the function of digestion, whereby the herbivore provides the substrate for fermentation and gains from the products of the process while the microbes get a constant supply of sustenance and a conducive environment to live and divide. However, the relationship may very well have been based on motivations other than nutrition, such as protection from ingested phytotoxins and mycotoxins (McSweeney & Mackie, 1997) or immunological or physiological benefits (Gaskins, 1997). If this were the case, then it would make sense if hindgut fermentation seen in many mammals preceded the foregut fermentation seen ruminants (Langer, 1991). This is supported further by the fact that ruminants still retain a significant capacity of hindgut fermentation (Hoover, 1978).

Scientists can only speculate on how herbivores first acquired gastrointestinal microbes since the fossil record cannot reveal such information. One theory is that the first microbes were picked up by detritivorous terrestrial amniotes as they foraged in decaying leaf litter (Hotton, Olson, & Beerbower, 1997). This material would contain bacterial already specialized in breaking down plant fibre. Another theory suggests that tetrapod herbivores acquired microbial endosymbionts from ingesting insects, which also harboured such microbes in their digestive system and for the same purpose (Sues & Reisz, 1998). In the latter case, the tetrapods would inherit microbes which were already adapted for both cellulose digestion and for living inside an animal’s body as an endosymbiont. It would presumably take a shorter time for the host and the symbionts to form a stable relationship. Animals that were originally

insectivores would become omnivores then herbivores. This is also consistent with the theory that hindgut fermentation developed before foregut fermentation (Mackie, Sghir, & Gaskins, 1999).

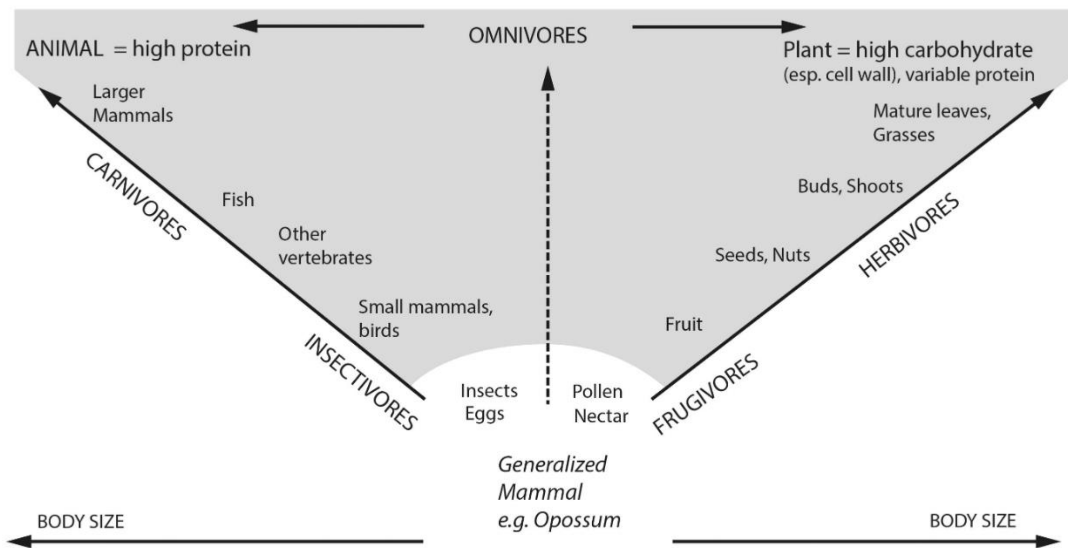


Figure 1.1: The Link between herbivory and body size (Clauss et al., 2013)

1.2.2 MICROBIAL ECOLOGY OF THE GUT

The microbial ecosystem found in the gastrointestinal tract is one of the most densely populated. The microbes found here are very diverse and have a complex relationship with each other and with the hosts. The three microbial domains *i.e.*, *Bacteria* (bacteria), *Archaea* (methanogens), *Eucarya* (protozoa and fungi) plus bacteriophages as outlined by Woese et al. (1990) and Klieve and Bauchop (1988) are all represented in this ecosystem. The rumen is perhaps the most complex site of interaction between animals and microbial endosymbionts. Contained in it are approximately 10^{11} cells/mL representing 200 bacterial species, 10^8 - 10^9 archaeal cells per mL, 10^4 – 10^6 protozoal cells per mL representing over 25 genera and 10^3 – 10^5 cells/mL of fungi representing five genera and 10^7 – 10^9 bacteriophage particles per mL (Mackie et al., 2000).

For a long time, the full complexity of this ecosystem was hidden from scientists since the only method available for identifying and differentiating microbes in the gastrointestinal tract was to attempt to isolate pure cultures. This was hampered by the realisation that only a small fraction of the total number of microbes found in the rumen could be recovered through culture methods (Amann, Ludwig, & Schleifer, 1995). This could be due to several factors. For instance, some bacteria may be

changing their metabolic status once they are removed from their preferred niche in the rumen and thus do not enter a multiplication state when put on artificial media. It could also be due to the inability of microbiologists to reproduce the exact *in vivo* environment for the microbes that are much more fastidious or have complex interactions within the ecosystem (Krause & Russell, 1996). Therefore this approach does not provide a representative picture of the ecosystem in regards to its members and their interactions (Tajima et al., 1999). These limitations would have stagnated the study of microbes not just in the gastrointestinal tract but in general were it not for advances made in molecular biology.

1.2.3 MOLECULAR BIOLOGY

The term "Molecular Biology" was first coined by William Astbury in 1950. He described Molecular biology as:

> "An approach from the viewpoint of the basic sciences with the leading idea of searching below the large-scale manifestations of classical biology for the corresponding molecular plan; and concerned particularly with the forms of biological molecules, and with the evolution, exploitation and ramification of those forms in the ascent to higher and higher levels of organisation" (Astbury, 1961).

In 1958, Francis Crick put forward the Central Dogma of Molecular Biology. A revised form was published in 1970 and stated that:

> "Molecular biology deals with the detailed residue-by-residue transfer of sequential information and such information cannot be transferred back from protein to either protein or nucleic acid" (Crick, 1970).

The central dogma has also been summed up as "DNA makes RNA and RNA makes protein". With time, the central dogma has been proven to be over-simplified in the face of more discoveries particularly regarding the elsewise unknown dynamic nature of RNA (Shapiro, 2009). For instance, since then, reverse transcription has been demonstrated to occur routinely in nature (Koonin, 2015). Furthermore, the discovery of prions suggests that information can be passed from protein to protein (Chien,

Weissman, & DePace, 2004). The possibility of reverse translation occurring in nature has also not been ruled out (Biro, 2014).

Molecular biology as a scientific approach was preceded and is still interdependent with biochemistry. The line between these two fields is blurred as their field of study heavily overlap (Papachristodoulou et al., 2014, p. 3). Due to its focus on DNA and RNA, molecular biology is related to genetics. Advances in technology are now allowing advanced biomolecular and biochemical methods. Automated DNA sequencing improved the speed and accuracy while mass spectrometry improved the study of proteins; gene and gene expression studies were improved by DNA microarrays. This has led to the rise of different branches of science such as proteomics and genomics and thus the dawn of the 'omics' age. These advances, particularly in DNA sequencing led to a flood of information through which scientists had to sift to make sense of it. This led to the setting-up of digital databases of DNA sequences and protein structures and the development of software in the public domain to enable researchers to search, analyse and share all the information; this field came to be known as bioinformatics and is closely related to molecular biology (Papachristodoulou et al., 2014, p. 13). Among the milestones achieved along the way are sequencing of the human genome (Schmutz et al., 2004); and those of the mouse, the rice plant and the *Drosophila* (fruit fly).

1.3 RUMEN MICROBIOLOGY

1.3.1 ESTABLISHMENT OF THE RUMEN MICROBIAL POPULATION

At birth, rumen has no microorganisms. Colonisation, however, occurs very early and the levels of microbes increase rapidly (Abecia et al., 2014; Yáñez-Ruiz, Abecia, & Newbold, 2015). In young ruminants and during rumen development, ingested microbes colonise and establish in a defined and progressive sequence. In lambs, at the end of the first week the population of these bacteria reached a level close to that generally observed in a mature rumen (Fonty et al., 1987). At birth, the ruminant animal is essentially a monogastric animal with a poorly developed foregut. The changes in the structural and physiological properties of the rumen with age are linked to the development of the rumen microorganisms, as their fermentation products are important for the development of the rumen wall villi (Jami et al., 2013). Ruminants naturally raised by their dams have been shown to have better rumen development compared to those artificially raised with colostrum alternatives and milk (Belanche, Cooke, et al., 2019). Maternal *versus* artificial rearing also shapes the rumen microbiota in early life and nutritional intervention can influence the initial rumen microbial community. However, the persistence of these effects later in life is weak (Belanche, Yáñez-Ruiz, et al., 2019).

Table 1.1: Establishment of the rumen microbial population from birth (Fonty et al., 1987; Jami et al., 2013; Yáñez-Ruiz et al., 2015)

Appear	Peak	Microorganisms
5-8 hrs	4 days	*Escherichia coli, Clostridium welchii*, *Streptococcus bovis*
½ week	3 weeks	*Lactobacilli*
½ week	5 weeks	Lactic acid-utilizing bacteria
½ week	6 weeks	Amylolytic bacteria, *Prevotella* (week 6)
1week	6 – 10 wks	Cellulolytic & Methanogenic bacteria *Butyrivibrio* (week 1), *Ruminococcus* (week 3), *Fibrobacter* (week 1)
1 week	12 weeks	Proteolytic bacteria
3 weeks	5-9 weeks	Protozoa
2-4 days	10-14 days	Methanogenic archaea
8-10 days	3 weeks	Anaerobic fungi
	9 - 13 wks	Normal population

For a long time, the composition of ruminal bacterial communities in the first days after birth had only been studied using culture-dependent methods, which allowed examination of only a small portion of the bacteria. However, application of molecular

techniques as in the cases of Li et al. (2012) and Jami et al. (2013) has provided a clearer picture by identifying and classifying microbes undetected by culture-dependent methods (Table 1.1).

1.3.2 RUMEN MICROBES IN ADULT RUMINANTS

The rumen microbiota in adult ruminants is diverse and complex. It is composed of anaerobic bacteria, protozoa and fungi. Each of these kingdoms has its own role in rumen function. Rumen microbial community composition varies with diet and host; with the former being the main driver of the difference. However, there is a core microbiome is found across a wide geographical range (Henderson et al., 2015). The rumen microbiome composition is also affected by the host factors including species, breed, individual specificity, host immunity and exposure to colonising microbes in early life. Species specificity of the microbial composition was demonstrated by Weimer et al., (2010); after a near-total exchange of ruminal contents between dairy cows and reindeer, the composition quickly returned to the original format. This was more so in terms of bacteria than for protozoa. This phenomenon has implications for likely success of attempts to modify the ruminal fermentation by targeted addition of microbes into the ruminant or into ruminant rations. Animals of the same species but of different breeds also show a difference in the microbial composition. There was a significant difference in the microbial population between lactating Jersey and Holstein dairy cows from a single herd fed the same diet and maintained under the same environmental conditions indicating a breed effect (King et al., 2011). Host specificity goes down to the individual, whereby even identical twin cows have different microbial composition (Weimer et al., 2010). It has been suggested that the animal exerts control over its ruminal chemistry through salivary buffering and through absorption and passage of volatile fatty acids (VFAs), and this is how the animal applies selective pressure on the microbial community (Weimer, 2015).

Apart from resilience and presence of a core microbiome, the rumen microbial composition is also highly redundant. This means that there is an overlapping distribution of physiological capabilities across multiple microbial taxa (Weimer, 2015). This has been suggested by the observation that considerable changes in community

composition often do not translate into changes in fundamental fermentation metrics such as pH, VFA concentrations or molar proportions of VFAs (Sandri et al., 2014). The high level of redundancy however may not translate to better production performance of the ruminant. For instance, Shabat et al. (2016) observed that milking cows with a lower number of microbial operational taxonomic units had a higher feed conversion efficiency. Metatranscriptomic profiling has revealed that rumen microbes may additionally play a role in the residual feed intake since the rumen microbiomes of inefficient cattle may have more diverse activities than those of efficient cattle (Li, 2017).

As mentioned earlier, the animal exerts some form of control over the composition of its rumen microbes. This may be *via* salivary buffering which controls the pH and VFAs concentration (Weimer, 2015). Saliva also contains immunoglobulin-A which remains active long enough in the rumen to interact with rumen microbes (Fouhse et al., 2017). When cattle were vaccinated with a methanogen protein, a strong antigen-specific IgG and moderate IgA responses were measured in the serum and saliva of the animals; the antibody was also detected in the rumen (Subharat et al., 2015). This outcome offers a possibility of vaccinating ruminants against specific rumen microorganisms (Yáñez-Ruiz et al., 2015). Another aspect of the host physiology is the rumen size; measurement of methane emissions from 160 mature ewes showed that sheep with smaller rumens and shorter rumen retention time have lower methane yield than those with a larger rumen (Goopy et al., 2014). This suggests that the selection of ruminants for smaller rumens may have important consequences for methane emissions.

Many approaches to modifying the rumen microbial community are hampered by its resilience and complex interaction with the host. This is particularly evident in adult animals. Interventions in the earliest parts of a ruminant's life may however confer lifelong change to this composition and offer an opportunity to mould it to one optimised for production performance (Abecia et al., 2014).

1.3.3 RUMEN BACTERIA

The rumen bacterial community is highly plastic. Community structure varies between ruminant species, between groups or herds, between individuals within a herd, under different environmental conditions, stresses and challenges and as a response to diet. However, there is a core community common across most ruminants (Henderson et al., 2015) (Figure 1.2). This diversity allows the plasticity. Despite this, each group of rumen bacteria have a specific role in rumen function.

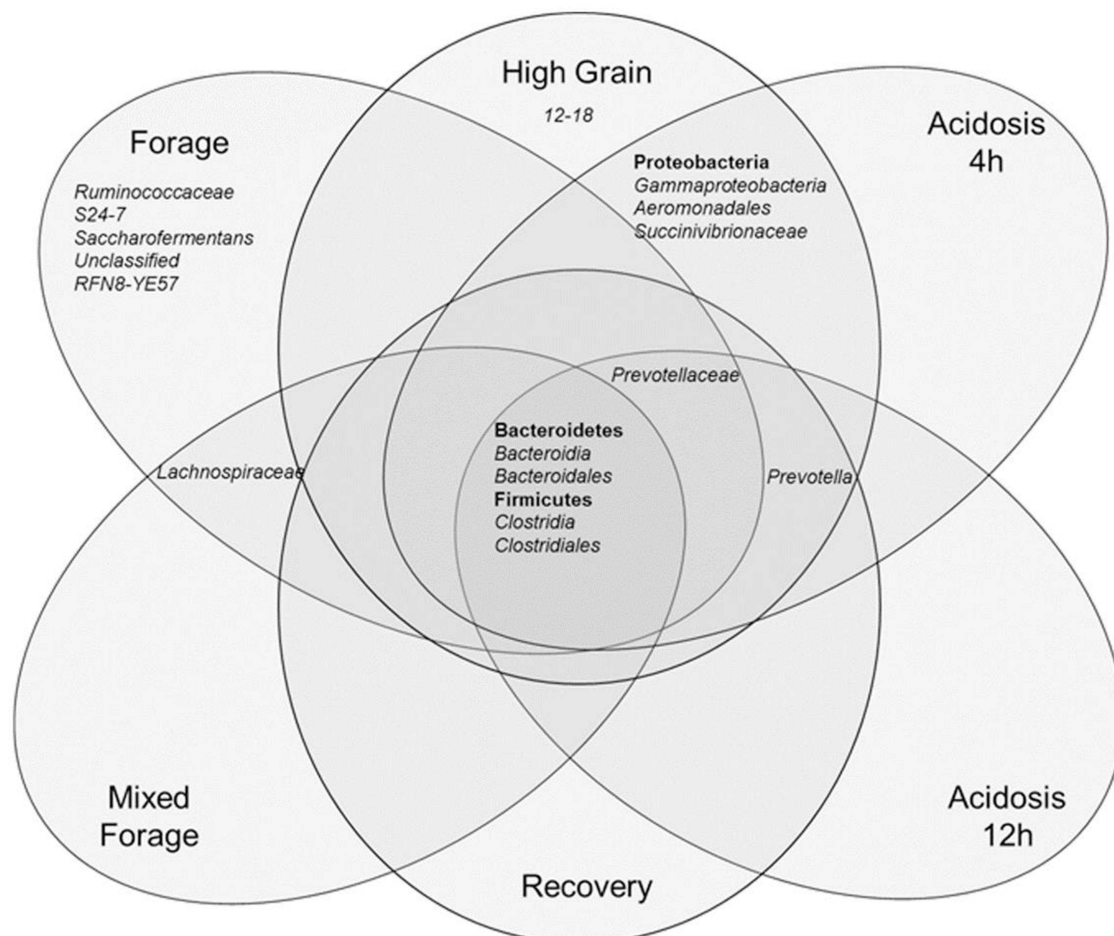


Figure 1.2: Venn diagram of the rumen core microbiome of 16 lactating heifers under different conditions. The microbiome is affected by type of feeds conditions. However, there is a core microbiome (in the middle) that is present under all feeding conditions. From Petri et al. (2013).

Previously, classical microbiological techniques (plating and counting) were used to study bacterial diversity. Nowadays, analysis of the 16S rRNA gene is used for this. The 16S RNA gene codes for part of the 30S ribosome components of the prokaryotic ribosome, which is the site of transcription. It is highly conserved across the bacteria and archaea allowing for the use of universal PCR primers enabling the amplification of

the majority of bacteria sequences. However, there are hypervariable regions (Figure 1.3) which allow the construction of phylogenies (Robert, 2012).

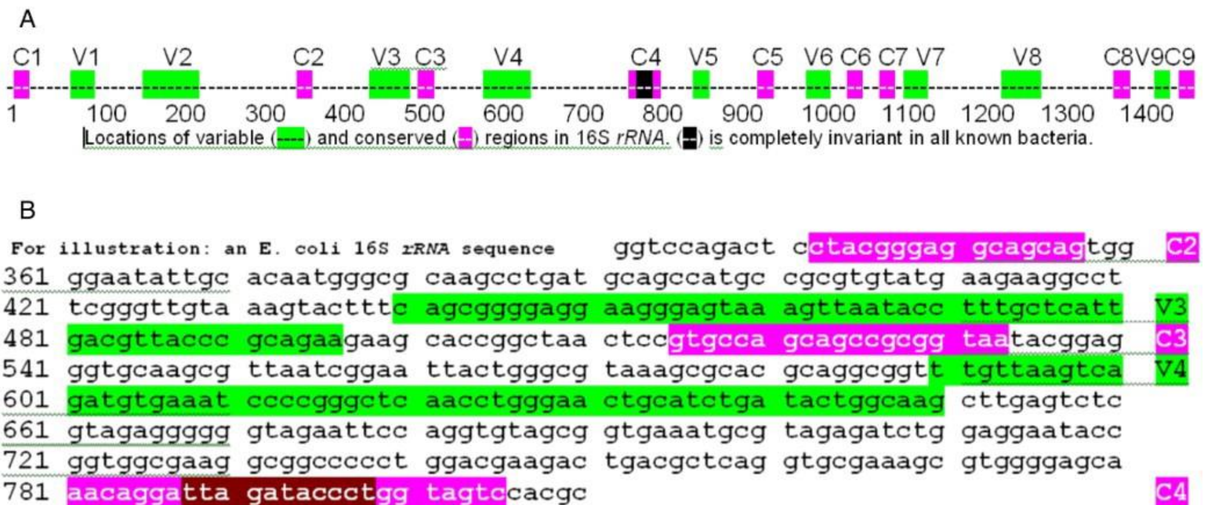


Figure 1.3: Location of variable and conserved regions in a typical bacterial 16S rRNA. A) Location of variable (Vi, green) and conserved (Ci, pink) regions in a typical bacterial 16S rRNA gene (complete gene). The black bases in C4 are completely invariant in all bacteria. B) Specific example of the C2 to C4 region in *Escherichia coli*, with a similar colour code to A. The C2, C3, and C4 conserved regions are the targets of universal bacterial ribosomal DNA primers. From Ram et al. (2011).

Phylogenetic studies based on 16S RNA gene sequencing has resulted in the Ribosomal Database Project (RDP), an online database specific for the rRNA of bacteria in many different ecosystems (Cole et al., 2009). A study specific for the rumen showed over 13,000 rumen bacterial sequences within the database. They estimated that although there are 7,000 rumen bacterial species in 20 existing phyla, the distribution is dominated by three phyla; 51% Firmicutes, 41.6% Bacteroidetes and 5.46% Proteobacteria (Jami & Mizrahi, 2012) (Figure 1.4). In addition, there are many uncultured and undescribed sequences. The potential to identify “unknown” and “uncultured” organisms is one advantage of molecular techniques over classical culturing methods. The sequences represent the actual composition of the rumen bacteria meaning that most of the bacteria living in the rumen are classified under one of these three phyla (Creevey et al., 2014).

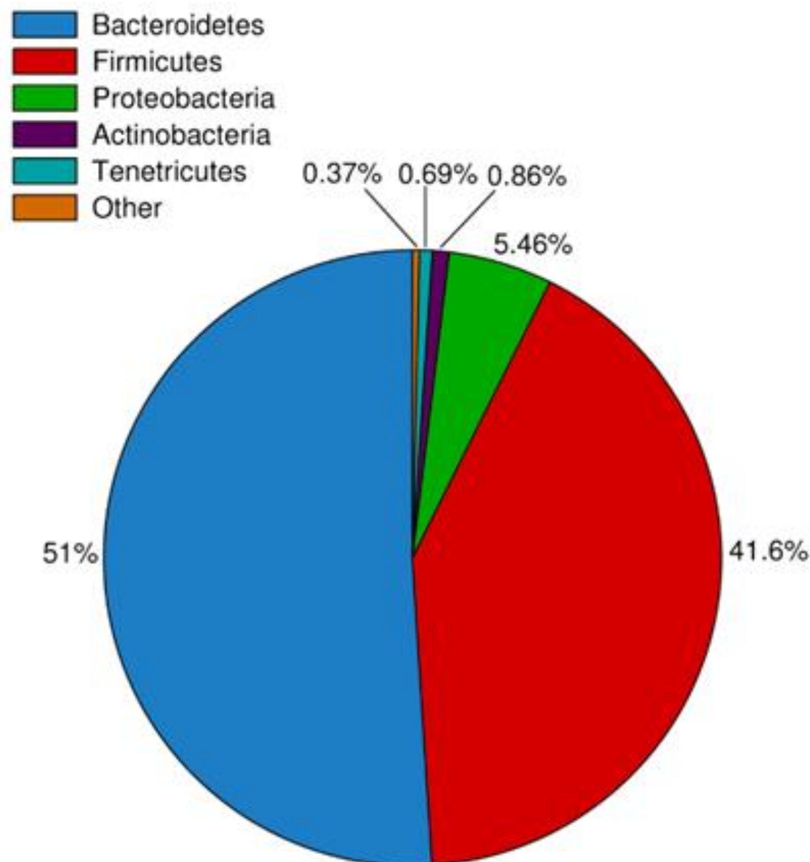


Figure 1.4: Composition and abundance of bacterial taxa in the bovine rumen, as determined by pyrosequencing of the 16S rDNA gene. From Jami and Mizrahi (2012).

For a long time, the dominant three phyla made it difficult to detect bacteria in the other seventeen even using molecular techniques. For example, to sample 99.9% of the bacterial sequences of the estimated 7,000 species, it is estimated that 78,218 sequences have to be read (Jami & Mizrahi, 2012). This is because of the scale of difference in relative abundances between the dominant and the minor phyla made detection of lower abundance organisms in a small sample more difficult. The advent of high-throughput next generation sequencing methods making it possible to collect hundreds of thousands of sequences spanning hundreds of samples in a short period has ameliorated this problem (Caporaso et al., 2011).

PHYLUM BACTEROIDETES

Members this phylum are gram-negative, anaerobic, non-spore-forming, rod shaped bacteria (Henthorne, Thompson, & Beaver, 1936). They are widely distributed in the environment, including in soil, sediments, and seawater, as well as in the guts and on the skin of animals. The classes in this phylum are Bacteroidia, Flavobacteria,

Sphingobacteria and Cytophaga (Mariat et al., 2009). Bacteroidia is the most important class in the rumen. Class Bacteroidia is broken down into two orders Bacteroidales and Prevotellacea. Members of this class are closely related and many species' classification switches between these two groups. *Prevotella* is the dominant species in the rumen (Stevenson & Weimer, 2007). The genus *Prevotella* is the most highly represented genus in the rumen, making up >50% of rumen bacteria sequences in databases (Jami & Mizrahi, 2012). The most important species are *Prevotella ruminicola*, *P. brevis*, *P. bryantii* and *P. albensis* (Tajima et al., 1999).These bacteria utilise starch, non-cellulosic polysaccharides and simple sugars and are major converters of succinate to propionate thus the maintenance glucose homeostasis in ruminants. There is substantial evidence to support the role of *Prevotella* spp. in protein (Wallace & Brammall, 1985; Wallace, Onodera, & Cotta, 1997) and peptide (Wallace & McKain, 1991) metabolism in the rumen. *Prevotella* spp. are considered to be among the prominent ruminal proteolytic bacteria (Griswold, White, & Mackie, 1999).

THE FCB GROUP

This is a superphylum originally proposed as an alternative phylum; it consists of the Fibrobacteres, Chlorobi and Bacteroidetes phyla. These three are very closely related, showing conserved proteins and gene structures. While underrepresented in databases, the group contains the important species *Fibrobacter succinogenes* (Gupta, 2004). Phylum *Fibrobacteres* contains only two species – *Fibrobacter intestinalis* and *F. succinogenes*. *F. succinogenes* is a major producer of succinate, again, key in glucose homeostasis and is considered one of the major cellulolytic rumen bacteria (Ransom-Jones et al., 2012).

PHYLUM FIRMICUTES

Most members of this phylum have a Gram-positive cell wall structure; a few, however, such as *Megasphaera*, *Pectinatus*, *Selenomonas* and *Zymophilus*, have a porous pseudo-outer membrane that causes them to stain Gram-negative (Pimentel, Micheletti, & Pace, 2013). Many produce highly resistant endospores (Galperin, 2013).

The classes in this phylum are Clostridia, Bacilli, Erysipelotrichia, Negativicutes and Thermolithobacteria (Zhang & Lu, 2015).

Class Clostridia is the largest group of this phylum found in the rumen. It is a highly diverse and polyphyletic class and accounts for >90% of sequences (Ludwig, Schleifer, & Whitman, 2009). It is broken down into orders Lachnospiraceae, Ruminococcaceae and Veillonellaceae (Kalia et al., 2011). There are several genera in this class that are of particular significance in the rumen. Genus *Butyrivibrio* is classified within the family Lachnospiraceae. Members of this genus break down hemicellulose in plant cells and can utilise wide range of sugars such as pentose and hexose sugars to butyrate, acetate and hydrogen. They are also involved in protein breakdown and lipid metabolism particularly in biohydrogenation of unsaturated fatty acids (Maia et al., 2010). Genus *Acetovibrio* is classified under the family Clostridiaceae; *A. cellulolyticus* has an important function of degrading crystalline cellulose with an elaborate multi-enzyme cellulosome. The cellulosome is located on the outer wall of the bacteria which attaches to the substrate to synergistically breakdown cellulose substrates (Dassa et al., 2012).

Genus *Ruminococcus* is found in the Ruminococcaceae family. It contains two of the three major cellulolytic species in the rumen *i.e. Ruminococcus albus* and *R. flavefaciens* (the third one is *Fibrobacter succinogenes* discussed earlier) (Koike & Kobayashi, 2001). Members of this genus also possess a cellulosome on the outer wall with both cellulase and hemicellulase activity; they produce succinate, acetate and some fumarate (Koike & Kobayashi, 2009). The genus *Succiniclasticum* is highly represented in sequence databases and is fairly common in the rumen (Li, Wu, et al., 2012). *S. ruminis*, the only member of the genus converts succinate to propionate as sole energy source; other bacteria require inputs such as hydrogen or the presence of other bacteria (Van Gylswyk, 1995).

Members of the class *Negativicutes* stain gram-negative unlike other *Firmicutes* since they have a mucous membrane on outer cell wall (Kojima et al., 2016). The class contains only two families, Acidaminococcaceae and Vaillonellaceae (Yutin & Galperin, 2013). The genus *Acidaminococcus* is classified under the family Acidaminococcaceae. Members of this genus are diplococcal bacteria capable of metabolising amino acids. The species *A. fermentans* is an important species since it oxidises trans-aconitate to

acetate therefore preventing accumulation of the highly toxic tricarballylate a tricarboxylic acid which chelates blood divalent cations and decreases their availability (Cook, Wells, & Russell, 1994). Decreases in blood magnesium in turn cause a potentially fatal disease known as grass tetany (Goff, 2014).

Under the family Vaillonellaceae, the genus *Veillonella* is highly represented in sequence databases. *Veillonella parvula* is a bacterium that ferments lactate to produce acetate (Prins & Van den Vorstenbosch, 1975). Genus *Selenomonas* is another member of the family Vaillonellaceae. It is an interesting motile crescent-shaped bacterium (Bryant, 1956). *Selenomonas ruminantium* often abundant in the rumen and is a major propionate producer (Sawanon, Koike, & Kobayashi, 2011). It also reduces trans-aconitate to tricarballylate but to a much lesser extent compared to *A. fermentans* (Cook et al., 1994). Lactic acid-producing genera *Streptococcus* and *Carnobacterium* are the most predominant within class Bacilli (Kim, Morrison, & Yu, 2011). Members of the genus Staphylococcus tend to be pathogens or introduced in the rumen as probiotics (Alves et al., 2009).

PHYLA PROTEOBACTERIA AND ACTINOBACTERIA

Members of each of the six classes of Proteobacteria are present in the rumen (Jami & Mizrahi, 2012; Tajima et al., 1999). However, there are species that are more important than others. *Succinivibrio dextrinosolvens* from the class Gammaproteobateria break down starch in high carbohydrate diets and a spike in population can be detected in animals changed to this type of diet (O'Herrin & Kenealy, 1993). *Desulfovibrio* are sulphate-reducing bacteria present in animals with sulphate in their diet (Howard & Hungate, 1976).

Members of the Phylum *Actinobacteria* represent a small but significant part of the rumen microbiome (Šuľák et al., 2012). Members of the family Coriobacteriaceae always show up in analyses (Robert, 2012). Members such as genera *Olsonella* and *Atopobium* key metabolisers of peptides and amino acids they further breakdown products from other bacteria are important for microbial protein synthesis. *Adlercreutzia*, *Atopobium* and other *Coribacteriales* have been shown to have role in milk lactose content and milk yield and big change in these can have a significant effect in milk yield (Jami, White, & Mizrahi, 2014).

OTHER PHYLA

Members the phylum Tenericutes are cocci bacteria which lack a cell wall. Bacteria of the order Anaeroplasmatales (family Anaeroplasmataceae) are commonly found in the rumen of cattle and sheep (Mao et al., 2015). Members of the phylum Spirochaetes are spiral motile bacteria. In the rumen the genus *Treponema* are cellulolytic and break down of pectin (Liu et al., 2015). In the phylum Synergistetes we can find *Synergistes jonseii* capable of degrading toxic pyridinediols present in some plants (Allison et al., 1992). The phylum Verrucomicrobia contains only a few described species. In the rumen, it is associated with polysaccharide degradation (Martinez-Garcia et al., 2012).

LACTIC ACID BACTERIA

Within the rumen the lactic acid producing genera that are most abundant are *Streptococcus* and *Carnobacterium* but also present are *Lactobacillus* and *Planococcus* (M. Kim et al., 2011). They are generally present in low numbers but the population spikes when diet allows lactic acid production to exceed lactic acid usage *i.e.* in conditions of acidosis (Jiao et al., 2017). They also have a role in pathogen control in young animals when the rumen is still developing (Vieco-Saiz et al., 2019). Milk fed animals inoculated with *Lactobacillus casei* or *L. acidophilus* show lower faecal counts of *Escherichia coli* and reduced mortality (Signorini et al., 2012). Lactic acid bacteria are also associated with conjugated linoleic acid synthesis (Ogawa et al., 2005).

1.3.4 RUMEN FUNGI

Rumen fungi make up 5-10% of the microbial biomass (Griffith et al., 2009). They are difficult to visualise since they are almost entirely attached to the fibre in the rumen. Rumen fungi are a relatively recent discovery. First described as fungi in 1975, zoospores had been previously mistaken for protozoa since they are motile (Orpin, 1975). It was thought no fungus could exist obligate-anaerobically; they have since been identified in caecum and colon of hindgut fermenters (Orpin, 1981).

Anaerobic rumen fungi are the primary colonisers of fibrous plant materials in the rumen and are able to degrade lignin-containing plant cell walls (Akin & Borneman, 1990). Colonisation of ingested fibrous materials can be affected by the type of pre-treatment the material undergoes. Chaudhry (2000) reported that chemically treating wheat straw with calcium oxide, sodium hydroxide and alkaline hydrogen peroxide improved colonisation and degradation by rumen fungi *in sacco* in the rumen of sheep. Mechanical processing of the forage also increases the activity of fungal populations in the rumen (Rezaeian, Beakes, & Chaudhry, 2006).

Rezaeian, Beakes, and Chaudhry (2005) observed a positive correlation between the *in vitro* enzyme activities of the anaerobic fungi their biomass concentration assessed by chitin assay. They concluded that chitin is a valuable index for the estimation of the fungal biomass *in vitro*.

CLASSIFICATION OF RUMEN FUNGI

Anaerobic gut fungi were first classified as Chytridiomycetes, in the order Spizellomycetales (Ozkose et al., 2001). Later, they were classified to a new order, Neocallimastigales and in the family, Neocallimastigaceae after sequence analysis of the small (18S) nuclear rRNA region and numerical taxonomic methods (Ho & Barr, 1995). The family is further classified into phyla based on the morphology of the thallus (vegetative state that forms after the zoospore germinates on the fibre material) (Gruninger et al., 2014).

The monocentric groups' thallus consists of single reproductive cell attached to substrate hyphae (Gruninger et al., 2014). On the other hand, the thallus of the polycentric group has many reproductive centres with interconnected rhizoids (Griffith et al., 2009; Ozkose et al., 2001). Therefore, the suggested subdivision of this family is

into three genera containing monocentric species, *Neocallimastix*, *Piromyces* (previously *Piromonas*) and *Caecomyces* (previously *Sphaeromonas*). Three polycentric genera have been described, *Orpinomyces*, *Anaeromyces* and *Cyllamyces* (Ozkose et al., 2001).

As with the bacteria, microscopic identification of anaerobic fungi is largely being replaced with molecular characterisation (Fliegerova, Hodrova, & Voigt, 2004). This approach will give a better estimate of the true percentage of microbes in the rumen (Denman & McSweeney, 2006). Other studies have estimated the fungal biomass to be up to 20% based on the determination in the amount of chitin in the rumen. However the chitin content of the rumen may not be a good indicator of fungal content as the chitin content of rumen fungal cell walls can vary according to the species, their age and conditions of growth (Sekhavati et al., 2009).

LIFE CYCLE OF RUMEN FUNGI

Life cycle lasts about 23-32 hours. The life cycle consists of an alteration between a motile, zoospore stage and a vegetative, zoosporangial stage. Flagellate zoospores are then released from a sporangium. The zoospore germinates to produce a germ tube, which later develops into rhizoids (Lowe et al., 1987; Orpin, 1977).

The zoospore is a microscopic, uninucleate, unicellular, flagellated spore lacking a cell wall. It is formed in a zoosporangium by a process involving mitosis and cytoplasmic cleavage (Moore-Landecker, 2001). Zoospores do not feed, and rely on endogenous energy reserves (Swafford & Oakley, 2018). Encystment seen in similar fungi in other ecosystems has not been described in rumen fungi yet but it believed to be the mechanism by which fungi end up in the faeces and can be transmitted from one animal to another (Gleason & Lilje, 2009).

ROLE OF ANAEROBIC FUNGI IN FIBRE DIGESTION

Role of rumen fungi in the degradation of plant fibre has been examined extensively (Ho & Abdullah, 1999). These fungi are better at penetrating plant tissue than are bacteria and protozoa. Penetration leads to faster and more complete degradation of forage that enters the rumen (Bauchop, 1981). While protozoa and bacteria can only colonise the cut ends where the animal has chewed it, fungi can penetrate unbroken

fibre using the rhizoid and extract nutrient from it (Akin & Borneman, 1990). This is important for the breakdown of particularly recalcitrant fibre (Borneman et al., 1990; Orpin, 1984). The greater ability of rumen fungi to weaken forage fibre may be important in enhancing forage utilization by the host animal (Akin & Borneman, 1990).

HYDROLYTIC ENZYMES

While rumen protozoa and bacteria have been shown to play a role in plant fibre degradation, rumen fungi display a higher potential for the degradation of more heavily lignified plant tissues. Apart from producing rhizoids (which physically penetrate the fibrous substrate), anaerobic fungi produce a wide range of hydrolytic enzymes degrade plant cell walls. This include a wide range of cellulases, hemicellulases, proteases, amylases and amyloglycosidases (Solomon et al., 2016). Of particular importance are feruloyl and *p*- coumaroyl esterases which fungi are thought to be the primary source of in the rumen (Borneman et al., 1990). Cellulose and hemicellulose are bound to lignin by ester bonds; rumen fungi therefore loosen up the fibre substrate that would otherwise be physically unavailable for other microbes to attack. Rumen fungi may be important for the initial colonisation of the ingested fibre substrate (Borneman et al., 1990). Rumen fungi also produce various disaccharidases and pectinases (Gordon & Phillips, 1992).

INTERACTION OF RUMEN FUNGI WITH OTHER RUMEN MICROORGANISMS

Anaerobic fungi in the rumen produce molecular hydrogen, which in turn stimulates the growth of methanogens (Ivarsson et al., 2016). The symbiotic cooperation between anaerobic fungi and methanogens in the rumen enhance the metabolic rate and growth of both resulting in formation of stable co-cultures with rumen methanogenic archaea. The archaea will remove the hydrogen and form methane thus allowing increase amount of fungal biomass and exhibit an increase in both the rate and extent of cellulose degradation (Bauchop & Mountfort, 1981). The downside to that is production of more methane. On the other hand, cellulolytic activity appears to be inhibited, when anaerobic fungi are combined in co-culture with the cellulolytic rumen bacteria most likely due to production of inhibitory factors (Dehority & Tirabasso, 2000). The relationship between protozoa and fungi is predation whereby

protozoa are able to both ingest and digest fungi (Theodorou & France, 1993; Williams & Coleman, 1992).

POTENTIAL BENEFITS OF RUMINAL ANAEROBIC FUNGI FOR IMPROVED ANIMAL NUTRITION AND PRODUCTIVITY

Improved fibre digestion and nutrient utilization leads to more feed intake and feed efficiency with increased body weight and improved milk production. These benefits are however hampered by the long lifecycle of fungi (24-36 hours) which is much longer than the average time the fibre substrate stays in the rumen in high producing cattle. However, rumen fungi may be an important aspect in animals fed a more recalcitrant fibre diet (Gruninger et al., 2014). Rumen fungi are associated with forage-based diets as observed by Belanche et al. (2012). They reported an increase in the ruminal abundances of anaerobic fungi (+59%) when cows consumed a fibre-rich diet compared with when they consumed a starch rich diet.

The superior ability of anaerobic fungi to degrade recalcitrant fibre in the rumen make them an attractive target for manipulation to improve rumen fermentation efficiency. At present, rumen fungi are difficult to exploit due to incomplete knowledge of how the fungi effect rumen function; their life cycle, cellular physiology, genetics, and cellulolytic metabolism (Haitjema et al., 2014). As such, there are not many publications on manipulation of rumen fungi. Additionally, the rumen ecosystem is very resilient and the resident microbes are very likely to revert back to pre-intervention composition (Yáñez-Ruiz et al., 2015).

Rumen fungi could be exploited as direct-fed probiotics or feed additives (Lee et al., 2004; Lee, Ha, & Cheng, 2000). However, they are anaerobic and present a difficulty in how to dose the animal. On the other hand, they could be used as novel silage inoculants (Lee et al., 2015). There's also potential for large-scale production of enzymes; enzymes have been cloned from rumen fungi particularly for the biofuel industry and production of bio-detergents (Dashtban, Schraft, & Qin, 2009; Ribeiro et al., 2016; Solomon et al., 2016).

1.3.5 RUMEN PROTOZOA

Rumen protozoa were first described as “animalcules” by Gruby and Delafond (1843). They are present at a density of 10^4 - 10^6/mL representing 100 species. Although they are fewer than bacteria, protozoa contribute 10 - 50% of microbial biomass due to their much larger size of 10-200μm (Williams & Coleman, 1992).

The interactions among protozoa species range from competition for substrate, specific predation, to same-species cannibalism (Martinele & D'Agosto, 2008). Protozoa also interact with bacteria and archaea in form of predation and mutualism with archaea. The latter relationship is important for methane production by ruminants. This active role means that protozoa have a significant effect on the metabolism of the ruminant host.

While protozoa make up a large portion of the rumen biomass, their role in ruminal fermentation and their contribution to the metabolism and nutrition of the host is still an area of substantial controversy (Newbold et al., 2015).

Protozoa are established very early in the animals life; they are passed *via* saliva (grooming), from drinking water and from pasture (Eadie, 1962). The process of colonisation is continuous and is enhanced by feeding of fibrous material. This increases the volume of the rumen, salivation and increases the pH allowing for the establishment of protozoa (Dehority, 1993b).

The morphology of rumen protozoa is quite distinctive; they have an oesophagus where the protozoa ingests substrate particles, macro- & micronucleus and contractile vacuoles, which allow for motility in the rumen. Certain species have skeletal plates where they store amylopolysaccharides but also for maintaining their specific shape (Dehority, 1993a). Protozoa are classified according to their stable and distinctive morphology; new attempts to classify them based on rRNA are still in development and must be validated. Currently, microscopy first proposed by Williams and Coleman (1992) (rather than PCR-based molecular methods) is the gold standard for studying ciliate protozoa (Newbold et al., 2015). This is because the majority of intestinal ciliates have been characterized morphologically while there is a lack of 18S rRNA gene reference sequences for many of the observed protozoa genera and species. Secondly, copy number variation of ribosomal RNA genes across the different genera or under

different growth conditions may skew the observed proportions of these genera in a sample (Newbold et al., 2015). However, efforts have been made to design and validate primers for next-generation sequencing to target the 18S rRNA genes of gastrointestinal ciliate protozoa (Ishaq & Wright, 2014); are being used to routinely identify and quantify rumen protozoa (Ishaq et al., 2017).

All the ciliates from the gastro-intestinal tract constitute a monophyletic group, which consists of two major taxa, *i.e.* Vestibuliferida and Entodiniomorphida (Moon-van der Staay et al., 2014). Under the taxon Vestibuliferida, we find the family Isotrichidae; these are commonly called Holotrich protozoa and they are covered by cilia all over their bodies (Dehority, 1993a). Under the taxon Entodiniomorphida is the family Ophryoscolecidae commonly called Entodiniomorphid protozoa; cilia only found close to the oesophagum. This group has three main subfamilies. Entodininae have one region of cilia, Diplodininae have two regions of cilia. The proportions of the different groups in the rumen vary according to the ruminant host; however, members of the *Entodinium* species are commonly found in the highest numbers representing up to 80% of the total population of the protozoa (Nagaraja, 2016).

Feeding habits affects the density and proportion of protozoa and can be distinctly different even in closely related species *e.g.* the goat and ibex (de la Fuente et al., 2009; Del Valle, De la Fuente, & Fondevila, 2008). Protozoa populations have been described according to the antagonism between the different species (Table 1.2).

Table 1.2: Rumen protozoa population types (Eadie, 1967; Imai, Katsuno, & Ogimoto, 1978)

Type A	Type B	Type O	Type K
Entodinium Holotrichs Ophryoscollex *Polyplastron multivesiculatum* *Metadinium affine* Diplodiniinae	Entodinium Holotrichs Epidinium *Eudiplodinium maggii* Ostracodinium Diplodiniinae	Entodinium Holotrichs	Entodinium Holotrichs *Elytroplastron bubali* Diplodiniinae

The most common ones are type A and B. Type A predominates in sheep while type B predominates in cattle. Type K is only found in in cattle. These different types of populations are antagonistic to each other; for instance, type A dominates over type B (predation). Experimental transfaunation between the A and B results in type A

population (Imai et al., 1978). Type O population is found in animals fed in animals fed mainly on soluble sugars (Eadie, 1967).

RUMEN PROTOZOA METABOLISM

Rumen protozoa have a slow generation time (6 to over 48h) meaning they must have a mechanism to allow them to propagate in the rumen without being washed out. They do this by being closely associated with feed particles, or in case of Holotrichs, exhibit chemotaxis moving to the back of the rumen when animals are eating before settling in ventral and cranial sacs (Abe & Iriki, 1989). Holotrichs near the rumen wall scavenge O_2 since they possess an organelle called hydrogenosomes (Belanche, de la Fuente, & Newbold, 2015). This structure make protozoa moderately aerotolerant and are also responsible for the role ciliated protozoa play in scavenging of oxygen to maintain rumen anaerobiosis (Nagaraja, 2016).

CARBOHYDRATE METABOLISM

Metabolism of carbohydrates in protozoa can be classified into two parts, digestion of soluble sugars and storage polysaccharides and digestion of structural polysaccharides. The metabolic pathway depends on the ability of each species to ingest cellulose or starch. The smaller protozoa such as Entodinia and Holotrichs are more specialised in taking in starch granules and degrading them at a slower rate than bacteria (Williams & Coleman, 1992). This helps to stabilise fermentation especially in diets rich in soluble carbohydrates (Mackie et al., 1978). The starch is degraded into VFAs or is stored as amylopectin (Coleman, 1992). The larger protozoa such as *Diplodinium* and *Epidinium* are specialised in taking in large fibrous material and breaking it down to VFAs (Fondevila & Dehority, 2001). As much as one-third of fibre digestion can be protozoal (Russell & Rychlik, 2001).

Protozoa store soluble carbohydrates as an amylopectin-like storage polysaccharide, which forms the skeletal plates. As protozoa are progressively starved, storage polysaccharides in other parts of the cell disappear first. As the protozoa are starved further, the amylopectin in the plates also disappears (Furness & Butler, 1983). These plates were originally thought only to be support structures but may also serve as a store of energy. This has benefits for both the protozoa and the host ruminant. To

protozoa, it maintains a constant energy source making them more resilient in the face of reduced nutrition. To animal, it stabilizes fermentation (Williams & Coleman, 1992).
Protozoa show a specificity in the way they store carbohydrates. In this regard, Holotrichs have a higher capacity to store sugars compared to Entodiniomorphs. However, Holotrichs have a comparatively lower capacity to store starch and insoluble plant particles. Consequently, Holotrichs will increase in population in animals given diets rich in soluble sugars and will decrease when digestible cellulolytic material is fed (Denton et al., 2015).
The presence of protozoa increases the digestion of fibre and organic matter. The cellulolytic activity depends on the species of protozoa. Large Ophyroscolecidae such as *Epidinium*, *Polyplastron* and *Eudiplodinium* have greater endoglucanase and xylanase activity while *Entodinium* spp. have only weak activity (Table 1.3). Similarly, *Dasytricha* has glucosidase and cellobiosidase activity but negligible fibrolytic activity (Newbold et al., 2015). Cellulolytic activity is dependent on the levels of relevant enzymes inside the protozoal cells (Dehority, 1990).

Table 1.3: Cellulolytic activity of some species of protozoa (Dehority, 1990, p. 335)

Species	Activity
Eudiplodinium maggii *Epidinium caudatum* *Ostracodinium dilobum*	High
Metadinium affine *Eudiplodinium bovis* *Ophryoscollex caudatus* *Polyplastron multivesiculatum*	Moderate
Diplodinium pentacanthum *Enoploplastron triloricatum*	Weak
Entodinium caudatum *Ostracodinium gracile*	Trace

NITROGEN METABOLISM

Nitrogen metabolism in the rumen affects both the efficiency of ruminant production and the environmental impact of excreta from ruminant livestock production. Inefficient nitrogen retention by rumen microorganisms is compensated in production terms by feeding excessive amounts of dietary protein to the animal to meet required output levels. This process leads directly to the excretion of nitrogen-rich wastes.

Engulfment and digestion of bacteria by protozoa is by far the most important cause of microbial protein turnover in the rumen. (Teferedegne, 2000).

Bacteria are the main source of proteins for rumen protozoa (Williams & Coleman, 1992). *In vitro* studies have shown the level of ingestion from hundreds to thousands of bacteria per hour. The level depends both on the volume of the protozoa and their activity. No selectivity has been demonstrated; it seems that bacteria with the highest density in the rumen are ingested at a higher rate (de la Fuente Oliver et al., 2011). Over 80% of bacterial breakdown in the rumen is carried out by protozoa. This leads to an increase in the amount of ammonia in the rumen since protozoa cannot utilise it for synthesis of amino acids. Thus, protozoa increase the amount of nitrogen recycling in the rumen and reduce the outflow of microbial proteins that flows to the abomasum (Eugène, Archimede, & Sauvant, 2004). For instance, Belanche et al. (2011) observed that the presence of protozoa decreased the bacterial-N flow through the abomasum by 33% when faunated and fauna-free lambs were compared. Because the level of bacteria breakdown is dependent on the numbers and activity of protozoa, it is apparent that removing ciliate protozoa from the rumen (defaunation) should prevent the recycling of nitrogen between bacteria and protozoa. The result would be to increase in the efficiency of nitrogen metabolism in the rumen and stimulate the flow of microbial protein from the rumen (Koenig et al., 2000).

Protozoa predation also affects the bacterial diversity in the rumen. Using protozoa-free animals that were progressively inoculated with rumen protozoa, Belanche et al. (2015) showed that the highest bacteria diversity is seen in animals when fewer protozoa are present. On the other hand, archaea community was not affected by absence/presence of protozoa.

LIPID METABOLISM

Rumen ciliate protozoa do not carry out biohydrogenation and the process is only slightly decreased following defaunation (Jenkins et al., 2008). In addition, protozoa ingest plant organelles and in particular chloroplasts (Huws et al., 2009). Protozoa therefore make a significant contribution to the flow of unsaturated fatty acids from the rumen (30–50 % of the fatty acids entering the duodenum of protozoal origin) (Yáñez-Ruiz et al., 2006). Therefore, while defaunation has been shown to improve

growth performance in ruminants, it might not be desirable in terms of producing a diet for optimal human health particularly in regards to the fatty acid composition of animal products (Yánez-Ruiz, Williams, & Newbold, 2007).

METHANE METABOLISM

The fermentation products of protozoa are VFAs and hydrogen (Newbold, Lassalas, & Jouany, 1995). As hydrogen donors, protozoa are closely associated with the archaea, which are the main producers of methane in the rumen. The association is either surface attachment on the protozoa or *via* endosymbiosis; between 9% and 25% of the rumen methanogens are thought to be associated with protozoa (Belanche et al., 2015). The increase in the rumen protozoa generally leads to an increase in methane production by the animal (Guyader et al., 2014) (Figure 1.5).

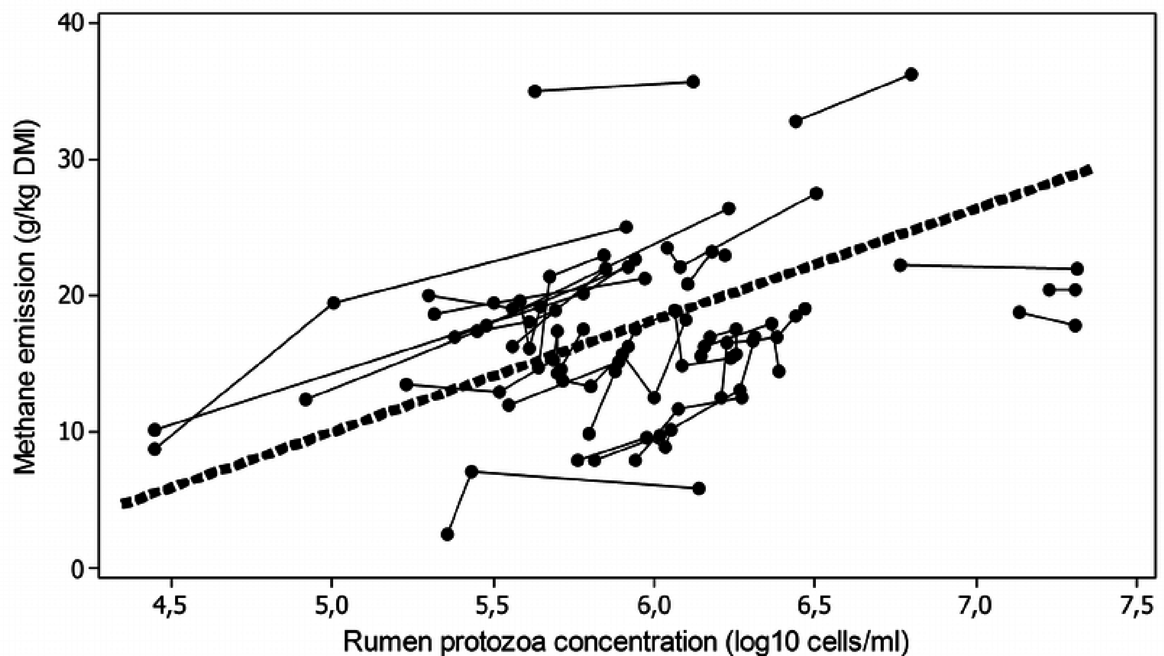


Figure 1.5: Relationship between methane emission and rumen protozoa concentration. Meta-analysis of raw data from 59 publications reporting data from 76 *in vivo* experiments. Solid lines represent within-experiment relationship. The black dashed line represents the average within-experiment relationship. From Guyader et al. (2014).

The specific contribution of the protozoal species is not very clear and has been studies using progressive inoculation of fauna-free animal. Using this approach Belanche et al. (2015) demonstrated a 60% increase in methane production in fauna-free animals inoculated with protozoa. The results suggested that Holotrich protozoa

have a greater impact on rumen methanogenesis than Entodiniomorphids. However, the relationship between the presence of protozoa in the rumen and methane production is not a simple causation since progressive defaunation does not always result in a reduction in methane production (Morgavi et al., 2010).

OTHER EFFECTS OF PROTOZOA

Protozoa can increase the pathogenesis of some bacteria such as *Salmonella*. The protozoa will engulf these bacteria and may remain undigested and can pass to the abomasum and releases in the duodenum to express hypervirulence (Rasmussen et al., 2005). On the other hand, protozoa are more active in detoxifying some compounds such as mycotoxins (Yiannikouris & Jouany, 2002). Rumen protozoa can also reduce the toxicity of nitrates and nitrites using lactic acid as a hydrogen donor (Nolan et al., 2016). Rumen ciliate protozoa may reduce the risk of chronic copper toxicity in ruminants. The presence of protozoa increases rumen production of sulphide (through increased breakdown of soluble proteins) which complexed part of the copper, making it unavailable for absorption and utilization (Ivan, Veira, & Kelleher, 1986). Rumen protozoa can also moderate of lactic acidosis especially in ruminants fed on high sugar diets (Nagaraja, Towne, & Beharka, 1992).

MANIPULATING RUMEN PROTOZOA NUMBERS

Considering the advantages and disadvantages of having protozoa in the rumen, there's potential to improve ruminant productivity by the manipulation of the protozoal presence, size or composition (Williams & Coleman, 1992). Dietary interventions can change the population; protozoa concentration increases with the level of concentrate, but it drops down with very high levels. This effect was observed by Metzler-Zebeli et al. (2013) where the number of Entodiniomorphid protozoa in goats was higher when the diet contained 30 and 60% barley grain diets as compared to 0% grain diet. Further, Hristov et al. (2001) reported that total protozoal numbers in ruminal fluid were 42% lower in cattle fed on a on 95% barley compared to a 62% barley diet. Additionally, frequent feeding increases protozoa concentration (Owens & Basalan, 2016). The presence and composition of rumen protozoa is also dependent on the age of the animal and the ruminant species.

Rumen protozoa are not essential to the animal to survive. The removal of protozoa from the rumen using a wide variety of chemicals and physical techniques (known as 'defaunation') and fauna-free animals have been used to study the role of ciliate protozoa in the rumen function (Williams & Coleman, 1992). Defaunated animals are different from fauna-free animals. The former involves the removal of protozoa from the rumen by use of chemicals or plant extracts. The latter involves separating new-born ruminants from their mothers to prevent colonisation with protozoa.

Defaunation studies suggest that fibre digestion in the rumen is a complicated task requiring the symbiotic collaboration of several fibrolytic microbes, including rumen protozoa, to carry out the initial stages of fibre colonisation and digestion. Therefore the absence of rumen protozoa seems to have a detrimental effect on this fibrolytic consortium and ultimately in fibre digestion (Newbold et al., 2015). The removal of protozoa results in the increase of concentration of bacteria and lactate in the rumen; there is a decrease in the concentration of ammonia and VFAs; and a drop in pH.

Degradation of organic matter, fibre and starch is also reduced by defaunation; and there's an increase of nitrogen to the duodenum (Koenig et al., 2000). The end effect is improved performance of the animal. However, with the lowered fibre degradation, ruminants fed on a high-fibre diet may not accrue these benefits. Additionally, there is a risk of acidosis in animals fed on a high-concentrate diet due to the drop in pH associated with defaunation (Williams & Coleman, 1992).

Co-incubation of protozoa with fungi has shown that the protozoa are able to both ingest and digest fungi (Miltko et al., 2016). Newbold and Hillman (1990) demonstrated a twofold increase in the fungal zoospore densities after the elimination of protozoa from the rumen of sheep. The observed effect could be related to the fungal predation by the protozoa (Miltko et al., 2016). More recent results have shown a contrasting picture such as a drop in the concentration of fibrolytic microbes such as anaerobic fungi following defaunation of sheep rumen (Newbold et al., 2015).

Defaunation has not been practicable in routine animal production systems due to the absence of a suitable defaunating agent. Most approaches to defaunation are experimental (Teferedegne, 2000); and rarely result in the total removal of protozoa from the rumen with their effectiveness largely dependent on diet composition. Treatments normally used to partially or completely defaunate the rumen include:

chemicals that are toxic to protozoa (copper sulphate, dioctyl sodium sulfosuccinate, alcohol ethoxy-late or alkanates, calcium peroxide), ionophores, lipids, and saponins (Newbold et al., 2015). Unfortunately, none of the methods has been without a negative effect either on the rest of the rumen microbes or on the host (Williams & Coleman, 1992, p. 115). Defaunation can also be achieved by emptying the rumen, carefully washing the rumen mucosa and treating the digesta by freezing before re-introducing it into the rumen, but obviously this is not a practical on-farm approach. A successful defaunation treatment for use in feeding practice would be one that could be applied continuously throughout the desired period and be safe to the rest of rumen microbes and host (Teferedegne, 2000).

1.3.6 RUMEN ARCHAEA

The importance of rumen archaea as a subject of study has increased due to their production of methane and its greenhouse effect. One dairy cow can produce 500-600 litres of methane per day (about 30L/kg dry matter consumed) (Grainger et al., 2007). About 15% of global methane production is from ruminant fermentation (Steinfeld et al., 2006). While methane has a much shorter lifetime in the atmosphere compared to CO_2 (~10 years compared to 100s of years), it is a more potent greenhouse effect than CO_2 (Jain et al., 2000). Therefore, increased emissions will produce more rapid impact on the global climate. On the other hand, any reductions in methane emissions will see a faster decrease in atmospheric concentrations compared to CO_2. Apart from the effects on the global climate, methane production accounts for 2-12% loss of gross energy from a ruminant's diet (Ramin & Huhtanen, 2013).

Methanogenic archaea occupy an ecological niche created by the production of hydrogen by fermentative bacteria; they consume this gas ensuring that its accumulation does not inhibit fermentation. There is a positive correlation between number of cellulolytic bacteria and that of methanogenic archaea (Morvan et al., 1996).

Archaea are strict obligate anaerobes; they utilise hydrogen to reduce carbon dioxide to methane. They are found in bogs, digesters, cockroach/termite gut and the gastrointestinal tracts of most vertebrates (Chaban, Ng, & Jarrell, 2006). In the rumen, they are free-living, or attached either on the surface of protozoa or as endosymbionts; between 9 and 25% of the rumen methanogens are thought to be associated with protozoa (Belanche et al., 2015). They are present at 10^8 per mL of rumen liquor. Over 150 species representing 29 genera have been reported. The majority (92.3%) of rumen archaea detected in total rumen contents can be placed in three genus-level groups. These are *Methanobrevibacter* (61.6%), *Methanomicrobium* (14.9%), and a large group of uncultured rumen archaea labelled as rumen cluster C, or RCC (15.8%) (Janssen & Kirs, 2008). The RCC clade of *Thermoplasmata* is a novel group of methylotrophic methanogens in the rumen (Poulsen et al., 2013). The methyl-coenzyme M reductase (MCR) gene pathway is common to all methanogenic archaea and is commonly used to characterise and quantify them in the rumen (Leahy et al., 2010).

Two mechanisms that determine the level of methanogenesis by rumen archaea have been proposed. On one hand, the abundance of the archaea rumen content has been shown to be correlated to methane emissions in cattle (Wallace et al., 2014). On the other hand, the patterns of VFAs (and thus hydrogen available for methanogenesis) produced can affect methane production because propionate formation consumes reducing equivalents, whereas acetate and butyrate formation generate H_2 for methanogenesis (Knapp et al., 2014). Defaunation and dietary manipulation have shown a weak relationship between the methanogen numbers and methane production (McAllister & Newbold, 2008; Wallace et al., 2014). The species of protozoa present in the rumen also affects the methanogen population; however, there's no correlation between the type of methanogen and the amount of methane produced (Belanche et al., 2015).

Protozoa associated methanogens are associated with approximately a quarter of the methane produced in the rumen and may offer a target for interventions for methane mitigation strategies (Belanche, de la Fuente, & Newbold, 2014). Defaunation has the possibility of reducing methane production by 20 – 26% (Morgavi et al., 2010). Apart from manipulating rumen protozoa, progress made in sequencing archaeal genomes is providing new targets for vaccines to target common archaeal proteins and design chemical inhibitors of methanogenesis (Leahy et al., 2013).

These contradictions in results highlight the substantial gaps which remain in our knowledge of the intricacies of hydrogen flow within the ruminal ecosystem (McAllister & Newbold, 2008).

1.4 THE CHALLENGE OF FEEDING RUMINANTS IN THE TROPICS

Rangelands in Africa cover 60% of the continent's land area (Mganga et al., 2015). In these areas, livestock are the main source of livelihood where over 1.9 and 0.6 tropical livestock units per square kilometre are produced in the nomadic pastoral and sedentary agropastoral areas respectively (Nyangito, Musimba, & Nyariki, 2008). Livestock also provide the main source of food among pastoral communities. The animals (cattle, camels, goats) provide milk and are also sold when cash is required to buy grains, pay school fees or meet other domestic requirements (Nyariki & Abeele, 2004). Rangelands in Kenya constitute 88% of the land surface area; people living in this area accounting for about 30% of the national human population, are mostly pastoralists who depend directly on the natural resource base for their livelihoods (Opiyo et al., 2011).

Globally, the livestock sector is economically valuable. Livestock contributes 1.4% of global gross domestic product providing employment for 20% of the global population in both developed and developing countries (Steinfeld et al., 2006). Livestock products provide an important component to diet especially in the developing world, providing key vitamins and nutrients. Neumann et al. (2003) reported that the consumption of meat was linked to both physical and mental development in Kenyan schoolchildren. Livestock, particularly the ruminant species, are a major component of Kenya's economy and distributed across all the production systems. According to the most recent (2009) census, the total population of ruminants was estimated at about 67 million, of which 3.4 million were dairy cattle, 14.1 million zebu cattle, 27.7 million goats, 17.1 million sheep, 2.9 million camels and 1.9 million donkeys (KNBS, 2010, p.438) . The livestock sub-sector contributes about 40% of the agricultural gross domestic product (GDP) and 10% of Kenya’s total GDP (Njarui et al., 2016). About 50% of the total labour force in the agricultural sector is said to be employed in livestock production. Over 70% of all the livestock in the country are found in the arid and semi-arid lands (ASAL) and the subsector employ 90% of the 7 million people living there and contribute 95% of their income. Among the pastoral communities ownership of livestock is recognized as indicator of wealth (Njarui et al., 2016).

Parts of Kenya that are not rangelands are dominated by mixed crop-livestock farming (Njarui et al., 2016). The system spans three climatic regions from humid and sub-humid to semi-arid. The humid and sub-humid regions are mainly in the central highlands in areas around Mt. Kenya and the Aberdare mountain range, Rift Valley, western highlands and a narrow strip along the coastal lowlands. These are high potential agricultural areas and are very densely populated. The land holding sizes are very small with an average of 0.9 - 2.0ha (Gitau et al., 1994). Natural grazing is no longer available so cattle have to be fed on crop residues, cultivated fodder and some concentrates (Orodho, 2006). Napier grass is the main fodder grown by over 70% of smallholder farmers in the region and normally provides over 40% of feed (Table 1.4). The semi-arid region is found in eastern Kenya and parts of central Rift Valley. The smallholder farmers account for over 80% of the farming community in these regions. Livestock serve a wide function; from social to subsistence, generates employment and income for thousands of smallholder farm households (Muriuki & Thorpe, 2001). They are source of food (milk, meat and egg) and contribute to health and nutritional security.

Livestock farmers in Kenya and in the tropics around the world are faced with problems of low livestock productivity. The low livestock productivity can be attributed to several factors. These include inadequate understanding of the ecology of tropical environments, particularly the temporal and spatial variability of rangeland production, range utilization and trophic interaction patterns, and the role of mobility in sustaining livestock production in these environments (Mganga et al., 2015).

However, inadequate supply of feeds, both in quantity and quality, is the main factor leading to low livestock productivity (Nyangito et al., 2008). This is primarily attributed to the seasonal fluctuation in forage availability and quality due to the modal rainfall patterns (Adjorlolo et al., 2016). Seasonal rainfall fluctuations notoriously and frequently lead to drought conditions in East Africa thereby affecting large numbers of households that predominantly depend on sufficient rainfall for sustaining their livelihoods through subsistence agriculture (Vrieling et al., 2016). Dry years can bring about a high mortality of their livestock due to reduced forage and water availability and outbreaks of epidemic diseases, especially if adverse conditions persist during multiple seasons (Megersa et al., 2014). During the dry season, forage yields and

quality reportedly decline drastically. Decline in forage yields has been reported in both natural and cultivated pastures during the dry season (Adjorlolo et al., 2016). In regards to quality, studies have indicated sharp changes in forage quality during the dry season. Warly et al. (2004) reported decreases in crude protein and increases in neutral detergent fibre of some forage legumes as the season changed from wet to dry. Fernandez et al. (2005) reported large seasonal changes in nitrogen and phosphorus concentrations in herbage and millet residues. The peak values of N and P in the standing herbage corresponded with the vegetative stage (in the wet season) after which the nutritive quality declined rapidly as the season ended. After the rapid fall, the feed quality, especially of grasses, remained constant and excessively low for most of the dry season (Fernández-Rivera et al., 2005).

Tropical grasses are also known to have a lower digestibility compared to their temperate counterparts (Minson & McLeod, 1970). This difference has been associated with the high temperatures of their growth environment. Tropical grasses grown under controlled conditions showed a negative correlation between increasing temperature and digestibility (Wilson, Taylor, & Dolby, 1976). This lower digestibility has been attributed to the different responses between tropical and temperate grasses to increased temperature. Tropical grasses show a lower level of cellulose and an increase in hemicellulose and lignin with increased temperature; temperate grasses do not show the same effect. Lignin appeared to be more closely associated with hemicellulose than with cellulose. This is coupled with a lowered digestibility (Ford, Morrison, & Wilson, 1979).

Table 1.4: Estimated annual production of feeds used in zero-grazing systems in Kenya. From Paterson et al. (1998)

Category	Feed	Annual production
Fodder DM (t/ha)	Napier grass	8.5 – 27.4
	Green maize	20.0 – 26.0
	Cassava tops	10.0 – 30.0
Crop residues DM ('000t)	Maize stover	7,500
	Sorghum/millet stover	676
	Wheat straw	187
	Rice straw	39
Agro-industrial by-products DM ('000t)	Cotton seed cake	5.4
	Sunflower seed cake	8.1
	Copra meal	5.1
	Coffee pulp	33
	Sisal pulp	41

Without appropriate supplementation, ruminants on range tend to lose weight during the dry season and in some cases reproductive wastage occurs. Fernández-Rivera et al. (2005) reported that the low availability and imbalances of nutrients during the dry season in the Sahel lead to poor animal performance. Depending on the animal physiological status, nutrient deficiencies and imbalances resulted in growth retardation, reproductive wastage, low milk yield, increased susceptibility to disease, lower ability to perform work and lower amounts of manure (Fernández-Rivera et al., 2005). Loss of livestock during drought often results in conflict between neighbouring pastoral communities as some turn to illegal and violent livestock raids to replenish their herds (Ember et al., 2012; Witsenburg & Adano, 2009).

To prevent these outcomes, supplementation of ruminant diets, especially during the dry season is therefore necessary. Such supplements need to have high enough crude protein to elevate dietary crude protein intake to levels that can support moderate production during the dry season. Use of fodder tree and shrub leaves as dry season supplements has yielded promising results (Adjorlolo et al., 2016).

1.4.1 FODDER TREES

Use of tree leaves in animal feeding has been practiced since antiquity; African farmers have fed tree foliage to their livestock for centuries using wild browse or trees that grow naturally on their farms (Baumer, 1992; Le Houerou, 1980). Most fodder trees are multi-purpose, providing products such as firewood and services such as soil erosion control. In many instances fodder may not be the tree's primary use (Franzel et al., 2014). Most of the leguminous fodders come from the genera *Acacia*, *Albizia*, *Calliandra*, *Desmanthus*, *Desmadium*, *Gliricidia*, *Leucaena*, *Prosopis* and *Sesbania*. Few non-leguminous tree species are also used for feeding to livestock (Mandal, 1997).

Since crude protein is a major liming nutrient in grasses during the dry season , tree leaves which are known to retain high crude protein content well into the dry season, become an important source for grazing ruminants (Adjorlolo et al., 2016). Utilisation of tree and shrub leaves in ruminant feeding has been extensively studied (Speedy & Pugliese, 1992). Leguminous fodder tree leaves important because they normally contain more crude protein than other ligneous species. However, the fodder

potential of trees and browses is determined not only by its crude protein content but also by its digestibility, palatability and the associative effects of other feeds (Smith, 1992).

NUTRITIONAL CONTENT OF FODDER TREES

Low-quality diets (hay and crop residues) are characterized by low animal productivity, resulting from the shortage of one or more essential nutrients required for rumen microbial activity, typically nitrogen. Supplementation is often necessary to correct this deficiency. Most forage tree have a nitrogen content higher than 13g/kg (Table 1.5). Therefore, supplementation of roughages with adequate quantities of rumen-degradable browses may alleviate nitrogen deficiency (Osuji, Fernandez-Rivera, & Odenyo, 1995). In addition, legumes with a high nitrogen content are likely to contain less NDF, and the total organic matter may be more easily fermented (Teferedegne, 2000). However, the potential uses of fodder tree as supplements to ruminants is often limited by the presence of anti-nutritive factors (El Hassan et al., 2000).

Table 1.5: Chemical characteristics of fodder trees. From Osuji et al. (1995)

	Low		High	
Nitrogen (g/kg DM)	*Dichrostachys cinerea*	13.9	Leucaena	40.5
Fibre (NDF) (g/kg DM)	Sesbania	206	*D. cinerea*	498
NDF-N (g/kg DM)	Sesbania	2.4	Tagasaste	92
Soluble tannins (g/kg DM)	*Carissa edulis*	21.5	*A. siberiana*	327
Condensed tannins (absorbance units/g NDF at 500nm)	Sesbania	13	Tagasaste	56
Phosphorus (g/kg DM)	Vernonia	0.7	Sesbania	2.7
Calcium (g/kg DM)	Tagasaste	10.5	Leucaena	20
Sulphur (g/kg DM)	Tagasaste	10.5	Leucaena	2.3
Iron (mg/kg)	Sesbania	360	Tagasaste	520
Manganese (mg/kg)	Leucaena	66	Tagasaste	200
Copper (mg/kg)	Leucaena	13	Vernonia	20
Zinc (mg/kg)	Leucaena	19	Tagasaste	39

NDF: Neutral detergent fibre.
NDF-N: Nitrogen bound to NDF.

ANTI-NUTRITIVE FACTORS IN FODDER TREE LEAVES

Plants produce a large number of chemicals, arbitrarily categorised as primary or secondary metabolites. These metabolites are thought to have a defensive role that ensures survival of the plant protecting them against insect predation or by restricting grazing of herbivores (McSweeney, Odenyo, & Krause, 2002). Thus, elimination or reduction of these compounds by breeding strategies may not be compatible with

high production. Small quantities of harmful secondary compounds can be tolerated especially where the offending plant is not used as the sole diet (McSweeney & Mackie, 1997). However, large amount of the metabolites might affect the availability of nutrients, palatability and feed intake (El Hassan et al., 2000). Common anti-nutritional factors which have been implicated in limiting the utilization of shrub and tree forages include non-protein amino acids (mimosine and indospecine), glycosides (cyanogens and saponins) and polyphenolic compounds (tannins and lignins) (Kumar, 1992). Among the polyphenolic compounds, tannins have received most attention in ruminant nutrition.

Saponins are glycosides that are generally considered as anti-nutritional factors. Recently, there has been increased interest in plant saponin as a possible means of suppressing or eliminating protozoa in the rumen. Since dietary saponins are poorly absorbed, their biological effects occur in the digestive tract (Das et al., 2012). The anti-nutritional effects differ depending on the digestive process of the ingesting animal. In ruminants, saponins are selectively toxic to rumen protozoa. The selective toxicity may be explained by the presence of cholesterol in eukaryotic membranes but not in prokaryotic cells (Klita et al., 1996). Saponins can form irreversible complexes with cholesterol in the cell membrane causing cell rupture and lysis (Wina, Muetzel, & Becker, 2005).

In vitro and *in vivo* studies have demonstrated antiprotozoal effect of fodder trees. Newbold et al. (1997) reported that foliage from *Sesbania sesban*, a multipurpose leguminous tree from sub-Saharan Africa, inhibited protozoal activity *in vitro* and transiently depressed the number of protozoa in the rumen of sheep. However, after day 9 of feeding, the protozoa counts in the rumen increased and reached the same level as the control. Ivan et al. (2004) also observed a rapid increase in the protozoal population in the rumen of sheep after day 14 of daily feeding with *Enterolobium cyclocarpum*. To avoid rapid microbial adaptation to saponin, Newbold et al. (1997) suggested that saponins should be fed intermittently. More recently, the *in vitro* antiprotozoal effect of saponins was enhanced by chemical modifications in their structure to produce sapogen-like analogs that were more resistant to degradation in the rumen allowing the antiprotozoal effect to persist over time (Ramos-Morales et al., 2017).

IMPROVING FIBRE UTILISATION AND PROTEIN SUPPLY IN ANIMALS FED POOR QUALITY ROUGHAGES

Many tropical native pastures and crop residues are poor quality roughages are bulky, high in fibre, poorly degraded in the rumen, low in nitrogen and minerals resulting in very low intakes (Osuji et al., 1995). This problem can be alleviated by supplementation with forage trees. The principal objective of supplementation is to increase the supply of nutrients, mainly energy and protein, such as to create favourable conditions in the rumen, which result in better fermentation and microbial protein supply. Supplementation with forage can do this in several ways.

Fodder trees can increase the amount of energy supplied by the basal feed by alleviating a deficiency hampering microbial fermentation of the basal feed, particularly nitrogen and sulphur deficiencies. The protein content of forage tree legume leaves (150 - 300g/kg) is usually high compared with that of hay and crop residues (30 - 100g/kg) (Teferedegne, 2000). *In vivo* trials have demonstrated that supplementation with nitrogen-rich legumes improve ruminant production. After carrying out on-farm feeding trials in Embu County, Kenya, Paterson et al. (1998) reported that feeding two kilograms of dried *Calliandra* provided an effective protein supplement to the basal feed of Napier grass and crop residues in dairy cows. Abule et al. (1995) reported that supplementation of Teff straw (*Eragrostis tef*) with graded levels of cowpea (*Vigna unguiculata*) or Lablab (*Lablab purpureus*) significantly increased microbial nitrogen supply in calves.

Supplementing with fodder trees can improve the rumen environment (*e.g.* pH, rumen NH_3 or rumen-degradable protein) to ensure increased fermentation of the basal roughage diet. Bonsi, Osuji, and Tuah (1995) reported that *Sesbania* and *Leucaena* supplementation promoted high levels of rumen ammonia, volatile fatty acids (propionate, butyrate, valerate) and minerals which probably enhanced microbial growth and proliferation. In addition, rumen pH recorded was at all times well above the cellulolytic threshold value of 6.2. This is a good attribute of fodder trees, compared with soluble carbohydrate-rich diets such as cereal grains and sugar-containing concentrates which tend to lower rumen pH, resulting in reduced cellulolytic activity. An improved rumen environment will increased numbers of

cellulolytic bacteria and in turn increase their invasion of and adhesion onto the fibrous feed (Osuji et al., 1995).

Inclusion of fodder trees as a supplement to a crop-residue base diet can improve the rate and extent of particle size reduction and thus increasing the passage rates of both liquid and particulate matter, leading to increased feed intake. Ethiopian Menz sheep fed with graded levels of *Sesbania sesban* and *Leucaena leucocephala* as supplements to an *ad libitum* diet of Teff straw showed increased DM intake and fractional rate of liquid outflow (Bonsi et al., 1994). Fodder trees are also a source of energy to ruminants ; during certain times of the year some fodder trees can form the sole feed (Osuji et al., 1995).

1.4.2 CROP RESIDUES AS ANIMAL FEED

In addition to the naturally growing and planted pastures, tropical farmers also produce crop residues which may be used to boost livestock feeding during the dry seasons and drought periods (Ndathi et al., 2011). Crop residues have been described as “Agriculture’s largest harvest” since they represent more than half of the world’s agricultural phytomass (Smil, 1999). This phytomass is composed of cereal and legume straws (tops & stalks), leaves and shoots of tuber, oil, sugar, and vegetable crops; and in prunings and litter of fruit and nut trees. Generally, countries do not maintain a yearly record of the crop residues produced by their respective agricultural sectors. However, there has been a renewed interest in this phytomass as a source of biofuels and for the production of biomass energy (Glassner, Hettenhaus, & Schechinger, 1999). It is estimated that the eight leading crops in the United States (corn, wheat, soybean, cotton, barley, oats and others) produce more than 500 million tons of residue each year (Andrews, 2006). Of these, corn is receiving the most attention due to its concentrated production area; because it produces 1.7 times more residue than other leading cereals, (Wilhelm et al., 2004) and due to sufficient quantity to support commercial scale production (DiPardo, 2000). Many other regions and countries do not keep any data on crop residue production and estimates rely on harvest index (HI) which is the ration of edible crop yield to the crop’s total aboveground phytomass (Figure 1.6). Harvest indices are of great interest to plant breeders since the great

improvements in crop yields witnessed in the 20th century can be largely attributed to the increase of harvest indices (Smil, 1999).

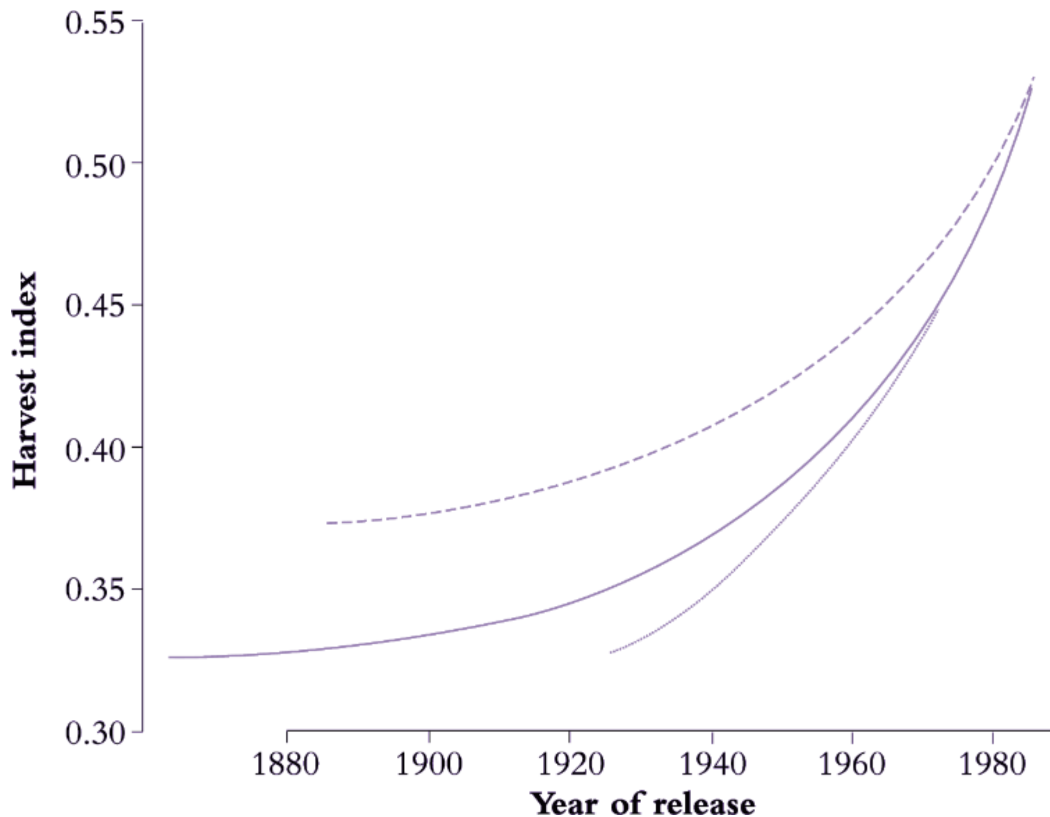


Figure 1.6: Effects of breeding and selection on harvest index (HI) for crop species including barley (dashed line), wheat (solid line) and rice (dotted line). Based on Evans (1993).

The introduction of genetically engineered dwarf varieties among cereals resulted in a higher increase of the HI limited only by the minimum amount of tissue required for photosynthetic activity and for structural support (Burgess, Gibbs, & Murchie, 2018; Long, Marshall-Colon, & Zhu, 2015). Some root crops can however achieve a HI as high as 0.80 (Hay, 1995). Estimating crop residue *via* HI, however, has its limitations as it does not take into account the variations seen in the same cultivar grown in different environments or production systems as demonstrated in sorghum (Roberts et al., 1993). Despite this increase in HI, estimates of global crop residue production were estimated at 3.75Gt constituting 1.4 times the size of the aggregate crop harvest in 1997 (Smil, 1999). These are field residues and not processing coproducts such as husks and bran (most of which are used as good-quality feed ingredients; 60% of these residues were produced in low-income countries and 45% specifically originated from the tropics (Smil, 1999).

Crop residues are composed primarily of fibre (cellulose, hemicellulose and lignin). However, they also contain other plant nutrients such as nitrogen and phosphorus (Noack et al., 2012). As such, they can ideally be used as a substitute for wood (in regard to cellulose), for petroleum (in regard to energy) and for synthetic fertilizers (for phosphorus and nitrogen). Several disadvantages limit the substitution of wood for crop residues, in particular, the low density of cereal straws (typically just 50–100kg/m^3, compared to 600–800kg/m^3 for wood). Additionally, crop residues have a scattered and seasonal availability resulting in high field collection and transportation costs (Smil, 1999). Regarding energy generation, crop residues contain approximately 18MJ/kg and could ideally substitute 1.5Gt of crude oil. Their use in this way is still also limited by low density and high cost of field collection thus making them uncompetitive. However, crop residues are currently being used as sources of energy, along with fuelwood and charcoal in many rural populations in Africa where access to modern energy services is limited (Cooper & Laing, 2007). Crop residues, particularly pulse straws retain a significant amount of nitrogen (about one third of the total amount taken by the plant); and phosphorus (approximately 30% of the total amount taken up by the plant) (Smil, 1999). The incorporation of crop residues into the soil recycles these nutrients and improves the soil structure by increasing the organic matter content thus reducing the need for chemical fertilizers (Nebiyu et al., 2014).

Despite the high level of lignification, Crop residues continue to form an important part of ruminant feedstuff in many developing countries (Table 1.4). There are other factors that limit digestibility in crop residues; for instance, in rice straw, silification is the most important limiting factors followed by lignification (Van Soest, 2006) (Table 1.6). Other factors which limit the degradation of roughage in the rumen are crystallinity of cellulose, degree of polymerization (DP), moisture content and available surface area (Hendriks & Zeeman, 2009).

Table 1.6: Composition and digestibility of various crop residues. Adopted from Van Soest (2006)

	SiO_2 (g/kg)	Lignin (g/kg)	NDF (g/kg)	ADF (g/kg)	Digestibility
Rice bran	50	30	250	140	0.70
Rice straw	130	52	820	531	0.45
Rice hulls	230	160	810	720	0.08
Barley straw	20	110	800	590	0.49
Oat straw	20–50	140	700	470	0.48
Wheat straw	10–50	85–140	828	540	0.44

NDF: Neutral Detergent Fibre ADF: Acid Detergent Fibre

REDUCING THE EFFECT OF LIGNIFICATION IN CROP RESIDUES

There are two main approaches for reducing lignin content in ruminant diets, pre-treatment of forages to remove lignin prior to feeding them to animals and breeding of new low-lignin genotypes of forage crops. High-lignin fodder can be physically, chemically or biologically pre-treated.

Physical pre-treatment

This involves mechanical and thermal treatment. The main form of mechanical treatment is milling which reduces the particle size and crystallinity of lignocellulose biomass (Hendriks & Zeeman, 2009). The result is an increase in the available surface area for hydrolysis and reduction in the degree of polymerisation (Palmowski & Müller, 2000). Milling has been shown to significantly increase the yield and the rate of hydrolysis of lignocellulosic biomass (Delgenes, Penaud, & Moletta, 2003). Milling as a method, however, suffers from the associated high energy costs thus limiting it as the ideal method for ventures with low input costs (Ramos, 2003).

Thermal treatment aims at solubilising hemicellulose and lignin therefore making the cellulose more accessible to bacterial enzymes. There are different methods of thermal treatment including steam, steam-explosion and liquid hot water treatments (Hendriks & Zeeman, 2009). Thermal treatment however carry a risk of condensation and precipitation of soluble lignin components, making the biomass less digestible; this is particularly in case where steam is used (Hendriks & Zeeman, 2009).

Chemical pre-treatment

Hemicellulose can also be solubilised by treating biomass with acid at ambient temperature. Stronger acids have a higher effect compared to weak ones. Alkali treatment on the other hand causes a solvation and saponification reaction resulting in swelling of the plant fibres. This makes them more accessible to bacterial digestion (Hendriks & Zeeman, 2009). Treatment with oxidising agents can also improve digestibility of lignified fodder. Peracetic acid selectively oxidises lignin with minimal loss of carbohydrates. A 21% solution was shown to increase hydrolysis of cellulose from 6.8% in untreated material to 98% in treated (Teixeira, Linden, & Schroeder, 1999).

Ammonia fibre expansion (AFEX) is an important pre-treatment technology that utilizes both physical (high temperature and pressure) and chemical (ammonia) processes (Bals, Rogers, et al., 2010). This approach increases the surface accessibility for hydrolysis and promotes cellulose decrystallization and partial hemicellulose depolymerisation thus reducing the lignin recalcitrance. AFEX offers several unique advantages over other pre-treatments, which include near complete recovery of the pre-treatment chemical (ammonia), nutrient addition for microbial growth through the remaining ammonia on pre-treated biomass, and not requiring a washing step during the process which facilitates high solid loading hydrolysis (Bals, Murnen, et al., 2010). While AFEX has been mainly used to generate feedstock for biofuel generation, a potential has been identified for production ruminant feed from low digestibility material. Bals et al. (2010) reported that AFEX treatment improved digestibility of corn stover and late-harvest switchgrass by 52% and 128% respectively over untreated material; additionally, the crude protein content of all treated samples increased to more than 100g/kg dry matter (Table 1.7).

Table 1.7: Neutral detergent fibre (NDF) digestibility after 48 hours *in vitro* fermentation of selected forages. Adopted from Bals et al. (2010)

	Digested (g NDF/kg biomass)		
	Untreated [b]	Treated	% Increase [c]
Corn Silage	208	275	32
Corn Stover	370	564	52
Alamo Switchgrass [a]	174	397	128
Wheat Straw	315	512	63
Miscanthus	51	132	159

a late harvest
b untreated samples prior to testing
c percent increase in treated sample over untreated sample

Biological pre-treatment

In ecosystems other than the rumen, lignocellulosic biomass is commonly broken down by fungi (Bomble et al., 2017). There has been interest in using these fungi to predigest recalcitrant fibre to improve digestibility before feeding it to ruminants. Such an approach would require an organism which combines high lignolytic activity with low degradation of cellulose and hemicellulose (Zadražil 2000). Brown-rot fungal species such as *Agrocybe aegerita* and *Flammulina velutipes* would not fit this bill since they attack cellulose and hemicellulose and leave the lignin aromatic rings intact (Mahesh & Mohini, 2013). This results in lowered digestibility. Soft-rot fungi such as *Chaetomium cellulolyticum* and *Kretzschmaria deusta* digests moist wood, causing it to decay into a watery soft material, which is not ideal for animal feed (Daniel & Nilsson, 1997). White-rot have been identified to have potential to generate high-quality animal feed from lignocellulosic biomass since the degrade lignin without destroying the important cellulose and hemicellulose (Zadražil 2000).

Examples of white rot fungi include *Abortiporus biennis*, *Agaricus bisporus* (one of the most commonly and widely consumed mushrooms in the world), *Dichomitus squalens* and *Pleurotus eryngii* (King Trumpet Mushroom, an edible mushroom native to Mediterranean regions of Europe, the Middle East, and North Africa, but also grown in many parts of Asia). Others include *Pleurotus pulmonarius* (Italian Oyster mushroom), *Pleurotus ostreatus* (Oyster mushroom), *Pleurotus flabellatus*, *Pleurotus floridanus*, *Phanerochaete chrysosporium*, *Ganoderma* sp. rckk02, *Crinipellis* sp. and *Coriolus versicolor* (Mahesh & Mohini, 2013).

White rot fungi have been investigated for use in solid-state fermentation to produce animal feed. Solid-state fermentation (SSF) is defined as:

> "A process, in which solid substrates are decomposed by known pure or mixed cultures of microorganisms (mainly fungi, which can grow on and through the substrate) under controlled conditions, with the aim of producing a high quality standardized product (different from composting)" (Zadražil 2000).

The substrate is characterized by a relatively low water content as opposed to liquid submerged fermentation, which is more suited for unicellular organisms such as bacteria and yeasts. Since much of the water is chemically or physically bound to the substrate, physical properties, *e.g*. porosity and density, are uniform (Roussos et al., 2013). The substrate is not mixed or moved during the process thus enabling the optimal development of filamentous fungi and allows the mycelium to spread on the surface of solid compounds among which air can flow. SSF is currently applied on an industrial scale for biomolecule manufacturing used in the food, pharmaceutical, cosmetic, fuel and textile industries; these biomolecules are mainly enzymes and secondary metabolites (Robinson, Singh, & Nigam, 2001). While the use of SSF for the production of biomolecules is a well-established industry, the same cannot be said regarding its use for the production of animal feed. This can partly be attributed to the fact that SSF does not necessarily increase the digestibility of the substrate. It has been noted that the *in vitro* digestibility of fungal substrates decreases at the beginning of colonisation by white rot fungi and increases afterwards (Zadražil & Brunnert, 1982). Additionally, the increase in digestibility depends on the fungal species, the cultivation time and temperature and on the preparation, water content, bulk density and composition of the substrate. The pattern of lignocellulose degradation by white rot fungi is influenced by the concentration of carbon dioxide, oxygen and fungal metabolites in the gaseous phase (Zadražil 2000).

SPENT MUSHROOM SUBSTRATE

The main constraints in optimizing biological upgrading of lignocellulosic biomass into ruminant feedstuff are the identification of appropriate fungal species, the understanding of factors controlling selective delignification by microorganisms and the development of an appropriate technology to achieve a cheap large-scale process. Lack of knowledge regarding the factors that regulate fungal development and lignin breakdown in solid fermentation has hampered the widespread adoption of this approach. If this is to happen, there need to exists cheap and simple low-cost technology.

Fortunately, similar processes have been applied for cultivation of human edible mushrooms grown on a lignocellulosic biomass substrate (Zadražil 2000). Mushroom production is the biggest solid state fermentation industry in the world (Grujić et al., 2015). After the mushrooms are harvested, the co-product left behind is commonly known as spent mushroom substrate (SMS) and is composed unutilised material and the mushroom mycelium (Phan & Sabaratnam, 2012).

Growth of the mushroom industry has been spurred by the increasing need for protein-rich food and the inefficient agricultural methods currently used to produce it from animals and plants (Phan & Sabaratnam, 2012). Edible mushrooms also offer an alternative source for vegetarian and gluten free diets, which are on the rise around the world (Sadler, 2004). In 2014, the global production of mushrooms (and truffles) was 10,378,163 tonnes with China producing 7,626,791 tonnes accounting for 73% of global production. In the same year, the production in the United Kingdom was 94,857 tonnes and the total production from Africa was 24,821 tonnes (FAOSTAT, 2014) (Figure 1.7).

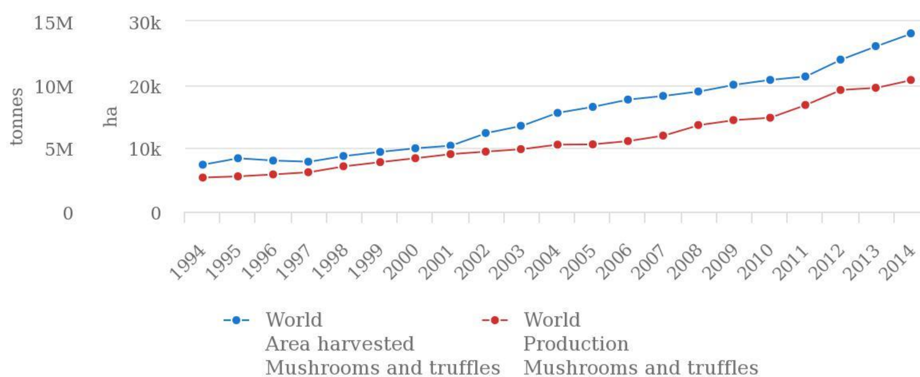


Figure 1.7: Production/Yield quantities of Mushrooms and truffles in World - 1994 – 2014. Source (FAOSTAT, 2014)

The production of 1kg of mushrooms results in the production of 5kg of SMS (Phan & Sabaratnam, 2012). As such, the industry produces a massive amount of organic waste with the accompanying challenges in storage and disposal (Grujić et al., 2015). The solution for this to increase the demand for SMS through exploration of new applications for utilisation. It would be more economical and favourable if SMS is to be recycled and re-used (Phan & Sabaratnam, 2012).

SMS is commonly composted and then marketed as marketed as "weathered" mushroom soil for application in agricultural fields to alter the organic matter and mineral content (Ribas et al., 2009). This however may not be the most economically rewarding use of the material. Recent advances in biotechnology has allowed for the recovery of enzymes including cellulase, xylanase, amylase and β-glucosidase (Grujić et al., 2015). Other uses for SMS are bioremediation, energy feedstock and animal feed.

SMS as animal feed

Substrates commonly used as a growth media to produce mushroom contain cellulose, lignin and little protein and thus declared unsuitable for animal feed (Phan & Sabaratnam, 2012). It is only after harvesting mushroom that the substrate could be more easily digested by ruminants owing to the enzymatic conversion processes during mushroom cultivation. Various studies have shown that white-rot fungi degrade lignin outside the rumen and improve the *in vivo* dry matter digestibility of lignocellulosic materials (Table 1.8). However, issues of high ash content and lowered voluntary intake have also been documented. The varying results indicate that the formulation of the diet of animal feed which includes SMS is not an easy task, as one must take into account factors like species of animal, mushroom strains, nutrition level of the SMS, cell wall component, digestibility and voluntary intake (Phan & Sabaratnam, 2012).

Table 1.8: Summary of feeding trials using spent mushroom substrate

Mushroom	Substrate	Trial	Findings	Reference
Pleurotus sp. *Agrocybe aegarita*, *Pleurotus eryngii* Pleurotus sp. and *Kuehneromyces mutabilis*	Sugarcane bagasse		The rate and specificity of degradation of a substrate dependent on: The fungal strain used for fermentation Substrate composition Different particle sizes of the same substrate.	Zadražil and Puniya (1995)
Lentinula edodes	Cotton-seed hulls 90%, wheat-bran 8%, sucrose 1%, gypsum 1%.	*In vitro*	The crude protein contents were increased from 24 to 32% and from 28 to 36% for *P. ostreatus* and *L. edodes* SMS respectively. The crude fibre contents of the composts were substantially decreased. After fermentation, the *in vitro* digestibility of crude protein was improved to 70%.	Zhang, Gong, and Li (1995)
Pleurotus ostreatus	Cotton-seed hulls 98%, gypsum 2%.			
Pleurotus florida	Wheat and paddy straw	Buffalo	There was an improvement in the N status of spent wheat and paddy straws. Paddy straw may not be as good as spent wheat straw for 100% replacement	Kakkar et al. (1990) Kakkar and Dhanda (1998)
Agaricus bisporus	Rice straw	Holstein beef cattle	SMS had 2 times the CP content and 22% lower NDF content on an OM basis compared to initial mushroom substrates. SMS had much higher predicted ruminal degradabilities and disappearances of DM and CP and a little lower predicted degradability and disappearance of NDF compared to raw rice straw. The general feed-nutritional value of spent mushroom substrates appeared to improve after cultivation of mushrooms	Y. Kim et al. (2011)
	Wheat straw	Mature castrated male sheep	SMS contained considerable amount of N and may be used as a ruminant feed. Utilisation in the diets of ruminants is limited because of high mineral content, which may reduce its acceptability and nutrient balances	Fazaeli and Masoodi (2006)
Pleurotus ostreatus	Wheat straw	Simmental heifers	Animals would not consume mixed ration with more than 17% DM from SMS.	Adamović et al. (1998)
	Sawdust	Post-weaning calves	Sawdust-based fermented SMS could be recycled after fermentation with three probiotic LAB strains as a feed supplement for post-weaning calves. Sawdust-based fermented SMS has the beneficial effects of an alternative to antibiotics for a growth enhancer in dairy calves.	M. K. Kim et al. (2011)
Pleurotus eryngii & *Pleurotus osteratus*	Rice straw	Hanwoo steers	SMS could be used as a forage source to replace 40% of rice straw without any negative effects on rumen fermentation and blood metabolites	Oh et al. (2010)

Pre-treatment with exogenous fibre-degrading enzymes

Supplementing dairy cow and feedlot cattle diets with fibre-degrading enzymes has significant potential to improve feed utilization and animal performance (Beauchemin et al., 2003). While many studies have explored the possibility of improving the nutritive value of forage for ruminants using exogenous fibrolytic enzymes (Beauchemin & Holtshausen, 2010), most of them have been conducted with mixed diets for high-producing ruminants or with medium- to high-quality forages. Consequently, there is little published information on how fibrolytic enzyme application can affect the digestion of low-quality forages (Dean et al., 2008; Elghandour et al., 2013; Krueger et al., 2008). Increasing the digestibility of low-quality forages using exogenous enzymes could lead to significant improvements in ruminant performance in many parts of the world, mainly in the tropics and subtropics. Tropical forages intrinsically have low nutritive value, but often constitute practically the whole diet of ruminants in tropical countries resulting in a low productivity of ruminant livestock in these areas (Díaz et al., 2015).

Several factors influence the effect of exogenous fibrolytic enzymes including type and dose of enzyme, type of diet fed to animals and enzyme application method (Beauchemin et al., 2003; Beauchemin & Holtshausen, 2010). The effectiveness of fibrolytic enzymes has also been shown to vary with feed (Colombatto et al., 2003; Wallace et al., 2001), enzyme application method (Wang et al., 2001; Yang, Beauchemin, & Rode, 2000)and the component of the diet to which the enzyme is added (Beauchemin et al., 2003).

A limited number of ruminant enzyme products are now commercially available, and this list of products is expected to grow. However, random addition of enzymes to diets without consideration for specific situations and substrate targets will only discourage or delay on-farm adoption of enzyme technology. As highlighted on Table 1.9, there is much variability of the response to treatment of fibrous feeds to enzyme treatment despite the much progress that has been made in advancing enzyme technology for ruminants. Considerable research is still therefore needed reduce this variability (Beauchemin et al., 2003). Additionally, further study is needed to investigate the mode of enzyme action in the different digestive sites, so that enzyme supplements can be adequately applied to the diets ruminants (Yang et al., 2000).

Table 1.9: Summary of studies on exogenous fibre-degrading enzymes

Enzyme	Substrate	Findings	Reference
Promote® Biovance Technologies (Xynalase, Cellulase, Endoglucanase)	Wheat straw	Alfalfa hay can be replaced with treated wheat straw in Naieni replacement ewe lamb diets	Jafari et al. (2005)
Promote®, Biocellulase X-20® (X-20), CA® (CA), and Biocellulase A-20®	NH_3 treated Coastal Bermuda grass hay (*Cynodon dactylon*) and Pensacola Bahia grass hay (*Paspalum notatum*).	Fibrolytic enzymes had negligible effects on the extent of fibre digestion and *in situ* DM degradation of the hays, but X-20 and A-20 treatment increased the initial and later phases of DM digestion. Ammoniation was more effective than any enzyme treatment at improving DM and fibre digestibility, and in situ degradability.	Dean et al. (2008)
Depol 740L, Biocatalyst, Pontypridd, containing high esterase (U/mL) activity	Pensacola Bahia grass, Coastal Bermuda grass, and Tifton 85 Bermuda grass	This enzyme enhanced *in vitro* digestion and ruminal *in situ* degradation of hays, but the pattern and extent of improvement was forage specific	Krueger et al. (2008)
ZADO® enzyme preparation mixture (7.1 units of endoglucanase, 2.3 units of xylanase, 61.5 units of α-amylase and 29.2 units of protease activity.)	*Saccharum officinarum* (Sugarcane), *Andropogon gayanus* (Gamba grass), *Pennisetum purpureum* (Napier grass) and *Sorghum vulgare* (straw).	The effectiveness of the enzyme preparation differed among the feeds being highest for *S. vulgare*, intermediate *A. gayanus* and *P. purpureum*, and lowest for *S. officinarum*. Increasing the enzyme dose, linearly increased ruminal gas production including fermentation rate and asymptotic gas production but decreased microbial CP production.	Elghandour et al. (2013)
22 commercial enzyme products	Alfalfa hay and Corn silage	Alfalfa - enzymes work by removing structural barriers that retard the microbial colonisation of digestible fractions, increasing the rate of degradation. Corn silage - enzymes seem to interact with ruminal enzymes to degrade the forage more rapidly.	Colombatto et al. (2003)
Xylanase from *Trichoderma longibrachiatum* (Biovance Technologies Inc., Omaha, NB, USA)	Steam-rolled barley grain and chopped alfalfa hay	Applying enzymes on feeds before feeding was more effective than dosing directly into the artificial rumen for increasing ruminal fibrolytic activity.	Wang et al. (2001)
Proprietary blend (Biovance Technologies Inc., Omaha, NE) with relatively high xylanase and low cellulase activities. Enzyme from *T. longibrachiatum*	Total mixed rations containing 24% corn silage, 14% alfalfa hay, and 62% concentrate	The method of enzyme application onto diets of cows is a crucial factor affecting exogenous enzyme action in dairy cows. Enzymes applied to the concentrate portion of the diet of cows in early lactation have the potential to increase milk production due to enhanced nutrient digestibility in the total tract. Applying enzymes to the TMR before feeding improved digestibility but had no effect on milk production. Exogenous fibrolytic enzymes added to high concentrate diets had no effect on digestibility in the total tract of lambs. Sheep may not be a suitable model to test effects of exogenous enzymes on the digestibility of feeds for dairy cattle because digestibilities are considerable higher for sheep than for cattle. Enzymes appear to enhance nutrient digestion by dairy cows because digestion is less than the potential digestion attainable in sheep or *in vitro*.	Yang et al. (2000)

1.5 JUSTIFICATION OF THE STUDY

The global demand for ruminant product is expected to increase in conjunction with population growth; by 2050 milk production is expected to rise from 580 to 1043 million tonnes and meat from cattle and sheep is expected to increase from 70 to 127 million tonnes (Kingston-Smith, Marshall, et al., 2013; Steinfeld et al., 2006). However, increasing output from livestock is hampered by limited resources such as availability of arable land, water and energy. Ruminants are capable of converting plant material that is otherwise unpalatable to humans to high value products such as milk and meat. Further, ruminant production can be undertaken in marginal lands that may not be useful for crop agriculture. However, rumen fermentation is inefficient resulting in loss of energy in form of methane and loss of nitrogen that would ideally be incorporated into useful products. Further, the deposition of methane and nitrogen compounds into the environment is significantly contributing to anthropogenic climate change and to the pollution of soil and water (Steinfeld et al., 2006). Due to limitations to the productivity and/or availability of land, the number of pastured ruminants cannot increase. Therefore efforts should be directed toward increasing the efficiency of rumen fermentation while avoiding an accompanying increase in nitrogen and methane emissions (Huws et al., 2018).

The rumen is a complex ecosystem which converts plant material to microbial protein *via* fermentation by a complex community of bacteria, anaerobic fungi, ciliate protozoa and methanogenic archaea. This complexity has been identified as the source of ruminal inefficiency (Kingston-Smith et al., 2010). The diversity of this ecosystem is only now becoming truly appreciated, due to the application of molecular techniques. Further, advances are being made in the understanding of the operation of the complex ecosystem that is the rumen, from where many feed use inefficiencies arise; and new insights into plant-microbe interactions during colonisation and plant degradation (Huws et al., 2014).

Molecular studies have shown that microbial colonisation of newly ingested plant material by rumen microbes is a prerequisite for ruminal degradation of plant biomass. Rumen microorganisms rapidly associate with and colonise ingested feed particles (Edwards et al., 2007). As the attachment of rumen microbiota to ingested

forage is central for utilization of plant nutrients (Kingston-Smith et al., 2010), understanding plant–microbe interactions is paramount in improving ruminant nutrient use efficiency (Huws et al., 2013). The dynamics of this process are poorly understood; earlier work demonstrated microbial colonisation in the rumen occurs *via* formation of biofilms that are similar to those seen in other ecosystems (Mayorga et al., 2007). Further work has shown that the colonisation in the rumen is a dynamic process (Huws et al., 2016; Huws et al., 2013); and that the patterns of colonisation are determined in part by the structure of the substrate surface (Huws et al., 2014). However, these have mainly investigated colonisation of temperate grasses such as perennial rye grass.

Little or no work has been done to investigate the colonisation pattern and the effect on the rumen microbes by tropical forages, crop residues and fodder trees. The increase in demand for livestock products projected to come mostly from developing countries due to an increase in affluence and population increases (Kingston-Smith et al., 2010). Many of these countries are located in the tropics where crop residues and fodder trees are routinely used for ruminant nutrition.

1.5.1 BROAD OBJECTIVE

To increase the understanding of the rumen microbial ecosystem to identify new targets for interventions to reduce the inefficiency of rumen fermentation for increased and sustainable ruminant production.

1.5.2 SPECIFIC OBJECTIVES

This study therefore proposes to carry out a series of experiments to determine:

1. The effect of fodder tree on rumen microbes.
2. The colonisation pattern of rumen microbes on crop residues.
3. The applicability of emerging molecular techniques to the study of rumen microbes.

1.5.3 RESEARCH QUESTIONS

1. Do selected fodder tree leaves have the nutritional composition to function as supplement for a crop residue base diet?
2. What is the composition and concentration of plant secondary metabolites found in selected fodder tree leaves?
3. What is the *in vitro* effect of selected fodder tree leaves on rumen bacteria?
4. Can the Oxford Nanopore MinION be a cheaper, faster and more accurate alternative to Ion Torrent sequencing for taxonomic investigation of the rumen microbial ecosystem?
5. What are the effects of chemical composition of rice straw on *in vitro* degradation?
6. What is the effect of mechanical treatment of rice straw on *in vitro* degradation?
7. Is the *in vitro* colonisation of rice straw by rumen bacteria uniform or is it dynamic over a 12-hour period?
8. What is the effect of chemical composition of rice straw on the *in vitro* colonisation by rumen bacteria?

CHAPTER 2: MATERIALS AND METHODS

2.1 INTRODUCTION

This chapter deals with the methods applied to accomplish the specific objectives of the study. It provides an introduction of the sample collection and various analytical methods used to determine their chemical compositions. Further it provides detailed account in vitro batch cultures used to estimate degradation and 16S metabarcoding to determine effect on rumen microbes. In addition, it provides details about the methods of data processing and statistical analyses adopted to meet the specific objectives.

2.2 WEST AFRICAN FODDER TREE LEAVES

2.2.1 PLANT MATERIAL

Leaf samples of *Acacia nilotica* (AN), *Azadirachta indica* (AI), *Guiera senegalensis* (GS), *Lannea acida* (LA), *Parkia biglobosa* (PB), *Piliostigma reticulatum*(PR) and *Ziziphus mauritiana*(ZM) used in the experimentation were collected in Katsina state, which is located in north-western part of Nigeria. The state is divided into three agroecological zones – Northern Guinea savannah, Sudan savannah and Sahel savannah described by Adenkule et al. (2005). Three sample collection sites were selected to represent each agroecological zone (Table 2.1); sampling was done between September and November 2016 coinciding with the end of rainy season. Samples from at least three plants were air-dried and pooled to form 21 samples representing seven species and three ecological zones. Sample collection and preparation was done by Muhammad Lawal (Federal College of Education, Katsina, Nigeria), as part of his MSc studies at Aberystwyth University.

Table 2.1: Locations of sample collection sites

No	Agro-ecological zone	Location
1	Sahel savannah	Daura (13.0315^{O}N, 8.3225^{O}E)
2	Sudan savannah	Katsina (12.5139^{O}N, 7.6114^{O}E)
3	Northern Guinea savannah	Bakori (11.5577^{O}N, 7.4246^{O}E)

2.2.2 PROXIMATE ANALYSES

The samples were ground to approximately 1mm^2 sized particles using a forage mill.

Dry matter (DM) was determined by drying in a ventilated oven with forced air circulation at 60°C for 48 hours (Purcell et al., 2011). The organic matter proportion (ash content) was determined according to the method used by Peyraud et al. (1997). The crucibles were labelled and oven-dried overnight at 100°C to remove any moisture that they might have absorbed at room temperature. The crucibles were weighed and approximately 0.5g of each of the milled samples added to the crucibles and placed in a muffle furnace overnight at a temperature of 500°C. The crucibles were removed from the furnace and cooled in a desiccator for 1 hour. The crucibles containing the ash were weighed and the weights of empty crucibles subtracted from the weights of crucibles plus samples in order to get the weight of the ash portions of the samples.

Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined sequentially using the Ankom Filter Bag method. NDF and ADF were determined according to the filter bag technique used by Vogel et al. (1999), using Ankom 220 Fibre Analyser (Ankom Technologies, New York, USA). Briefly, 0.5g of milled sample was weighed into labelled and weighed filter bags (F57, Ankom Technologies) in duplicate. The bags were heat-sealed, weighed and loaded onto the sample trays. The sample suspender was inserted into the fibre analyser to submerge the samples in 2L of NDF solution (Table 2.2).

Table 2.2: Preparation of NDF solution

Reagent	Amount
Sodium dodecyl sulphate	30g
Sodium borate	6.81g
Ethylene-diamine-tetra-acetic disodium salt	18.61g
Sodium phosphate	4.56g
Triethylene glycol	10mL
Distilled water	1000mL

The reaction vessel was sealed to maintain positive pressure and NDF extraction was done for 75mins at 100°C with agitation. After the extraction period, the solution was drained from the reaction vessel, and the reaction vessel was filled with 2L of 94°C tap water. The top was left open and the samples were agitated for 5 min. The hot water

rinse was repeated three times. After the final rinse, the bags were removed and gently squeezed to press out excess water. The bags were then placed in a 250mL beaker and covered with acetone. After 5min of soaking, the bags were again squeezed to remove the acetone, air dried in a hood, and then dried at 100°C for the final drying step. The dry bags were then weighed.

ADF analysis was done sequentially using the dried residue left in filter bags after NDF extraction and using the same Ankom 220 fibre analyser. The bags were submerged in 2L of acid detergent solution (20g of cetyl trimethylammonium bromide (CTAB) in 1L of standardized sulphuric acid). Extraction was done 60 minutes at 100^oC with agitation and positive pressure. Rinsing, drying and weighing was performed as previously described.

ADL was determined using 72% H_2SO_4 (Van Soest, 1963). ADL extraction was done sequentially using the dried residue left in the filter bags after ADF extraction. The bags were placed in a 3L beaker and completely covered with 72% H_2SO_4 (approximately 250mL). To keep the bags submerged, a 2L volumetric flask with water was used to weigh them down. Bags were agitated at the start and at 30min intervals by gently pushing and lifting the 2L flask up and down approximately 30 times. Extraction was done for 3hrs after which the bags were rinsed with water to completely remove the acid. Further rinsing with acetone, drying and weight proceeded as previously described.

Crude protein (CP) was determined using the Dumas sample burning technique (Elementar auto sampler, Vario Max Cube analyser, Hanau, Germany) at an internal temperature of 900^oC with aspartic acid as the internal standard and helium as the carrier gas (Belanche, Kingston-Smith, & Newbold, 2016).

Crude fat (CF) content was determined using the filter bag technology and Ankom XT-15 extractor (ANKOM Technologies, Macedon, NY). Briefly, filter bags (XT4, Ankom Technologies) were labelled and weighed in duplicate; 0.5g of the sample was added into the bags and the combined weight recorded. The bags were heat-sealed to encapsulate the sample and placed in a drying oven at 100°C for 3 hrs. The bags were cooled, weighed and then placed in the Ankom XT-15 extractor with diethyl ether as the solvent. Fat extraction was run at 90^oC for 60 minutes. At the end of the extraction the bags were dried for 30 minutes in the oven at 100°C, cooled and weighed.

2.2.3 ANALYSIS OF FLAVONOIDS AND TANNINS

EXTRACTION

Free phenolics were extracted with solvents of different increasing polarity; 100% Acetone, 75% HPLC grade ethanol (75mL ethanol + 25mL water) and distilled water. An aliquot of 1g of sample was placed in a Hungate tube in duplicate, 5mL of solvent was added and left overnight at room temperature. Supernatant was transferred to a separate Hungate tube. Solvent was evaporated in a fume chamber (water was evaporated using a heat block). The solid residues transferred into 1.5mL microcentrifuge tubes.

The solid residues were then re-dissolved as follows; the acetone extract was dissolved in 1mL of 100% HPLC grade methanol, the ethanol extract was dissolved in 1mL 50/50 solution of methanol and ultra-pure water, the water extract was dissolved in 1mL water. The samples were vortexed to dissolve then centrifuged at 17g for 5min to remove undissolved portions. Supernatant was transferred to fresh, labelled 1.5mL microcentrifuge tubes.

SOLID PHASE EXTRACTION

Solid phase extraction was carried out to separate sugars and proteins from the extract. Discovery® DSC-18 3mL 500 mg solid phase extraction (SPE) tubes (SUPELCO, Bellefonte, USA) were used in conjunction with 10mL syringes and SPE tube adapter for syringes (Sigma-Aldrich). The SPE tubes were washed by passing 10mL of methanol *via* a syringe and then washed twice with 10mL of water before each filtration. The filtrate was collected in a labelled test-tube. The water filtrate was frozen at -80˚C and then freeze-dried. Residue was re-suspended in 1mL water and transferred into labelled Eppendorf tubes. The methanol filtrate was evaporated in a water bath at 40˚C in a fume hood until approximately 1mL was left; and was also transferred into separate labelled Eppendorf tube. In preparation for analysis the samples were centrifuged at 17,000 x *g* for 3 minutes. An aliquot of 400µL of the supernatant was transferred into 2mL HPLC vials.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) - PHOTODIODE ARRAY DETECTION (PDA) - ELECTROSPRAY IONIZATION (ESI) – TANDEM MASS SPECTROMETRY (MS/MS)

HPLC/MS^n analysis was performed as described previously (Parveen et al., 2014) using a Thermo Finnigan LCMS System (Thermo Electron Corporation, USA). The system comprised a Finnigan Surveyor PDA Plus detector and a Finnigan LTQ (linear trap quadrupole) with an ESI source. Chromatography was performed on a Waters C_{18} reverse-phase Nova-Pak column. The column temperature was constant at 30˚C and the temperature of the autosampler tray was maintained at 5˚C. The sample injection volume was 10µL; the detection wavelength was set at 240–400nm with a flow rate of 1mLmin^{-1}. Purified water-formic acid (A; 100:0.1 v/v) and HPLC-grade methanol-formic acid (B; 100:0.1) together formed the mobile phases. The initial condition was A:B (95:5 v/v) and the percentage of B increased linearly over time from 5% to 75% in 70 min. Interface and MD parameters were as follows; sheath gas 30 arbitrary units, auxiliary gas 15 units, spray voltage –4kV, capillary temperature 320˚C, capillary voltage –1.0V and tube lens offset –68 V.

Instrument control and data processing were carried out by Barbara Hauck (IBERS, Gogerddan) using Xcalibur 3.0 software (Thermo Fisher Scientific, USA).

DATA ANALYSIS AND STATISTICS

Compounds were identified based on the characteristic MS^2 fragmentation patterns consistent with literature and the Riken tandem mass spectral database (ReSpect) (Sadiq et al., 2015; Sawada et al., 2012). Volumes were recorded throughout all process to calculate total concentrations of phenols in the samples. Quantifications were calculated using response factors. The abundances of phenols in the various extracts were determined by measurement of peak area in the HPLC chromatogram. Tannins were quantified using 5-caffeoylquinic acid (8.74×10^{-7}µg/area unit) and expressed per unit of dry matter (Belanche, Jones, et al., 2016). Flavonoids were quantified with the response factor of luteolin (5.32×10^{-7}µg/area unit) (Parveen et al., 2014).

Statistical analyses were done on Genstat 19th Edition (VSN International Ltd., Hemel Hempstead, UK). Analysis of chemical composition was done using two-way ANOVA,

with chemical composition as a dependent variable and tree species and ecological zone as the fixed factors. Multiple comparisons of the means (Fisher's protected LSD, p=0.05) was done as the *post hoc* test.

2.2.4 *IN VITRO* GAS PRODUCTION

In order to determine the degradability of the forage in the rumen, gas production over 96 hours was determined using the method of Theodorou et al. (1994) which relies on an inverse relationship between gas accumulation and degradation of the biomass.

RUMEN FLUID AND MEDIA

Rumen fluid was taken from four cannulated mature sheep fed on *ad libitum* hay and 100g sugar beet pulp per day (normal diet) under the authority of Licenses under the UK Animal Scientific Procedures Act, 1986. Rumen fluid was strained through two layers of muslin. The fluid was mixed with anaerobic incubation buffer from Lowman et al. (1999) at a ratio of 1:2 (rumen fluid: buffer) and held under CO_2 at 39°C.

INCUBATION AND GAS PRESSURE MEASUREMENTS

The biomass was incubated in gas-tight 120mL Wheaton bottles, thus enabling gases to accumulate in the headspace as the fermentation proceeded. An aliquot of 300mg of each sample weighed into bottles and 30mL of the inoculum was added to each bottle which were then capped and sealed. Buffer (5mL) + rumen fluid mixture from each sheep was retained and stored at -20°C to be used as a baseline for later analysis of volatile fatty acid production. Headspace gas pressure in each bottle was adjusted to ambient pressure at time zero. Bottles were then incubated at 39°C.

A detachable pressure transducer (Bailey & Mackey Ltd., Birmingham, UK) attached to a Tracker 220 Universal Input Digital Panel Meters (calibrated by the manufacturer to read units of pressure (psi); Data Track Process Instruments, Christchurch, Dorset, UK) was used to measure gas pressure. Measurements were done at 0, 1, 2, 3, 4, 6, 8, 12, 16, 24, 48, 72 and 96 hours after the start of the fermentation.

CURVE FITTING AND STATISTICS

An exponential model (Ørskov & McDonald, 1979) was used to describe the kinetics of the accumulated gas production profiles:

$$Y = a + b\ (1 - e^{(-ct)})$$

> Where "y" is the accumulated gas produced over time in mL; "t" is time in hours; "a" and "b" parameters explained the potential of fermentation ("a + b" reflects the maximum potential of fermentation), and "c" parameter explained the speed/slope of the curve of fermentation.

Curve fitting was done on SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, USA). Statistical analysis was done using two-way ANOVA, with rate and extent of degradation as the respective dependent variables and tree species and ecological zone as the fixed factors. Multiple comparisons of the means (Fisher's protected LSD, p=0.05) was done using Genstat 19th Edition (VSN International, Hemel Hempstead, UK) as the *post hoc* test.

METHANE PRODUCTION MEASUREMENT

Measurement of methane production was done at 24 and 48 hours immediately after measurement of gas pressure. A gas sample (1mL) was removed from each bottle and analysed for CH_4 and H_2 by gas liquid chromatography as described by Lopez et al. (1999), using PYE 4500 Unicam GC containing a 4mm x 3m glass column packed with Porapak Q mesh 60–80 (Waters Associates Inc., Milford, MA, USA). The oven temperature was 65^oC and the carrier gas (He) flow rate was 30mL/min; a katherometer detector was used. Peaks were identified by comparison with gas standards of known composition (Newbold et al., 2005). Instrument control and data processing were carried out by Susan Girdwood (IBERS, Penglais)

2.2.5 VOLATILE FATTY ACID (VFA) ANALYSIS

Volatile fatty acid analysis was done on the separated supernatant from all the bottles and the previously frozen inoculum plus a sample of the rumen fluid. Supernatant was preserved with 20% orthophosphoric acid (containing 20 mM 2-ethyl butyric acid as an internal standard) at a ratio of 1:4 (acid: supernatant) in microcentrifuge tubes, plus 3 additional micro centrifuge tubes for the 'non-fermented' rumen fluid (samples

collected after mixing rumen fluids with buffer) and three from the rumen fluid. The contents were left in the cold room for 24 hours to settle and then filtered into 30 labelled 2mL GC glass vials using separate 0.45 µm pore PES Membrane syringe-driven filters (Jet Bio-Filtration Co. Ltd, Guangzhou, China). All GC glass vials were airtight sealed with crimp caps. Samples were analysed using Varian CP 3380 gas chromatograph (Middelburg, Netherlands) and Varian CP 8400 automatic sampler. Hydrogen was maintained at 5psi and flowing at 20mL per minute as a carrier gas. Short chain fatty acids were detected *via* by the flame ionisation detector (FID). An external standard volatile fatty acid mix (10mM Supelco 46975-U) comprising of propionic, acetic, N-butyric, iso-butyric, iso-valeric, N-valeric, iso-caproic, heptanoic and formic acids was used to create the calibration curve. Final analysis of VFAs data was done using the Varian Galaxie chromatography workstation software (version 1.9.3.2). Total volatile fatty acid was computed as the total of individual short chain fatty acids detected by the FID of the gas chromatograph. N-butyric and iso-butyric acids were summed to get the overall value of butyric acid. N-valeric and iso-valeric acids were also summed to get the overall value for valeric acid. The GC values of different VFAs were corrected for the values of volatile fatty acids produced by the mean of the three blanks. They were also be corrected for the VFAs produced by the three non-fermented ruminal fluids representing the status of volatile fatty acids yields before incubation. Instrument control and data processing were carried out by Susan Girdwood (IBERS, Penglais).

2.2.6 ION TORRENT SEQUENCING

DNA EXTRACTION

DNA was extracted using a CTAB/Chloroform method adapted from Yu and Morrison (2004). Samples were lyophilized at -40°C for 96 hours (Lyotrap freeze dryer, LTE Scientific Ltd, UK). Cell lysis was achieved by bead-beating 20mg (DM) of sample in a Mini Beadbeater™ (Biospec Products, Bartlesville, OK, USA) with a block chilled to -80°C, for 45s using 8mm glass beads. Lysis was done in the presence of 600µL lysis buffer composed of 4% (w/v) sodium dodecyl sulphate (SDS), 500mM NaCl, and 50mM EDTA on a rack prechilled to -80°C. The buffer was designed to protect the released

DNA from degradation by DNAses, which are very active in rumen and gastrointestinal samples (Flint & Thomson, 1990). The samples were incubated for 10 minutes at 95°C on a heat block (Techne Driblock® DB-2D, Bibby Scientific, Staffordshire, UK) to maximize microbial lysis (Belanche, Pinloche, et al., 2016). Most of the impurities and the SDS were removed by precipitation following mixing in 60µL 3M potassium acetate and incubating on ice for 5 minutes (Yu & Morrison, 2004). The precipitate was removed by centrifuging at 17000 x *g* for 5 minutes after which 400µL of the supernatant was recovered to a sterile 1.5mL microcentrifuge tube. Nucleic acid was further purified with 100µL CTAB buffer prepared by mixing equal volumes of 5M NaCl and 10% hexadecyltrimethylammonium bromide (CTAB) in 0.7M NaCl. The tubes were vortexed and incubated for 10 minutes at 60°C in a water bath (with a vortexing at the half point of this time). The final extraction was performed with 300µL of chloroform: isoamyl alcohol (24:1), and layer separation occurred by centrifugation at 17000 x *g* for 5 min at room temperature. The upper aqueous phases were transferred to fresh 1.5mL microcentrifuge tubes. Precipitation of DNA was achieved by adding the upper phase from the last extraction step to 300µL of isopropanol (Minas et al., 2011). Samples were centrifuged at 17000 x *g* for 10 min at room temperature, and supernatants were discarded. Finally, DNA pellets were washed two times in 500µL of 70% (v/v) ethanol. The final pellet was air-dried and re-suspended in 50µL of molecular biology grade water (Fisher Scientific).

Concentration and quality of genomic DNA was assessed by spectrophotometry (NanoDrop ND-1000, Thermo Scientific, USA). The NanoDrop is designed for measuring nucleic acid concentrations in sample volumes of 1µL and overcomes the need for cuvettes when making measurement. The concentration was estimated from the absorbance at 260nm and the purity by verifying that the ratio 260/280nm and 260/230nm were between 1.8 and 2.2 (phenolic compounds absorb around 230nm and proteins around 280nm) (Desjardins & Conklin, 2010).

The CTAB purification step was repeated in extracts with low A260/280 ratios to bring them closer to the recommended cut-off of 1.8 (Sambrook & Russell, 2001, p. A8.20). In high ionic strength solutions (>0.7M NaCl), CTAB form complexes with proteins and with all but most acidic polysaccharides, but will not precipitate nucleic acids (Sambrook & Russell, 2001, p. 6.62).

CLEAN UP OF DNA EXTRACTS WITH LOW A260/280

Attempts were made to improve the purity of extracts with low A260/280 measurements.

1. CTAB method with 4% polyvinylpyrrolidone (PVP) added to the CTAB buffer (Rawat et al., 2016).
2. CTAB method with 2% β-mercaptoethanol added to the extraction buffer (Rawat et al., 2016).
3. CTAB method with 4% β-mercaptoethanol added to the extraction buffer (Khan et al., 2007).
4. CTAB method with 4% PVP (in CTAB buffer) and 4% β-mercaptoethanol (in extraction buffer)(Khan et al., 2007).
5. Precipitation of impurities with two volumes of 22% polyethylene glycol (PEG) – the pellet was resuspended in 10µL of molecular grade water. An aliquot of 20µL of 22% PEG was added and homogenised; the mixture was incubated at room temperature for 30 minutes and then spun at 3000rpm at 4°C for 30 minutes in an attempt to recover a clean DNA pellet.
6. Repeating the CTAB buffer clean-up on the resuspended pellet after the last step of extraction. This approach produced the most acceptable result.

LIBRARY PREPARATION AND SEQUENCING

The rumen bacterial community was studied using Ion Torrent Sequencing as used previously by de la Fuente et al. (2014) and Morales et al. (2018). Amplification of the V1–V2 hypervariable regions of the 16S rRNA gene was carried out using bacterial primers (27F and 357R) tagged with Ion Torrent adaptors sequences (Morales et al., 2018). Forward primers were barcoded with 10 nucleotides to allow sample identification. PCR was carried out on a 25µL reaction containing DNA template (1µL), 0.2µL reverse primer, 1µL forward primer, 5µL buffer (PCR Biosystems Ltd., London, UK), 0.25µL BioHiFi polymerase (PCR Biosystems, UK) and 17.6µL molecular-grade water. Amplification conditions were 95˚C for 1min, and then 22 cycles of 95˚C for 15s, 55˚C for 15s and 72˚C for 30s (T100 Thermal Cycler, BioRad Laboratories). For samples where amplification failed due to contamination with polyphenols, bovine serum albumin (BSA Acetylated, Promega, UK) was added to the PCR mix to a concentration

of 500µg/µL (Sharma et al., 2017) and the amplification repeated. To assess quality of amplifications, resultant amplicons were visualised on a 1% agarose gel.

PCR products were then purified using Agencourt AMpure XP beads (Beckman Coulter Inc., Fullerton, USA). The PCR product was mixed with 0.8µL/µL of room temperature beads and incubated to 10 minutes at RT. The beads were precipitated for 5 minutes on a DynaMag™ 96 side magnet (Life Technologies, Oslo, Norway); the supernatant was discarded, and the pellet was washed twice with 200µL of 70% ethanol. The pellet was air-dried and resuspended in 20µL TE buffer for 2 minutes to elute the nucleic acid. The beads were precipitated on the magnet for 2 minutes at RT; the supernatant was recovered to a fresh PCR plate. DNA concentration was determined using an Epoch Microplate Spectrophotometer fitted with a Take 3 Micro-Volume plate (BioTek, Potton, UK) to enable equimolar pooling of samples with unique barcodes (Morales et al., 2018).

Libraries were further purified using the EGel system with 2% agarose gel (Life Technologies Ltd., Paisley, UK). Purified libraries were assessed for quality and quantified on an Agilent 2100 Bioanalyzer with High Sensitivity DNA chip (Agilent Technologies Ltd., Stockport, UK). Template preparation consisting of emulsion PCR, enrichment of beads containing template and chip loading was carried out using the Ion Chef system (Life Technologies UK Ltd) and the Ion PGM™ Hi-Q™ Chef kit. Sequencing was done using the Ion Torrent Personal Genome Machine (PGM) system on an Ion PGM Sequencing 318™ Chip v2 BC.

BIOINFORMATICS

Following sequencing, sample identification numbers were assigned to multiplexed reads using the MOTHUR software package. Data were denoised by removing low-quality sequences, sequencing errors and chimeras (quality parameters: maximum 10 homopolymers, qaverage 13, qwindow 25 and erate = 1; Chimera check, both *de novo* and database driven using Uchime). Sequences were clustered into OTUs using the Uparse pipeline at 97% identity (de la Fuente et al., 2014; Morales et al., 2018). Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II (Q. Wang et al., 2007). The number

of reads per sample was normalised to the sample with the lowest number of sequences. Raw sequences reads from the bacterial libraries were deposited at the European Nucleotide Archive (PRJEB31014).

Template preparation, chip loading and bioinformatics were carried out by Eleanor Jones (IBERS, Penglais).

STATISTICAL ANALYSES

The effect of tree species on the relative abundance of different bacteria taxa was analysed by ANOVA (7 treatments plus blank). P values were adjusted for multiple testing using the method proposed by Benjamini & Hochberg (1995) to decrease the false discovery rate. When effects were detected, treatment means were compared by Fisher's unprotected LSD test. Findings with $p<0.05$, $p<0.10$ when applying Benjamini & Hochberg correction, were regarded statistically significant. Genstat 18th (VSN International, Hemel Hempstead, UK) was used.

Principal coordinate analysis (PCO) ordination on a Bray-Curtis distance matrix was done to identify patterns in the data. Permutation multivariate analysis of variance (PERMANOVA) was used to determine overall significant differences in community data. Relative taxa abundance data were subjected to square root transformation and Bray-Curtis distance matrices calculated. PERMANOVA was carried out using default settings with unrestricted permutations. All tests were carried out using the PAST v3 statistical package (Hammer, Harper, & Ryan, 2001).

2.2.7 OXFORD NANOPORE SEQUENCING

DNA EXTRACTION AND AMPLIFICATION PCR

Amplification of the nearly-full-length 16S rRNA genes within a sample of microbial DNA was carried out using bacterial primers (27F and 1492R) (Frank et al., 2008). PCR was carried out on a 50µL reaction containing DNA template (2µL), 2µL reverse primer, 2µL forward primer, 10µL buffer (PCR Biosystems Ltd., London, UK), 0.5µL BioHiFi polymerase (PCR Biosystems) and 33.5µL molecular-grade water. Amplification conditions were 95˚C for 1min, and then 22 cycles of 95˚C for 15s, 55˚C for 15s and 72˚C for 30s. To assess quality of amplifications, resultant amplicons were visualised on a 1% agarose gel. PCR products were then purified using Agencourt AMpure XP beads (Beckman Coulter Inc., Fullerton, USA). Concentration and quality of genomic DNA was assessed again by spectrophotometry (Nanodrop ND-100, Thermo Scientific, USA).

16S RAPID AMPLICON BARCODING FOR THE MINION™ USING SQK-RAB204

Barcoding amplification was carried out on a 50µL reaction containing 25µL LongAmp® *Taq* 2X Master Mix (New England Biolabs), 14µL nuclease-free water, 1µL 16S barcoding kit (SQK-RAB204, Oxford Nanopore Technologies) and 10µL template in 0.2mL thin walled PCR tubes. Amplification conditions were initial denaturation 1 min @ 95°C (1 cycle), denaturation 20 secs @ 95°C (25 cycles), annealing 30 secs @ 55°C (25 cycles), extension 2 mins @ 65°C (25 cycles) and final extension 5 mins @ 65°C (1 cycle). At the end of the PCR, a 1% gel was run to confirm amplification.

LIBRARY PREPARATION

After barcoding, 45µL from each sample was pooled in a low-bind microcentrifuge tube (total 500µL). An aliquot of 200µL of Ampure beads (at a ratio of 0.4) was added. The tube was gently inverted and incubated at room temperature for 5 minutes. The beads were sedimented on a magnet and the supernatant was discarded. The pellet was then washed three times in 200µL of 80% ethanol was added and air dried at room temperature. Nucleic acid was eluted in 12µL of TRIS buffer (from Qiagen kit). 1µL the rapid adaptor (RPD) (from the flow-cell priming kit) was added and mixed by

plunging. The mixture was incubated at room temperature for 5 minutes. 12µL of the sample was added to the library to the library loading mix just prior to loading the flow-cell.

PRIMING THE MinION FLOW CELL

The Oxford Nanopore Technologies (ONT) MinION™ portable sequencer was used in conjunction with the MIN106 flow cell (ONT) and the EXP-FLP001 Flow-cell Priming Kit (ONT). The sequencing platform was connected to a laptop *via* USB and the blank flow-cell was removed. The selected flow-cell was installed and was checked for the number of open pore (must be ≥800) using MinKNOW (the software that runs the sequencing platform). After the check, the bubble was removed using a micropipette. An aliquot of 800µL of running buffer (RBF) from the kit was slowly pipetted into the cell through the priming port.

FLOW CELL LOADING AND SEQUENCING

The SpotON sample port cover on the flow-cell was gently lifted to make the SpotON sample port accessible. 200µL of the RBF was loaded into the flow cell *via* the priming port (not the SpotON sample port), avoiding the introduction of air bubbles. The library was prepared just prior to loading by gently mixing the components by pipetting up and down. An aliquot of 80µL of the library was added to the flow cell *via* the SpotON sample port in a dropwise fashion ensuring that each drop flowed into the port before adding the next. The SpotON sample port cover was gently replaced, making sure the bung entered the SpotON port; the priming port was closed, and the MinION lid was replaced. The sequencing run was started on the MinKNOW software after selecting the appropriate setting for the barcodes and the flow-cell used.
16S Rapid amplicon barcoding, library preparation, flow cell priming and loading; and sequencing was done by Arwyn Edwards (IBERS, Penglais) as a tutorial to the author.

BIOINFORMATICS

At the end of the sequencing, base-calling and demultiplexing were performed on a 1TB SSD 32GB i7 laptop using Albacore v2.1.3 and the default command structure c:\ac\read_fast5_basecaller.exe --input C:\data\reads\ --recursive --worker_threads 6

--save_path C:\data\outputs --flowcell FLO-MIN106 --kit SQK-RAB201 --barcoding --output_format fastq. This processing produced folders for reads from each sample as discrete, multi-read .fastq files. Each fastq had a maximum of 4000 reads.

Demultiplexed .fastq were size-filtered to only those reads between 1300 and 1600bp and quality-filtered to a mean quality score of 25 using the BASH informatics language and Filtlong (https://github.com/rrwick/Filtlong). Passed reads were then mapped with minimap2 (https://github.com/lh3/minimap2) to the RefSeq 16S database (https://www.ncbi.nlm.nih.gov/refseq/targetedloci/). Mapped reads were then clustered together on a per-sample basis, assigned to their respective taxonomy and transformed to an abundance matrix format using custom python scripts developed by André Soares (IBERS, Penglais). The single-sample abundance matrices were finally collated into a final one containing all samples, detected taxa and their counts by means of an R script.

Bioinformatic analysis was done by André Soares and Arwyn Edwards (IBERS, Penglais).

STATISTICS

Principal coordinate analysis (PCO) ordination on a Bray-Curtis distance matrix was done to identify patterns in the data. Relative taxa abundance data were subjected to square root transformation and Bray-Curtis distance matrices calculated. The tests were carried out using the PAST v3 statistical package (Hammer et al., 2001).

2.2.8 METHOD DEVELOPMENT FOR ISOLATING PURE DNA FROM FODDER TREES LEAVES

The isolation of pure, intact and high-quality DNA is the most important step in any molecular study (Tan & Yiap, 2009). This makes it useful for downstream protocols such as PCR and sequencing (Minas et al., 2011). A major problem encountered with samples containing fully developed and mature leaves is accumulation of polyphenolics and tannins (Coley & Barone, 1996). When in oxidized form, polyphenolics and tannins covalently bind with DNA and making it resistant to restriction enzymes. They also give the extracted DNA a brown colour (Katterman & Shattuck, 1983). Over the years, various methods have been developed to counter this problem especially by scientists seeking to isolate plant DNA. However, since the biochemical composition of each plant and tree species is significantly varied, it is not possible to develop a single protocol which would universally solve this problem for all plant species (Sharma et al., 2017). Scientists in the plant field have found that even closely related species require different protocols to acquire pure DNA (Rawat et al., 2016). This problem was encountered during this study, manifesting as DNA pellets that were brown to black in colour due to high contamination with polyphenolics. In addition, the A260/280 values was less 1.6 which is lower than the recommend cut-off of 1.8 (Sambrook & Russell, 2001, p. A8.20). To overcome the problem, several the extraction methods used were including the CTAB method, CTAB method with varying concentrations of Polyvinylpyrrolidone (PVP) and β-mercaptoethanol (Khan et al., 2007; Rawat et al., 2016). PVP purges and forms complex hydrogen bonds with polyphenols release during the lysis steps and gets precipitated and can then be separated from DNA by centrifugation(Maliyakal, 1992). β-mercaptoethanol acts as strong reducing agent in higher concentrations and helps in reducing the polyphenols (Khanuja et al., 1999).

Extraction was also done using a QIAamp DNA Stool Mini Kit (Qiagen Ltd., Crawley, UK). The protocol used was according to the manufacturer's instructions, but with the incubation temperature increased to 95˚C for 10 minutes to maximize microbial lysis (Belanche, Pinloche, et al., 2016). The CTAB protocol has been shown to produce microbial DNA from rumen samples of sufficient quantity and quality for use in PCR

amplification. Additionally, when compared to the commercially available kits, DNA extraction using the CTAB protocol is more cost efficient (Minas et al., 2011).

Eventually a modified CTAB/Chloroform method was settled on, with aspects of it adopted from Yu & Morrison (2004); and previously used by (Morales et al., 2018). Addition of a purification step improved the quality of the extracts, albeit at much lower concentrations. Despite this success, some samples were still contaminated at levels that inhibited PCR amplification. For this samples, 500µg/µL of bovine serum albumin was added to the PCR mix (Sharma et al., 2017). BSA has been used as an additive in various applications, including restriction enzyme digestions as well as in PCR amplification of templates from environmental samples that contain potential inhibitors such as phenolic compounds(Farell & Alexandre, 2012). Phenols are known to bind to proteins by forming hydrogen bonds with the oxygen of peptide bonds; BSA has been widely used during isolation of organelles and enzymes from plants to scavenge endogenous phenolic compounds that can bind to and inactivate proteins of interest (Loomis, 1974). The mechanism of action of BSA in enhancing PCR reactions has been suggested as through scavenging a variety of substances and thereby prevent their binding and inactivation of *Taq* DNA polymerase (Kreader, 1996). Such inhibitors include tannic acids (one type of plant phenolic compound); humic and fulvic acids are mixtures of polyphenolic substances produced during the degradation of plant material. Faeces, especially from herbivores, which contain copious quantities of degraded plant material have these inhibitors as well. BSA is also known to bind lipids *via* hydrophobic forces and anions by virtue of its high lysine content (Loomis, 1974). Despite these interventions, 21 of the 88 samples did not amplify and as a result were excluded from the experiment. An unbalanced ANOVA was used to take to account these missing samples.

2.3 RICE STRAW DEGRADATION AND COLONISATION

2.3.1 PLANT MATERIAL

Plant material was kindly donated by Prof Sue Hartley at University of York. Samples originated from the BBSRC-GCRF project titled, "Developing rice resources for resilience to climate change and mitigation of carbon emissions". The goal of the project was to identify existing rice varieties with low silica or highly digestible straw to investigate and demonstrate the advantages of using straw with better quality for applications as animal feed and for biofuel production. The samples were sent to Aberystwyth University (IBERS) for to investigate the effect of silica content on degradation by rumen microbes.

GROWTH OF PANELS

A total of 215 rice germplasm accessions were planted in two adjacent fields partitioned into three blocks at the experimental field of the Philippine Rice Research Institute in Munoz, Nueva Ecija, Philippines. Each block/replicate has 5 columns with a total of 215 plots (43 plots per column) randomly assigned to each of the rice accessions. Each column had a size of 86 m (L) x 2.5 m (W) and the distance between columns was 0.75 m. Each plot within the columns had a dimension of 2.5 m x 2 m (5 m2). A planting distance of 20 cm x 25 cm (row to hill) was used; therefore, there were 10 rows x 10 hills per plot and a population size of 100.

Sowing of seeds was done on December 20, 2016 and transplanting was done on January 10, 2017 (dry season). Harvesting began on March 22, 2017 (dry season) and completed on May 12, 2017 considering that some materials have late maturity. A total of 25 primary tillers per rice accession in triplicate (75 tillers per accession) were collected for analysis. After collection, the straws were sundried for one or two days before drying by incubation at 50°C – 60°C. The rice straws were sent in York University in July 2017 and thereafter subsamples were donated to Aberystwyth University. The different parts of the plant *i.e.* flower-head, stems and leaves were separated, and the experiments described in this thesis involved only using the leaves.

Six accessions each with three replicates were selected for experimentation (Table 2.3). The selection was based on stem Si content to give a high, medium and low Si comparison.

Table 2.3: Accessions selected for experimentation

ID	Replicate	Plot no.	Column	Plot No. in block	15WS entry code	Designation
L-8	1	77	2	10	CB 20	PSB Rc56 (DAPITAN)
	2	285	7	27		
	3	639	15	37		
L-12	1	204	5	32	CB 28	NSIC Rc19 (MALAGKIT 4)
	2	323	8	22		
	3	432	11	2		
L-94	1	153	4	20	CB 256	Pokawan
	2	307	8	38		
	3	509	12	8		
L-103	1	115	3	29	CB 275	CSR 89-IR-15
	2	252	6	7		
	3	561	14	42		
L-117	1	92	3	6	CB 305	PR 38732-B-B-1
	2	368	9	24		
	3	547	13	31		
L-123	1	141	4	32	CB 320	PR41035-B-B-17-2-5-1
	2	371	9	27		
	3	516	12	1		

2.3.2 PROXIMATE ANALYSIS

The samples were ground to approximately 1mm^2 sized particles using a forage mill. Dry matter, organic matter, NDF, ADF and ADL analysis was carried out as previously described (section 2.1.2) in triplicate.

2.3.3 ANALYSIS OF SILICA CONTENT USING X-RAY FLUORESCENCE SPECTROMETRY (XRF)

Silica content in the rice straw samples was analysed at York University according to the method described by Reidinger et al. (2012).

PELLET PREPARATION

X-ray fluorescence emitted from light elements such as Si and P is of low energy and has low penetrating power, and hence the sample surface must be tight, flat and of equal density to obtain a repeatable photon flux from the sample to the XRF detector. Approximately 0.5g of dried and ground material were pressed at 10 tons for 2s using a manual hydraulic press and a 13mm diameter die(Specac, Orpington, UK) (Figure

2.1), resulting in a cylindrical pellet of approximately 3mm thickness. Samples were prepared in triplicate.

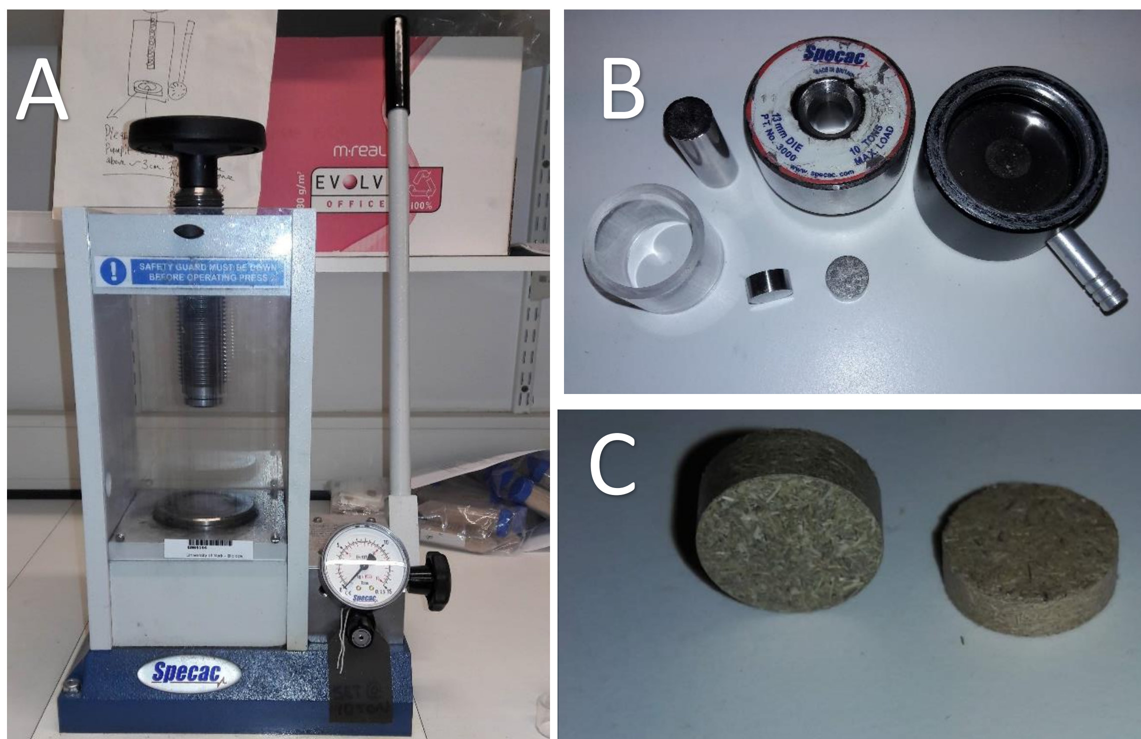


Figure 2.1: A. Manual hydraulic press. B. 13mm die assembly C. Pressed pellets

XRF SPECTROMETRY

Analyses was done using a commercial P-XRF instrument (Niton XL3t900 GOLDD Analyzer; Thermo Scientific, Winchester, UK) in conjunction with a test stand (Portable Test Stand, Thermo Scientific, Billerica, MA, USA), which increases the instrument's performance when analysing light elements with low energy fluorescence such as Si and P (Reidinger et al., 2012) (Figure 2.2). To avoid signal loss by air absorption, the instrument was connected to a (portable) gas cylinder containing low-grade helium, and all measurements were carried out in a helium atmosphere with a flow rate of 70cLmin^{-1}. P-XRF. Analyses can be conducted without helium; however, inclusion of helium may increase the value of the detection limit of the method, particularly for light elements such as Si or P (Reidinger et al., 2012). Samples were analysed randomly and on both surfaces of the pellet to obtain an average.

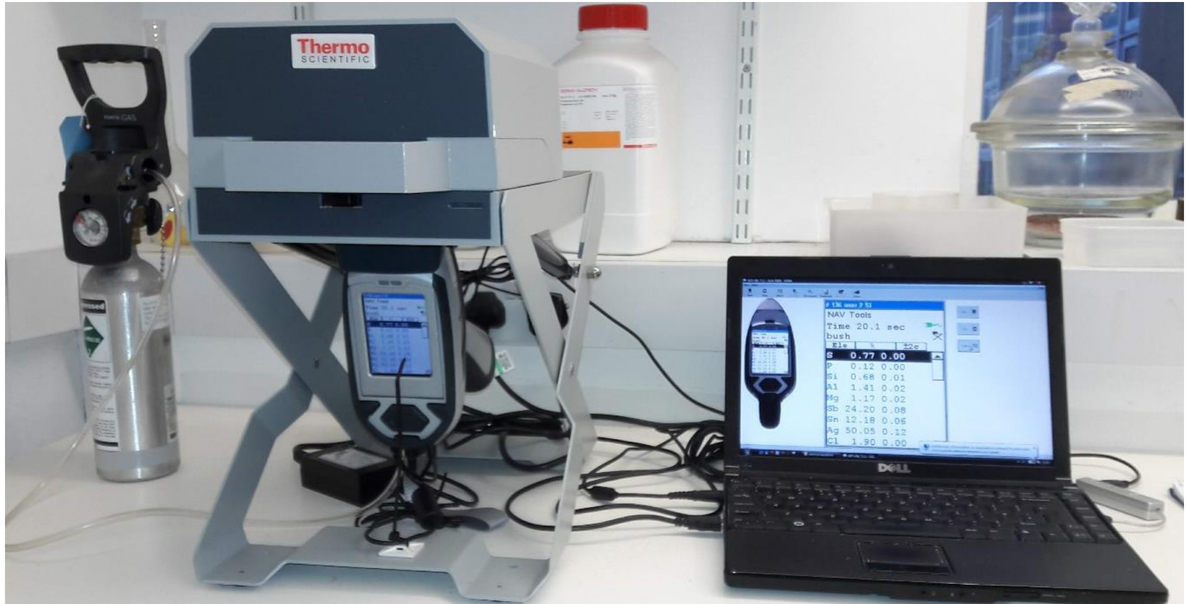


Figure 2.2: P-XRF spectrometer system

2.3.4 *IN VITRO* GAS PRODUCTION

DONOR ANIMAL SPECIES

The rumen fluid used in the preceding experiment (detailed in section 2.2) was collected from fistulated adult sheep maintained by the Aberystwyth University. The rumen fluid used in the rest of experiments detailed in the sections below was collected from adult fistulated cows also maintained at Aberystwyth University. Although the rumen environment is not uniform between the two species, rumen fluid from sheep can replace that from cattle or *vice versa* as rumen inoculum as the two sources were comparable under tropical feeding conditions (Bueno et al., 2015). Muetzel et al. (2014) also reported no difference in total gas production when they compared rumen fluid from cattle and sheep. A close association for gas production at 24 and 48h for incubations with rumen fluid from cows and sheep was also observed by (Cone, Van Gelder, & Bachmann, 2002).

On the other hand, rumen fluid from sheep shows a higher fermentation rates and extent of degradation of fibre-rich substrates such as straw and hay compared to buffalo (Calabrò et al., 2005). The microbial ecology of rumen samples between ruminant species may vary even among animals housed in close contact and fed a similar diet. The differences in microbial ecology between sheep and goats housed and fed together have been attributed to differences in dentition, eating and ruminating behaviour, digestive tract physiology and ruminal retention time (Ammar

et al., 2008). Differences due to feeding behaviour and diet composition can to some extent be overcome by the collection of rumen samples before morning feeding, when the effect of diet composition on rumen metabolites or microbiota are likely to be minimized (Martínez et al., 2010).

However, the most dependable way to avoid large differences in rumen fluid is collect from animals of the same target species fed a diet containing the same feedstuffs (Yáñez-Ruiz et al., 2016). The choice between sheep and cows in this dissertation was based on availability. The animals are frequently used for *in vivo* studies and the switch from sheep to goats was to avoid collecting rumen fluid from animals on an experimental diet. For each species, rumen fluid was collected from four animals to provide a representative source of rumen inoculum (Yáñez-Ruiz et al., 2016).

ANKOM^{RF} GAS PRODUCTION SYSTEM

The ANKOM^{RF} Gas Production System (Ankom Technology, Macedon, NY) was used for measurement of *in vitro* gas production. Each unit consisted of a Duran bottle (capacity: 274 ± 1.1mL) equipped with an ANKOM pressure sensor module (pressure range: –69 to +3447 kPa; resolution: 0.27 kPa; accuracy ± 0.1% of measured values) including a microchip and a radio sender (Cornou et al., 2013).

Rumen fluid was taken from four cannulated mature cows fed on hay under the authority of Licenses under the UK Animal Scientific Procedures Act, 1986. Rumen fluid was strained through two layers of muslin. The fluid was pooled and mixed with anaerobic incubation buffer from (Lowman et al., 1999) at a ratio of 1:2 (rumen fluid: buffer) and held under CO_2 at 39°C. Rice straw from each treatment was chopped to 1cm length and 1g was weighed into 250mL Duran bottles in quadruplicate. Inoculant (100mL) was added to each bottle under CO_2; the bottles were sealed and incubated at 39˚C for 96 hours. During the incubations, pressure changes in the headspace of the bottles were measured as a difference with respect to concurrently measured atmospheric pressure. The readings were transmitted *via* a radio frequency to a PC at intervals of 5 min. Gas accumulating in the headspace of the bottles was automatically released when the pressure inside the units reached 6.9kPa above ambient pressure (Cornou et al., 2013). The cumulative gas pressure (psi) was converted to volume of gas (mL/g DM) produced over time as per the operator's manual (Ankom, 2018). An

aliquot of 5mL of the buffer + rumen fluid inoculum was retained and stored at -20°C and used as a baseline for analysis of volatile fatty acid production (four replicates).

IN VITRO GAS PRODUCTION AND VFA ANALYSIS (WHEATON BOTTLES)

The biomass was incubated in gas-tight 120mL Wheaton bottles, thus enabling gases to accumulate in the headspace as the fermentation proceeded.

Rumen fluid was taken from four cannulated mature cows fed on *ad libitum* hay and 100g sugar beet pulp per day (normal diet). Rumen fluid was strained through two layers of muslin. The fluid was pooled and mixed with anaerobic incubation buffer from (Lowman et al., 1999) at a ratio of 1:2 (rumen fluid: buffer) and held under CO_2 at 39°C. Rice straw from each treatment was chopped to 1cm length and 300mg of each treatment weighed into bottles and 30mL of the inoculum was added to each bottle. Bottles were capped and sealed; gas was released to zero all bottles at time zero. 5mL of the buffer + rumen fluid mixture from each cow was retained and stored at -20°C to be used as a baseline for later analysis of volatile fatty acids production. Bottles were incubated at 39°C and the headspace gas pressure in each adjusted to ambient pressure prior to and just after inoculation with the microbial suspension.

A detachable pressure transducer (Bailey & Mackey Ltd., Birmingham, UK) attached to a Tracker 220 Universal Input Digital Panel Meters (calibrated by the manufacturer to read units of pressure (psi); Data Track Process Instruments, Christchurch, Dorset, UK) was used to measure gas pressure. Measurements were done at 0, 1, 2, 3, 4, 6, 8, 12, 16, 24, 48, 72 and 96 hours after the start of the fermentation. At the end of the incubation, the pH was measured using a Jenway pH probe (Cole-Parmer Instrument Company Ltd, Staffordshire, UK) attached to a SevenEasy pH meter (Mettler-Toledo GmbH, Schwerzenbach, Switzerland). Curve fitting for gas production was done as described previously in Section 2.1.4. At the end of the incubation, the supernatant was sampled, and volatile fatty acid analysis was performed on samples from all the bottles and the frozen inoculum as previously described in Section 2.1.5.

2.3.5 TEMPORAL COLONISATION OF RICE STRAW

RUMEN FLUID AND MEDIA

A colonisation study was be carried out according to Mayorga et al. (2016). Rumen fluid was taken from four cannulated mature cows fed on *ad libitum* hay and 100g sugar beet pulp per day (normal diet) under the authority of Licenses under the UK Animal Scientific Procedures Act, 1986. Rumen fluid was strained through two layers of muslin. The fluid was pooled and mixed with anaerobic incubation buffer from (Lowman et al., 1999) at a ratio of 1:9 (rumen fluid: buffer) and held under CO_2 at 39°C.

***IN VITRO* COLONISATION**

Rice straw from each treatment was chopped to 1cm length and 600mg was weighed into 120mL Wheaton bottles and 60mL of the inoculum added to each bottle. Rumen fluid (20mL) from each cow was flash frozen in liquid nitrogen and stored at -80°C to be used as a baseline during analysis microbial population. Bottles were sealed and incubated in a horizontally rotating rack at 100rpm and 39˚C (ISF-1-W Incubator Shaker, Kühner, Switzerland). Bottle contents were harvested at 2, 4, 6, 8 and 12 hours. At each time interval bottle contents were harvested by filtration through a strainer and lightly washed with water to remove loosely attached bacteria. Separated material was blotted with tissue paper to drain excess water and wrapped in labelled aluminium foil. The samples were flash-frozen in liquid nitrogen and stored at -80°C until further use.

2.3.6 ION TORRENT SEQUENCING

RNA EXTRACTION (HOT PHENOL METHOD)

RNA was extracted using a hot phenol method (Huws et al., 2016; Ougham & Davies, 1990). Frozen material (250mg) was ground into a fine powder in a pre-cooled mortar under liquid nitrogen. Ground material transferred to 15mL sterile Greiner centrifuge tube; 2mL of room temperature aquaphenol and 2mL of buffer solution (Table 2.4) warmed to 65°C were added and the mixture was homogenised.

Table 2.4: Extraction buffer

Reagent	Volume (6 samples)
Sterile Distilled Water	12.18mL
0.5M EDTA pH8.0	300µL
3M NaAc pH5.2	1mL
10% SDS	1.5mL

The tubes were incubated in a water bath at 65°C for 30 minutes and then vortexed for 10s. To this 2mL of chloroform was added and the tubes were vortexed for 10s. The tubes were centrifuged at 20°C, 5000 x *g* for 30min; the lower layer (phenol phase) was removed using a sterile Pasteur pipette. A further 2mL of chloroform was added and vortex for 10s and the tubes centrifuged at 20°C, 5000 x *g* for 20min. The upper layer was removed into a new 15mL sterile Greiner centrifuge tube. 2mL chloroform-isoamyl (24:1) was added, vortexed for 10s; tubes were centrifuged at (20°C, 5000rpm for 20min). The volume of upper layer was measured into a sterile microtube and 10M LiCl to a final concentration of 2M LiCL (*i.e.*, ¼ volume measured in the preceding step). Tubes were mixed and stored overnight at 4°C.

The tubes were subsequently centrifuged (4°C, 30min, 13000 x *g*). The supernatant was discarded and 0.5mL ice cold 2M LiCL was added and mixed to re-suspend the pellet. The tubes were centrifuged at 4°C, 15min 13000 x *g*. The process was repeated from addition of LiCl to ensure all DNA was removed. After the supernatant was discarded, the pellet was resuspended in ice-cold 80% ethanol and centrifuged (13000 × *g*, 15 min, 4°C), this was repeated twice before the pellet was air-dried and re-suspended in molecular grade water.

Quality and quantity of retrieved RNA was checked using the Qubit© 2.0 Fluorometer (Invitrogen, Life Sciences, California). Briefly, 2µL of extract was mixed with 198µL of

Qubit RNA assay buffer containing 1µL of dye (Qubit™ RNA BR Assay Kit, Invitrogen). The quality of isolated RNA was determined using absorbance at 230, 260, and 280nm. The possible protein contamination with RNA was measured using a 260/280 ratio, and the salt and organic solvent contamination was measured using a 260/230 ratio.

DNA/RNA CO-EXTRACTION

Nucleic acid was extracted by method modified from Griffiths et al. (2000).

Nucleic acids were extracted from 250mg (wet weight) of frozen sample ground into a fine powder in a pre-cooled mortar under liquid nitrogen. The material was transferred into 2mL capped micro-tubes (Sarstedt, Nümbrecht, Germany) together with 8mm glass beads and 300mg of 0.1mm glass homogenisation beads (Biospec Products, Bartlesville, OK, USA). Extractions were performed by the addition of 0.5mL of hexadecyltrimethylammonium bromide (CTAB) extraction buffer and 0.5mL of phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0, Fisher Scientific). CTAB extraction buffer, modified from the method of Kowalchuk et al. (1998), was prepared by mixing equal volumes of 10% (wt/vol) CTAB in 0.7M NaCl with 240mM potassium phosphate buffer, pH 8.0 (Sambrook & Russell, 2001, p. A1.5). Samples were lysed for 45s in a Mini Beadbeater™ (Biospec Products, Bartlesville, OK, USA) with a block chilled to -80°C. The aqueous phase containing nucleic acids were separated by centrifugation (16000 x *g*) for 5 min at 4°C. The aqueous phase was then extracted, and phenol was removed by mixing with an equal volume of chloroform-isoamyl alcohol (24:1) followed by repeated centrifugation (16000 x *g*) for 5 min at 4°C. Total nucleic acids were subsequently precipitated from the extracted aqueous layer with 2 volumes of 30% (wt/vol) polyethylene glycol 6000 (Fluka BioChemika)–1.6 M NaCl for 2 h at room temperature, followed by centrifugation (18000 x *g*) at 4°C for 10 min. Pelleted nucleic acids were then washed in ice cold 70% (vol/vol) ethanol and air dried prior to resuspension in 50mL of RNase free Tris-EDTA buffer pH 7.4 (Fisher Scientific).

CTAB DNA EXTRACTION

DNA was extracted using a CTAB/Chloroform method described in Section 2.1.6 with the following changes. Nucleic acids were extracted from 25mg (wet weight) of frozen sample ground into a fine powder in a pre-cooled mortar under liquid nitrogen. Cell lysis was achieved by bead-beating ground material in a Mini Beadbeater™ (Biospec Products, Bartlesville, OK, USA) with a block chilled to -80°C, for 45s using 8mm and 300mg of 0.1mm glass homogenisation beads (Biospec Products, Bartlesville, OK, USA). The rest of the extraction proceeded at previously described.

LIBRARY PREPARATION AND SEQUENCING

The rumen bacteria community was studied using Ion Torrent sequencing. Library preparation and sequencing was carried out as described in Section 2.1.6 using the Ion Torrent PGM system on an Ion PGM Sequencing 316™ Chip v2 BC.

SEQUENCE DATA PROCESSING

Sequence data were quality checked and demultiplexed using MOTHUR (v. 1.31.2; (Schloss et al., 2009)). Sequences with mismatching barcode and primer sequences (length less than 100bp and with an average Phred score less than 20) were discarded. Sequence files were dereplicated and singletons discarded as recommended in Tedersoo et al. (2010), and operational taxonomic units (OTUs) assigned using USEARCH/UPARSE (v7 (Edgar, 2013)) at 97% clustering; clusters containing less than 2 sequences were discarded. A taxonomy was assigned to each OTU (operational taxonomic unit) using the Naïve Bayesian Classifier (Q. Wang et al., 2007), against an adapted RDP v11 database with a cut-off of 0.6. Where genus was not assigned by the classifier (but only assigned to family or order, due to confidence being lower that the threshold), an OTU identifier was assigned to that cluster. Data were then rendered in Excel and standardized by dividing the number of reads in each taxonomic unit by the total number of reads in each sample to give relative abundances of the assigned taxa for each quadrat.

Principal coordinate analysis (PCO) ordination on a Bray-Curtis distance matrix was done to identify patterns in the data. Permutation multivariate analysis of variance (PERMANOVA) was used to determine overall significant differences in community

data. Relative taxa abundance data were subjected to square root transformation and Bray-Curtis distance matrices calculated. PERMANOVA was carried out using default settings with unrestricted permutations. All tests were carried out using the PAST v3 statistical package (Hammer et al., 2001).

Template preparation, chip loading and bioinformatics were carried out by Andrew Detheridge (IBERS, Penglais).

2.3.7 REAL-TIME POLYMERASE CHAIN REACTION (qPCR)

The amplification of bacterial DNA was done according to (Belanche et al., 2014).

STANDARD PREPARATION

Absolute concentrations of DNA from total bacteria were determined using qPCR and serial dilutions (10^{-2} to 10^{-7}) of specific DNA standards. Briefly, rumen liquid-associated bacteria were obtained from each animal by sequential centrifugation (Cecava et al., 1990) and pooled to generate a bacterial DNA standard. Two protozoal DNA standards were generated by pooling DNA from all Holotrich fractions (for Holotrich protozoa quantification) and from all total protozoa fractions but F < 5 (for total protozoa quantification). Then, their true protozoal DNA concentration was determined by subtraction of the bacterial and methanogens DNA contamination from the genomic DNA concentration measured of the protozoal standards. Finally, a methanogens DNA standard comprised the methyl coenzyme-M reductase (mcrA) gene inserted into the PCR-TOPO plasmid (Invitrogen).

qPCR PROTOCOL

All the primers were obtained from Eurofins Scientific. They were rehydrated to 50picomoles per microliter (50µM) in molecular grade water and stored frozen at -20°C until required. The primers were diluted to a final concentration of 400nM in a total reaction volume of 12.5µL. The reactions were performed in triplicate using a LightCycler® 480 System (Roche, Mannheim, Germany). The total reaction volume was prepared to a total volume of 12.5µL. The reagents were thawed on ice, mixed and spun down solutions before use to recover the maximum amount. 7.5µL (per sample) of the master mix was prepared by mixing 5.625µL of PCR Biosystems 2X qPCRBIO SyGreen Mix Lo-ROX, 0.09µL of each of the primers (forward primer 5' –

GTGSTGCAYGGYTGTCGTCA – 3’; and reverse primer 5’-ACGTCRTCCMCACCTTCCTC-3’ (Maeda et al., 2003)) and 1.695µL of molecular grade water (Table 2.5). 7.5µL of master mix and then 5µL of sample DNA was added to each well of a white qPCR plate. A column for the ladder (7 dilutions + 1 negative template) in triplicate was also included. The plates were spun down before amplification. Amplification conditions were 95°C for 5min, then 45 annealing cycles at 61°C for 30s, 72°C for 30s and 95°C for 15s. The C_T value was determined during the exponential phase of amplification, and a final melting analysis was performed to check primer specificity. Finally, efficiencies of PCR amplification were determined by serial dilutions of DNA samples (Belanche et al., 2014).

Table 2.5: qPCR reaction mix preparation

Reagent	Volume (µL)	Final concentration
2X qPCRBIO SyGreen Mix Lo-ROX	5.625	1X
Forward primer (50 µM)	0.09	400nM
Reverse primer (50 µM)	0.09	400nM
Template (DNA extract diluted 1/10)	5	0.1X DNA
Molecular Water	1.695	
Total volume	12.5	

CHAPTER 3: *IN VITRO* RUMINAL FERMENTATION KINETICS AND METAGENOMIC CHARACTERISTICS OF LEAVES OF SEVEN WEST AFRICAN FODDER-TREE SPECIES

3.1 INTRODUCTION

Livestock farmers in many parts of sub-Saharan Africa (SSA) contend with low production caused by shortage of feed, low quality forages or both. This situation is compounded by increasingly erratic weather attributed to climate change. During the dry season, livestock lose body-condition and in some cases, die. During that period, farmers are forced to sell their animals at below market prices to avoid total loss (Kurukulasuriya et al., 2006).

The use of fodder trees and shrubs has received increasing attention as a source of highly nutritious feed to supplement bulk feeds during the dry seasons. This fodder can serve as a rich source of crude protein and minerals especially during the dry season. Fodder trees are planted as part of an agroforestry system as seen in many parts of the East African highlands (Franzel et al., 2014). In the Sahel, wild tree seedlings are allowed to grow on farmland so that they can be used as fodder once they mature (Boffa, 1999).

Fodder trees may help reduce methane emissions (per unit of production) by improving livestock productivity and by reducing carbon footprint of industry by substituting for commercially manufactured concentrates (Franzel et al., 2014). Few studies were found that explicitly quantify the contributions of fodder trees to climate change adaptation or mitigation. Pal et al. (2015) showed that leaves of tropical fodder trees (including *Acacia nilotica and Azadirachta indica)* produced less methane per unit of degradable organic matter had generally greater organic matter degradability and favoured production of microbial biomass compared to the control diet. Further work needs to be done to determine the effect of including fodder trees in ruminant diets on methane production.

There are significant variations in the chemical composition and ruminal degradability in the tree species (Tefera et al., 2008). A considerable amount of work has been done demonstrating these differences; however, the sheer variety of the species being used

across the continent means that a lot more work remains to be done. In Kenya for instance, there are more than 160 species of trees and shrubs being used as fodder (Franzel et al., 2014). This number increases significantly if the entire continent is considered.

Browse species often contain an abundance of protein and other nutrients. They are however known to contain secondary metabolites such as tannins, saponins and non-protein amino acids, which are either toxic to rumen microbes or to the animal, or produce toxic metabolites upon degradation (El Hassan et al., 2000). The presence of secondary metabolites can reduce the availability of nutrients after ingestion. The presence of terpenes, tannins, and phenolics in browse species has been implicated in the reduction of dry matter and/or protein digestibility and nitrogen retention (Kaitho et al., 1997; Ngugi, Hinds, & Powell, 1995). Belanche, Jones, et al. (2016) observed that while brown seaweeds (*Ascophyllum nodosum*) had 11.7% protein content, most of it was likely to be bound to phenols through oxidative and nucleophilic reactions. The ability of tannins to bind and precipitate protein can also shift site of protein digestion from the rumen to the abomasum and nitrogen excretion from urine to faeces in ruminants resulting in a net loss of nitrogen (Mueller-Harvey, 2006). Goats fed shrubs containing tannins showed reduced serum urea concentration attributed to low nitrogen availability (Silanikove et al., 1996). The effect of high tannin levels in *A. nilotica* on CP digestibility has been reported (Barman & Rai, 2008). Tannins also bind carbohydrates to some extent, which may also have implications for fermentation and nutritional value of some diets (Estell, 2010; Mueller-Harvey, 2006). Therefore, apart from proteins, tannins may reduce ruminal degradation of other dietary components through substrate privation, enzyme inhibition and direct inhibition of certain rumen microorganism (Belanche, Jones, et al., 2016; McSweeney et al., 2001).

Conversely, the protein-binding effect of tannins may have a positive effect on nitrogen metabolism in ruminants. Protein-phenol complexes are not targeted by bacteria; the presence of tannins may therefore reduce the degradation of forage proteins in the rumen, without reducing the amount of microbial protein synthesized resulting in an increase the absorption of essential amino acids from the small intestine (Belanche, Jones, et al., 2016; Min et al., 2003). Min et al. (2003) proposed that moderate concentrations of condensed tannins can be used to increase the

efficiency of protein digestion and to improve animal health under grazing, producing more sustainable grazing systems. The effects produced are not the same for all plant secondary metabolites (PSM), but rather depend upon the concentration and structure of the compounds (Min et al., 2003). This may limit their palatability or inclusion rate in the ration. It is therefore important to undertake *in vitro* and *in vivo* studies to accurately measure of the degradation of fodder trees in the rumen.

3.1.1 PHENOLICS IN LEAVES OF FODDER TREES

Tree fodders leaves often contain phenolic compounds that may act as anti-nutritive factors generally resulting in a reduction in the nutritive value when consumed by ruminants. High total phenol levels may be associated with poor quality feeds (Wood et al., 2000). Phenolics are secondary products that contains a phenol group *(i.e.* a hydroxyl functional group on an aromatic ring). They are ubiquitous in the plant species but their distribution at the plant tissue, cellular and subcellular levels is not uniform. Insoluble phenolics are linked to various cell components and contribute to the mechanical strength of cell walls and play a regulatory role in the plant growth and morphogenesis. Examples included lignin, which is the second most abundant compound in plants.

Soluble phenolics are present within the vacuoles and are essential to the plant's physiology being involved in diverse functions such as structure, pigmentation, pollination, pathogen, and herbivore resistance, as well as growth and development (Sengupta, Gaurav, & Tiwari, 2018). Major soluble phenolics are simple phenolics (*e.g*. coumarins), flavonoids (have two aromatic rings *e.g*. anthocyanins and flavones/flavonols) and tannins (condensed and hydrolysable).

In some plants, biosynthesis of these secondary plant metabolites, although largely constitutive, can be induced and enhanced by biotic and abiotic environmental stress factors such as herbivory or moisture stress (Mnisi & Mlambo, 2017). The concentration and biological activity of phenolic compounds in leaves are known to vary with factors such as tree species, growth environment and grazing/browsing pressure, among other factors (Estell, 2010; Harborne, 2001).

FLAVONOIDS

Flavonoids are extensively distributed in the plant kingdom, particularly in photosynthesizing plant cells; over 5000 different flavonoids have been isolated and identified from plant sources (Havsteen, 2002; Middleton, Kandaswami, & Theoharides, 2000). Flavonoids are synthesised from phenylalanine and are the most important plant pigments for flower coloration, producing yellow or red/blue pigmentation in petals designed to attract pollinator animals. They are primarily responsible for the many colours present in flowers, fruit, and leaves (Kawser et al., 2016). In higher plants, flavonoids are involved in UV filtration, symbiotic nitrogen fixation and floral pigmentation. They may also act as chemical messengers and physiological regulators. They regulate plant growth by inhibition of the exocytosis of the auxin indolyl acetic acid, as well as by induction of gene expression (Havsteen, 2002). Flavonoids are composed of a 15-carbon (C6–C3–C6) skeleton and two benzene rings (A- and B-rings) joined by a linked by a heterocycle, C ring (Parveen et al., 2010).

Flavonoids can be classified into six groups based on the substitution patterns of the ring C (Table 3.1). Flavonoids within the same group differ by the substitution of A and B (Middleton et al., 2000) (Figure 3.1). There are six major subgroups of flavonoids (Parveen et al., 2010).

Table 3.1: Classification of flavonoids based on specific structural characteristics and chemical properties

Group	Examples
Flavonols (3-hydroxyflavans)	Quercetin, Kaempferol, and Myricetin
Flavanones	Eriodictyol, Hesperetin, and Naringenin
Isoflavones	Daidzein, Genistein, and Glycitein
Flavones	Apigenin and Luteolin
Flavans-3-ol	Catechin, Epicatechin gallate, Epigallocatechin, Epigallocatechin gallate, Proanthocyanidins, Theaflavins, Thearubigins
Anthocyanidins	Cyanidin, Delphinidin, Malvidin, Pelargonidin, Peonidin, and Petunidin

Flavonols (3-hydroxyflavans) are widely distributed in the plant kingdom. Quercetin is reported to be the most widely distributed and has a wide range of biological activities in mammals (Crozier, Clifford, & Ashihara, 2008). Generally, the flavonols are found as *O*-glycosides mostly 3-glucosides, 3-galactosides, 3-rhamnosides, and 3-glucuronides (Ferreres, Llorach, & Gil-Izquierdo, 2004; Parveen et al., 2010). Flavones are

structurally related to flavonols, with the exception of the hydroxy group at position 3 of the C-ring (Crozier et al., 2008) (Figure 3.1). Apigenin and luteolin are the most common flavones in the plants, mostly angiosperms (Parveen et al., 2010). Flavanones are formed from flavones by reduction of the double bond in the central C-ring. They are highly reactive and readily undergo hydroxylation, glycosylation, and *O*-methylation reactions. Flavanones are less widely dispersed than flavones and flavonols (Parveen et al., 2010). Isoflavones are isomers of the flavones that are generally characterized by attachment of the B-ring at the C-3 position of the central heterocycle. The most common isoflavone compounds are daidzein, genistein, formononetin, and biochanin A (Parveen et al., 2010). They are reported to be weakly estrogenic and hence are termed phytoestrogens (Booth et al., 2006).

Flavan-3-ols (catechins) are possibly the most complex subclass of the flavonoids. Their structures range from the simple monomers (+)-catechin and its optical isomer (-)-epicatechin, to the oligomeric and polymeric proanthocyanidins, (condensed tannins) (Parveen et al., 2010). Anthocyanidins are highly abundant in the plant kingdom; in fruit and flower tissues, they contribute to red, blue, and purple colours (Crozier et al., 2008). They protect plants by absorbing light and are susceptible to oxidation because of their oxidizable hydroxy groups in ring B. Common examples of anthocyanidins are pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin, with cyanidin being the most widely distributed (Parveen et al., 2010). In plant tissues, these compounds occur as glycosides known as anthocyanins.

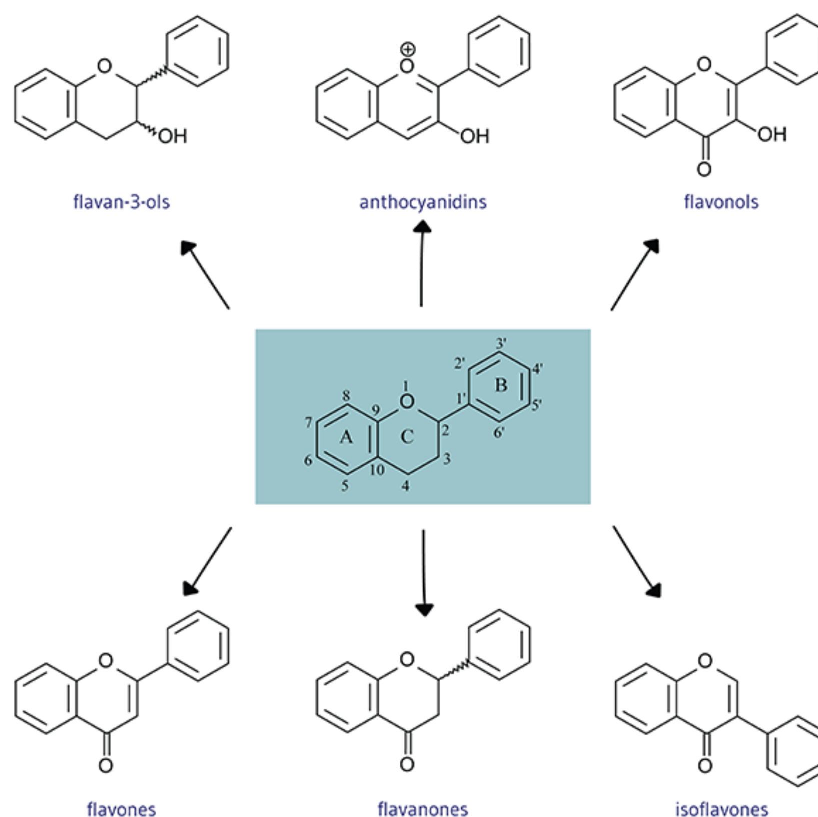


Figure 3.1: Classification of flavonoids and their chemical structures. Flavonoids are classified into six groups, including flavonols, flavanone, isoflavone, flavone, flavan-3-ols, and anthocyanin. From Kawser et al. (2016).

TANNINS

Tannins are a heterogeneous group of polyphenols widely distributed in plants with wide-ranging molecular weights (500–30000Da) (Falcão & Araújo, 2014). They are characteristically capable of precipitating proteins and other organic compounds including amino acids, alkaloids and polysaccharides (Schofield, Mbugua, & Pell, 2001). This precipitation exerts an inhibitory effect on many enzymes and may contribute a protective function in barks and heartwoods(Hart & Hillis, 1972). They also play an important role in the mechanism of hydrogen transfer in plant cells, probably due to their high affinity for oxygen (Roberts & Sarma, 1940). The term “tannin” refers to the use of oak and other bark in tanning animal hides into leather due to their precipitating effect (Falcão & Araújo, 2014). Tannins result from the condensation of gallic acid (with glucose) or flavan-3-ol (Pereira et al., 2009). Tannins are classified according to specific structural characteristics and chemical properties (Khanbabaee & van Ree, 2001)(Table 3.2,Figure 3.2).

Table 3.2: Classification of Tannins based on specific structural characteristics and chemical properties

<table>
<tr><td colspan="5">Classification of tannins</td></tr>
<tr><td colspan="4">True tannins (high molecular weight compounds)</td><td rowspan="4">Pseudotannins (low molecular weight compounds e.g. Gallic acid, Flava-3,4-diol)</td></tr>
<tr><td colspan="2">Hydrolysable tannins (Pyrogallol)</td><td rowspan="2">Condensed tannins (catechol)</td><td rowspan="2">Complex tannins</td></tr>
<tr><td>Gallitannins</td><td>Ellagitannins</td></tr>
</table>

True tannins are complex phenols of high molecular weight ranging from 1000 to 5000. They display the general properties of tannins and are precipitated by gelatine in 1% solution. True tannins are classified into hydrolysable tannins, condensed tannins and complex tannins (Mole & Waterman, 1987).

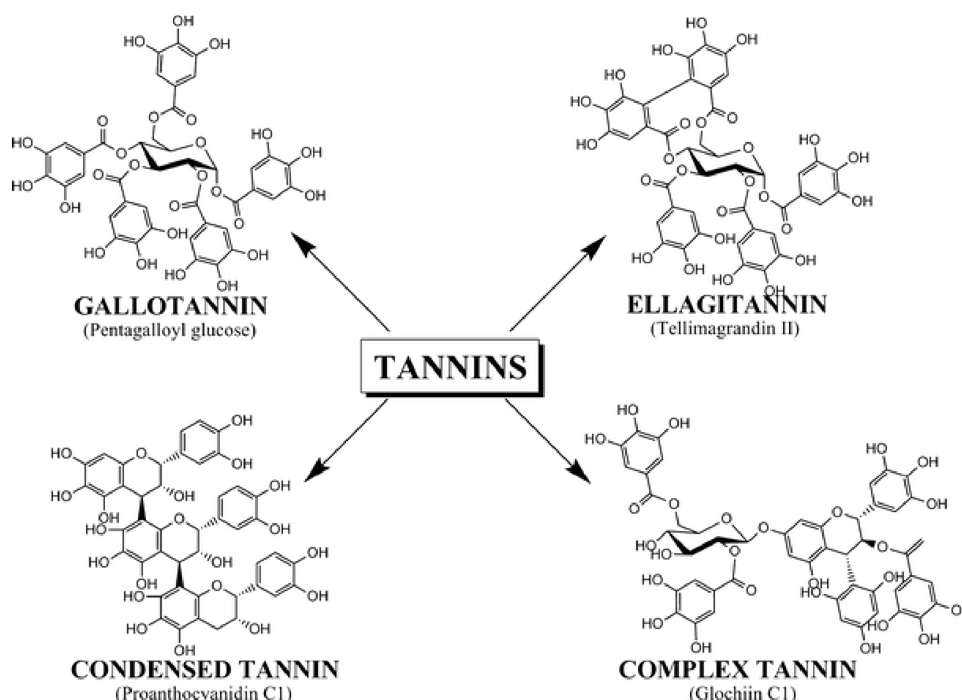
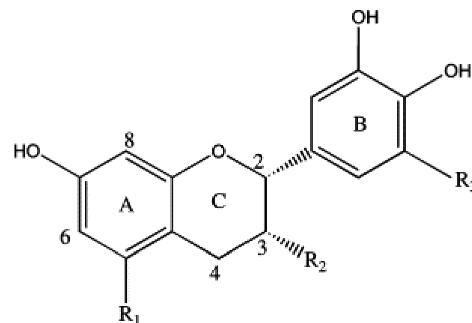


Figure 3.2: Classification of tannins. From Chávez-González et al. (2012)

Hydrolysable tannins can be hydrolysed by acids or enzymes (*e.g*. tannase). They are formed from several molecules of phenolic acids united by ester linkages to a central sugar (mainly glucose) molecule. The two principal types of hydrolysable tannins are gallitannins and ellatannins which are composed of gallic acid and hexahydroxydiphenic acid units respectively (Chávez-González et al., 2012; Naumann et al., 2017). Tannase is an enzyme that catalyses the reaction "digallate + $H_2O \leftrightharpoons 2$ gallate". This enzyme belongs to the family of hydrolases, specifically those acting on the carboxylic ester bonds. The systematic name of this enzyme is "tannin acyl hydrolase" (TAH) (Chávez-González et al., 2012). Tannase is present in a diverse group of microorganisms including rumen bacteria (Chávez-González et al., 2012).

Condensed tannins are also known as non-hydrolysable/catechol tannins/proanthocyanidins. Unlike hydrolysable tannins, these are not readily hydrolysable to simpler molecules and they do not contain a sugar moiety (Figure 3.3). They are related to flavonoid pigments and have polymeric flavan-3-ol structures (usually di- and trimers) (Figure 3.4). Complex tannins are tannins in which a catechin unit is bound glycosidically to a gallotannin or an ellagitannin unit (Khanbabaee & van Ree, 2001).



R_1	R_3	Class
OH	H	Proanthocyanidin
OH	OH	Prodelphinidin
H	H	Profisetinidin
H	OH	Prorobinetinidin

Figure 3.3: The basic repeating unit in condensed tannins. If, R1=R2=OH, R3=H, then the structure is that for (-)-epicatechin. The groups at R1 and R3 for other compounds are indicated below the structure. R2=O-galloyl in the catechin gallates. From Schofield et al. (2001).

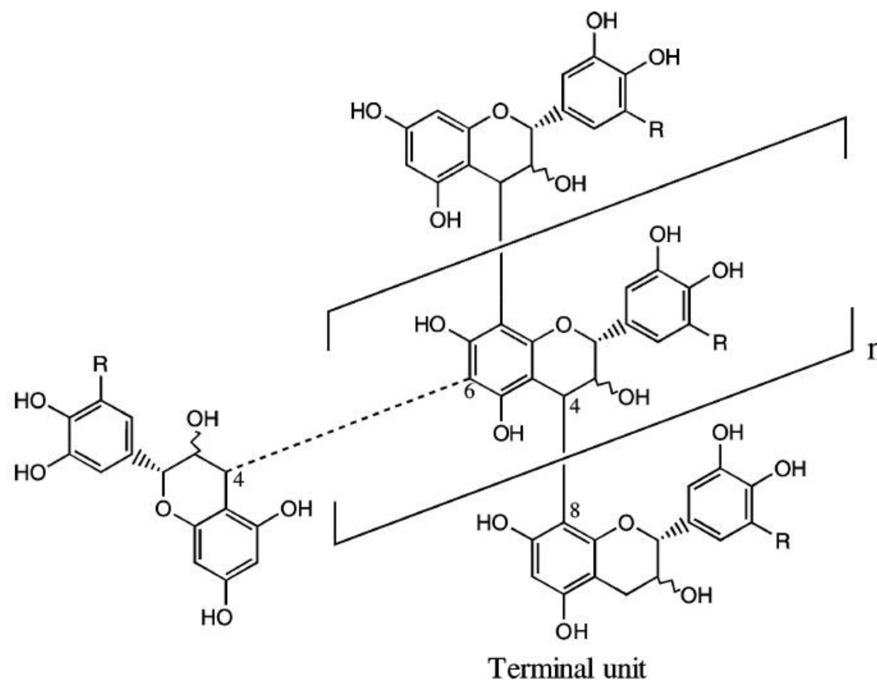


Figure 3.4: Model structure for a condensed tannin. If R=H or OH then the structure represents a procyanidin or prodelphinidin. The 4→6 linkage (dotted line) is an alternative interflavan bond. The terminal unit is at the bottom of such a multi-unit structure. From Schofield et al. (2001).

STRUCTURAL ANALYSIS OF FLAVONOIDS

Determination of the absolute structure of flavonoids is a complex task requiring the combination of advanced analytical techniques and large amounts of adequately purified sample (Fossen & Andersen, 2005). Modern mass spectrometry (MS) techniques combined with electrospray ionization (ESI) and high-performance liquid chromatography (HPLC) allows the direct analyses of the raw extracts and notably reduce the initial sample volume. In addition, they provide important information about the structure of the flavonoid aglycone, the type of sugar moieties and interglycosidic linkages (Ferreres et al., 2004). High performance liquid chromatography coupled with photodiode array detection and electrospray ionization–tandem mass spectroscopy (HPLC/PDA/ESI/MS/MS) analysis has been previously applied to identify and quantify phenolic compounds from plant crude extracts (Belanche, Jones, et al., 2016; Parveen et al., 2011; Parveen et al., 2014).

Mass Spectrometry has proved to be one of the most effective techniques particularly when the samples are complex biological matrices (Herebian et al., 2009). The main advantages of MS are its high sensitivity thus allowing analysis of compounds present in the µg scale and its high specificity enabling the separation of molecules of the same molecular weight but different atom composition, and sometimes even to differentiate stereoisomeric compounds. Additionally, it is easy to couple MS to separation techniques such as liquid and gas chromatography (Pinheiro & Justino, 2012).

Gas Chromatography (GC) is one of the key techniques for the separation of organics and, coupled to MS, one of the most common techniques of structural identification. However, flavonoids are largely non-volatile, and need be derivatized; also, they are usually thermally unstable (Pinheiro & Justino, 2012). This has resulted to the adoption of Liquid Chromatography (particularly High Performance - HPLC), as the fundamental separation technique for flavonoids (Vukics & Guttman, 2010). Consequently, LC-MS coupling is routinely used for the overall structure elucidation of flavonoids (Fossen & Andersen, 2005). Electrospray Ionization (ESI) has been used for the analysis of flavonoids (Belanche, Jones, et al., 2016; Lin et al., 2000; Parveen et al., 2011). The ions are generated by solvent evaporation under a high voltage potential, and can be applied directly, by infusion of the sample with a flow-controlled syringe,

or coupled to separation techniques such as LC or capillary electrophoresis (Pinheiro & Justino, 2012). In both cases, a steady liquid stream enters the system, allowing multiple analyses to be performed over a relatively large period. ESI interfaces are mostly coupled to quadrupole mass spectrometers; both are simple and robust equipment, able to produce either positive or negative ions (Pinheiro & Justino, 2012).

Tandem mass spectrometry (MS/MS, or MS^n for n^{th} order fragmentation) is a method that involves two or more stages of mass analysis. Mass analysis is the second step in mass spectrometry. Following ion generation, mass analysers measure the mass-to-charge ratio (*m/z*) of the ions, by using a combination of electromagnetic fields (Pinheiro & Justino, 2012). In a typical MS^2 experiment, a mass analyser is used to isolate a precursor ion, which is then fragmented to yield product ions (and, eventually, neutral fragments) that will be detected in the second mass analysis. This can conceptually be expanded with further successive modification and detection steps, giving rise to MS^3,… , MS^n. However, since only a small fraction of ions detected in one analyser makes it to the following analysers, MS^3 is usually the highest order achieved (Pinheiro & Justino, 2012). This spatial arrangement of equipment, analyser-modified-analyser, corresponds to tandem MS in space, where ions are treated in different regions of space.

Alternatively, tandem MS can be performed in time, with analysers such as ion traps, Orbitraps or Fourier-transform ion cyclotron resonance (FT-ICR), where the same analyser performs different tasks in succession (Pinheiro & Justino, 2012). In an Orbitrap mass analyser, ions are trapped in an electrostatic field between an inner and outer electrode (Makarov, 2000). As the ions rotate around the inner electrode, they precess along its axis with a frequency characteristic of their mass-to-charge *(m/z*) ratio. Acquisition of transients and the Fourier transformation of that signal yields frequencies and their intensities. A simple relationship converts frequencies into *m/z* values. The Linear Trap Quadrupole(LTQ)-Orbitrap hybrid mass spectrometer is a high mass accuracy, high resolution mass analyser, capable of achieving resolving power > 100,000 and having mass accuracy ≤ 5 ppm (Makarov et al., 2006).

FRAGMENTATION OF FLAVONOIDS

Theoretically, MS fragmentation varies with the instrument used. In practice however, main fragmentation paths of flavonoids are apparently independent of the applied ionization mode or the type of analyser (Vukics & Guttman, 2010). Fragments containing intact A- and B- rings of free aglycones are labelled ${}^{i,j}A_0$ and ${}^{i,j}B_0$, respectively, where superscripts *i* and *j* indicate the broken C-ring bonds. Glycoside fragments, with retained charges on the carbohydrate portion, are designated as A_i, B_i, and C_i, where *i* represents the number of broken bonds, counted from the terminal sugar unit. On the other hand, ions containing the aglycone are labelled as X_j, Y_j, and Z*j*, where *j* is the number of the cleaved interglycosidic bond, counted from the aglycone. The glycosidic link to the aglycone is numbered as 0.

In the case of *C,O*-glycosides *O*-glycosylated on the C-glycosyl moiety, the first glycosidic bond does not connect to the aglycone, therefore the numbering starts at the C-glycosyl moiety (Ferreres et al., 2007). In addition, for C,O-glycosides, the Y_0 or Z_0 ions represent fragments, resulting from the loss of the C-glycosyl moiety. The product ions generated through the cleavages of the sugar ring's C–C bonds are labelled with ${}^{k,l}A_i$ and ${}^{k,l}X_j$, where *k* and *l* indicate the broken sugar ring bonds (Figure 3.5) (Vukics & Guttman, 2010).

O-, C-, and *C,O*-glycosides can be distinguished by their positive or negative ionization spectra. For *O*-glycosides, the application of low or medium fragmentation energy results in heterolytic cleavage of their hemi-acetal O–C bonds, yielding distinctive Y_i fragments (Cuyckens & Claeys, 2004; Ferreres et al., 2004). For *C*-glycosides, low fragmentation energy does not provide adequate fragmentation. At medium fragmentation energy, however, characteristic ${}^{i,j}X$ and E_i fragments result from intraglycosidic cleavages and from losses of water molecules, respectively (Vukics & Guttman, 2010). On the other hand, the application of higher fragmentation energy leads to intraglycosidic cleavages of the sugar units of O-glycosides and produces Y_i fragments for C-glycosides rendering spectral interpretation difficult, sometimes misleading (Vukics & Guttman, 2010).

Generally, by LC–MS^n analyses no information can be obtained about the stereochemistry of the glycan part of the flavonoid glycosides. The sugar type can be, however, easily determined, since the A_i, B_i, and C_i fragments appear at different *m/z*

values in the case of hexoses, deoxyhexoses, and pentoses. Although these ions are usually not present in the actual spectra, their masses can be indirectly deduced from the difference of the mass of the parent ions and the masses of the corresponding X_i, Y_i, and Z_i fragments, respectively (Ferreres et al., 2004; Vukics & Guttman, 2010).

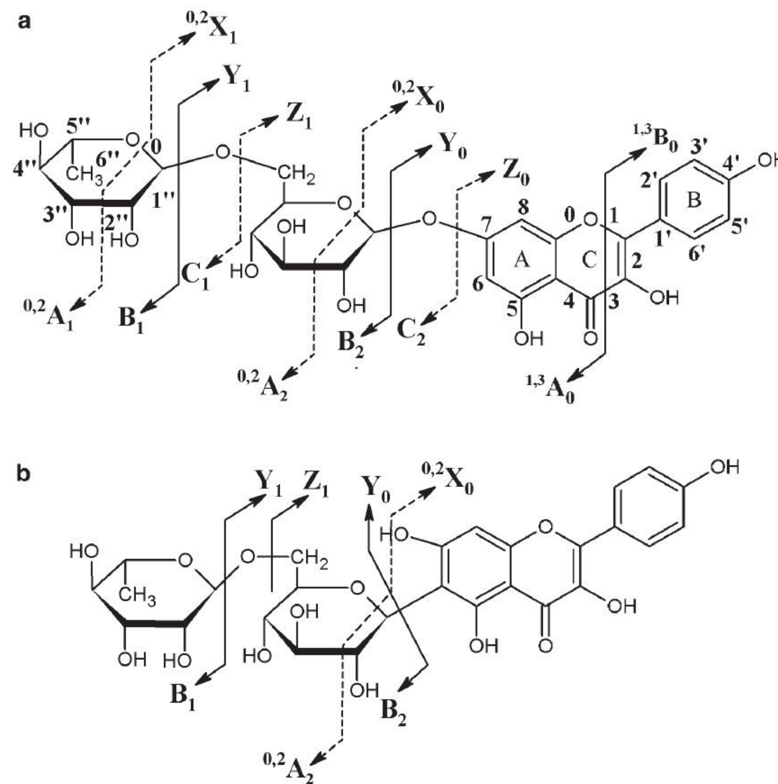


Figure 3.5: Fragment nomenclature commonly applied for (a) *O*-glycosides and (b) *C,O*- and *C*-glycosides. From Vukics and Guttman (2010).

3.2 AIMS AND OBJECTIVES

The aim of this study was to investigate the chemical composition of leaves of seven species of tree fodders commonly used in Nigeria and any significant difference between specimens harvested in the different ecological zones. Forages were assessed for standard measures of feed quality and digestibility but also for more detailed evaluation of phenolic content. By relating the chemical composition to measurements of fermentation (using the gas production kinetics methodology), it would be possible to identify factors in the fodders that made them suitable as feeds. Characterisation of the rumen bacterial community would indicate if changes in fermentation were due to alterations in the community structure.

3.3 EXPERIMENTAL DESIGN

The species investigated were *Acacia nilotica (AN), Azadirachta indica (AI), Guiera senegalensis (GS), Lannea acida (LA), Parkia biglobosa (PB), Piliostigma reticulatum (PR)* and *Ziziphus mauritiana (ZM).* The samples in this study were collected in Katsina state, located in north-western part of Nigeria. The state is divided into three agroecological zones – Northern Guinea savannah, Sudan savannah and Sahel savannah described by Adenkule et al. (2005). Sample collection and preparation was done by Muhammad Lawal (Federal College of Education, Katsina, Nigeria) as part of his MSc studies at Aberystwyth University.

The experiment and statistical analysis were carried out in a 7 x 3 factorial design with the seven fodder tree species and three ecological zones being the factors. Chemical composition (section 2.1.2 and 2.1.3), *in vitro* degradation (section 2.1.4 and 2.1.5) and bacterial composition (section 2.1.6) were determined as described in Chapter 2.

Statistical analyses were done on Genstat 19th edition (VSN International Ltd., Hemel Hempstead, UK). Analysis of chemical composition and *in vitro* degradation measurements was done using two-way ANOVA, with chemical composition as a dependent variable and tree species and ecological zone as the fixed factors. Multiple comparisons of the means (Fisher's protected LSD, p=0.05) was done as the *post hoc* test.

Relative bacterial taxa abundance data were subjected to square root transformation. Statistical analysis was carried out using unbalanced ANOVA to account for missing samples. Principal coordinate analysis (PCO) ordination on a Bray-Curtis distance matrix was done to identify patterns in the data. Permutation multivariate analysis of variance (PERMANOVA) was used to determine overall significant differences in community data. PERMANOVA was carried out using default settings with unrestricted permutations. All tests were carried out using the PAST v3 statistical package (Hammer et al., 2001).

3.4 RESULTS

3.4.1 CHEMICAL COMPOSITION OF LEAVES OF FODDER TREES

There was both a species effect and an ecological effect on the content of OM (and ash). The highest ash content was found in *A. indica* at 10% and the lowest in *G. senegalensis* at 3.7% (Table 3.4). Crude protein (CP) analysis showed both a species effect and an ecological zone effect. The CP content was highly variable in *A. indica* between the ecological zones ranging between 14% in Sudan savannah and 22% in the Sahel savannah. The differences were smaller in the other species. There was no significant difference in CP content found in *G. senegalensis* between the three zones. Analysis of crude fat content of the samples revealed levels ranging from 1.3% to 3.9%. There were statistically significant differences between species in the same region and within the same species in different regions (Table 3.3).

Table 3.3: Crude protein and Crude fat percentage of leaves of *A. nilotica, A. indica, G. senegalensis, L. acida, P. biglobosa, P. reticulatum,* and *Z. mauritiana* harvested from Sahel savannah, Sudan savannah and Northern Guinea savannah ecological zones of Nigeria.

	Crude protein %				Crude fat %			
	Zone							
	Sahel	Sudan	Guinea	Mean	Sahel	Sudan	Guinea	Mean
A. nilotica	11.06^{a1}	15.09^{a2}	15.13^{a2}	13.76^{c}	3.04^{a1}	2.01^{a2}	3.56^{a3}	2.87^{b}
A. indica	15.33^{b1}	14.08^{b2}	22.23^{b3}	17.22^{e}	2.79^{b1}	2.63^{b2}	2.53^{b3}	2.65^{ab}
G. senegalensis	11.13^{a}	11.97^{c}	11.61^{c}	11.57^{a}	1.30^{c1}	2.39^{c2}	2.47^{c3}	2.05^{ab}
L. acida	11.62^{a1}	13.86^{b2}	8.94^{d3}	11.47^{a}	3.03^{d1}	3.49^{d2}	2.15^{d3}	2.89^{bc}
P. biglobosa	14.58^{b1}	13.98^{b1}	13.09^{e2}	13.88^{c}	3.74^{e1}	3.92^{e2}	3.82^{e3}	3.83^{c}
P. reticulatum	11.28^{a1}	14.30^{bd2}	12.77^{e3}	12.78^{b}	1.48^{f1}	1.77^{f2}	1.86^{f3}	1.70^{a}
Z. mauritiana	17.70^{c1}	13.74^{be2}	17.72^{f1}	16.38^{d}	1.83^{g1}	2.14^{g2}	1.30^{g3}	1.76^{a}
s.e.d	Species			0.1641				0.00
	Zone			0.1075				0.00
	Species x Zone			0.2843				0.00
p value	Species			<0.001				<0.001
	Zone			<0.001				<0.001
	Species x Zone			<0.001				<0.001

Superscripts a, b, c, d, e, f and g depict significant differences in the columns and 1, 2 and 3 depict differences within a row ($P < 0.05$). Entries with at least one shared letter superscript are not significantly different.

Table 3.4: Organic matter and Neutral detergent fibre (NDF) percentages of leaves of *A. nilotica, A. indica, G. senegalensis, L. acida, P. biglobosa, P. reticulatum,* and *Z. mauritiana* harvested from Sahel savannah, Sudan savannah and Northern Guinea savannah ecological zones of Nigeria.

	Organic matter %				Neutral detergent fibre %			
	Zone							
	Sahel	Sudan	Guinea	Mean	Sahel	Sudan	Guinea	Mean
A. nilotica	96.67^{a1}	94.70^{a2}	95.73^{a3}	95.7^{a}	76.18^{a}	73.09^{a}	73.48^{a}	74.26^{f}
A. indica	89.4^{b1}	91.53^{b2}	89.66^{b1}	90.19^{a}	60.84^{b}	63.78^{b}	63.53^{b}	62.71^{e}
G. senegalensis	96.28^{a1}	96.45^{c1}	95.29^{c2}	96.0^{e}	33.89^{c}	30.36^{c}	32.17^{c}	32.16^{a}
L. acida	92.88^{c1}	94.05^{d2}	90.68^{d3}	92.54^{b}	54.59^{d1}	44.20^{d2}	57.42^{d1}	52.08^{d}
P. biglobosa	92.0^{d1}	92.99^{e2}	94.19^{e3}	93.07^{c}	36.93^{ce}	38.71^{e}	35.87^{e}	37.18^{b}
P. reticulatum	96.20^{a1}	93.84^{d2}	95.41^{ac3}	95.15^{d}	30.45^{cg1}	42.02^{df2}	37.98^{e2}	36.85^{b}
Z. mauritiana	92.79^{c1}	92.28^{f1}	94.80^{f2}	93.29^{c}	43.19^{f}	46.02^{dg}	51.31^{f}	46.85^{c}
s.e.d	Species			0.101				0.957
	Zone			0.066				0.626
	Species x Zone			0.176				1.657
p value	Species			<0.001				<0.001
	Zone			0.553				0.003
	Species x Zone			<0.001				<0.001

Superscripts a, b, c, d, e, f and g depict significant differences in the columns and 1, 2 and 3 depict differences within a row (P < 0.05). Entries with at least one shared letter superscript are not significantly different.

Generally, there was a statistically significant difference in NDF, ADF and ADL between species in the same ecological zone and no difference was detected in samples of the same species in different ecological zones (Table 3.4, Table 3.5). Thus, there was no ecological zone effect on the fibre content of the sample. The samples generally showed high levels of lignin (2.29—23.21) relative to levels found in commonly used bulk feeds; this was particularly pronounced in *G. senegalensis* (Table 3.5).

Table 3.5: Acid detergent fibre (ADF) and Acid detergent lignin (ADL) percentages of leaves of *A. nilotica, A. indica, G. senegalensis, L. acida, P. biglobosa, P. reticulatum,* and *Z. mauritiana* harvested from Sahel savannah, Sudan savannah and Northern Guinea savannah ecological zones of Nigeria.

	Acid detergent fibre %				Acid detergent lignin %			
	Zone							
	Sahel	Sudan	Guinea	Mean	Sahel	Sudan	Guinea	Mean
A. nilotica	10.86^{a}	13.34^{a}	12.94^{a}	12.37a	6.43^{a}	6.97^{a}	6.07^{a}	6.48^{a}
A. indica	14.60^{a}	12.82^{a}	15.98^{ab}	14.49^{ab}	14.55^{b}	12.43^{b}	14.24^{a}	13.73^{b}
G. senegalensis	16.43^{ac}	21.68^{bc}	15.41^{ab}	17.81^{bc}	23.21^{c}	18.57^{b}	23.07^{b}	21.63^{c}
L. acida	13.77^{a}	17.71^{ac}	15.15^{ab}	15.55^{ab}	10.98^{ab}	13.70^{b}	9.140^{a}	11.27^{ab}
P. biglobosa	22.56^{b}	19.10^{ac}	18.97^{bc}	20.23^{c}	10.01^{ab}	14.50^{b}	2.63^{a}	9.1^{ab}
P. reticulatum	20.95^{c}	18.90^{a}	22.38^{cd}	20.72^{c}	2.92^{a}	17.50^{b}	15.66^{a}	11.92^{b}
Z. mauritiana	34.50^{d}	30.68^{d}	26.32^{d}	30.5^{d}	11.15^{abd}	10.75^{a}	11.09^{a}	10.99^{ab}
s.e.d	Species			1.17				1.662
	Zone			0.766				1.088
	Species x Zone			2.027				2.879
p value	Species			<0.001				<0.001
	Zone			0.369				0.131
	Species x Zone			0.008				0.003

Superscripts a, b, c, d, e, f and g depict significant differences in the columns and 1, 2 and 3 depict differences within a row (P < 0.05). Entries with at least one shared letter superscript are not significantly different.

3.4.2 PRESENCE OF PHENOLIC COMPOUNDS IN FODDER TREE LEAVES

Tannins were quantified using the response factor of 5-caffeoylquinic acid (8.74×10^{-7}μg/area unit) and the flavonoids with the response factor for luteolin (5.32×10^{-7}μg/area unit) and expressed per unit of dry matter (Belanche, Jones, et al., 2016; Parveen et al., 2014). There was a significant effect of both the tree species and the ecological zone within species. The highest levels of phenols (9.4%) were recorded in *A. nilotica* samples from N. Guinea savannah. All *A. nilotica* samples showed total phenol levels higher than 5%. Other tree species showed much lower levels of total phenols (Table 3.6).

Table 3.6: Total phenolics (mg/g DM) recovered from extracts of leaves of seven tropical fodder trees collected in three different ecological zones of Nigeria. The abundances were determined by measurement of peak area in the HPLC chromatogram. Tannins were quantified using with the response factor of 5-caffeoylquinic acid (8.74×10^{-7}μg/area unit); flavonoids were quantified with the response factor of luteolin (5.32×10^{-7}μg/area unit) and expressed per unit of dry matter.

	Sahel	Sudan	N. Guinea	Mean
A. nilotica	79.53	55.54	94.17	76.41^{c}
A. indica	7.34	4.88	10.25	7.49^{a}
G. senegalensis	7.72	12.57	11.97	10.75^{ab}
L. acida	6.80	13.76	17.90	12.82^{ab}
P. biglobosa	5.43	6.76	10.80	7.67^{a}
P. reticulatum	4.53	10.47	11.49	8.83^{ab}
Z. mauritiana	12.24	14.07	14.48	13.6^{b}
s.e.d.	Species			187.4
	Zone			122.7
	Species x Zone			324.6
p	Species			<0.001
	Zone			<0.001
	Species x Zone			<0.001

The results of the HPLC/PDA/ESI/MS/MS analysis of phenolic compounds are summarised in Table 3.7. Eighteen compounds were tentatively identified including flavonols, flavan-3-ols and tannins. Compounds identification was based on the MS/MS fragmentation and literature comparison.

Compound 1 was detected in the *A. nilotica* and *A. indica* extracts. It had a molecular ion *m/z* 441 $[M - H]^-$. In addition molecules of *m/z* 883 $[2M - H]^-$, 1324 $[3M - H]^-$, and 1768 $[4M - H]^-$ were detected representing dimers, tetramers and quatrimers. Fragmentation in negative ion mode ESI-MS/MS yielded MS^2 base ion *m/z* 289 $[(M - H) - 152]^-$. The loss of 152amu suggests a gallic acid *via* by Retro-Diels-Alder (RDA) fragmentation of the C-ring of a monomeric subunit (Saldanha, Vilegas, & Dokkedal, 2013). The molecule was therefore tentatively identified as epicatechin gallate based

on fragmentation characteristics and supported by literature (Sun et al., 2007; Wang et al., 2008).

Compound 2 was detected in extracts of *A. nilotica*. In negative mode, the compound's molecular ion had with *m/z* 593 $[M - H]^-$. Additional molecules were also detected including *m/z* 296 $[M – H, + 297]^-$, 890 $[(M – H) + 297]^-$, 1188 $[2M - H]^-$, 1485 $[(2M – H) + 297]^-$, 1781$[3M - H]^-$ (Figure 3.6). Fragmentation of the molecular ion 593 $[M - H]^-$ in negative ion mode ESI-MS/MS yielded MS^2 base ion *m/z* 441 $[(M – H) - 152]^-$ suggesting the loss of gallic acid and corresponding to epicatechin gallate (Figure 3.7). The molecule was tentatively identified as epicatechin-3,5-digallate (Wang et al., 2008).

Table 3.7: Phenolic compounds in extracts of leaves of seven *A. nilotica, A. indica, G. senegalensis, L. acida, P. biglobosa, P. reticulatum*, and *Z. mauritiana*. Extracts were analysed using HPLC-ESI-MS^n. Compounds were tentatively identified based on the characteristic MS^2 fragmentation patterns consistent with referenced literature and the Riken tandem mass spectral database (ReSpect).

Peak (Cpd) No.	Plant	HPLC t_r (min)	λ_{max}(nm)	*m/z*	MS^2 Fragmentation (relative density %)	Tentative identification	References
1	A. nilotica A. indica	11.4	283	441 $[M - H]^-$ 883 $[2M - H]^-$ 1324 $[3M - H]^-$ 1768 $[4M - H]^-$	**289** $[(M - H) - 152]^-$	Epicatechin 5-gallate	Sun et al. (2007) Wang et al. (2008)
2	A. nilotica	13.2	284	593 $[M - H]^-$	**441** $[(M - H) - 152]^-$	Epicatechin - 3,5-gallate	Wang et al. (2008)
3	A. nilotica	8.7	271	197 $[M - H]^-$	**169** $[(M - H]) - 28]^-$ 125 $[(M – H) – 28 – 44]^-$	Ethyl gallate	Sadiq et al. (2015) Wyrepkowski et al. (2014)
4	A. indica P. reticulatum	13.2	260, 293, 352	463 $[M - H]^-$	**301** $[(M - H) - 162]^-$	Quercetin 3-O-glucoside	Ablajan and Tuoheti (2013)
5	A. indica P. reticulatum	14.3	260, 306, 348	447 $[M - H]^-$	**301** $[(M - H) - 146]^-$	Quercetin-3-O-rhamnoside	Ablajan and Tuoheti (2013) Zhou et al. (2014)
6	A. indica	14.8	286, 341	593 $[M - H]^-$	**285** $[(M - H) - 308]^-$	Kaempferol-3-O-rutinoside	Kumar, Singh, and Kumar (2017) Wang et al. (2008)
7	A. indica	13.3	263, 290, 354	609 $[M - H]^-$	**301** $[(M - H) - 308]^-$	Quercetin-3-O-rutinoside	Ablajan and Tuoheti (2013) Cuyckens and Claeys (2004) Zhou et al. (2014)
8	G. senegalensis L. acida P. biglobosa	12.5	262, 350	463 $[M - H]^-$	**317** $[(M - H) - 146]$	Myricetin-3-O-rhamnoside	Barbosa et al. (2006)
9	L. acida	21.5	258, 272, 348	313 $[M - H]^-$	**298** $[(M - H) - CH_3]^-$	Methylated flavonoid	Justesen (2001)
10	L. acida	21.9	260, 356	343 $[M - H]^-$	**327** $[(M - H) - CH_3]^-$	Trimethylated flavonoid	Justesen (2001)
11	L. acida	11.5	267, 303, 355	493 $[M - H]^-$	**317** $[(M - H) - 176]$	Myricetin 3-O-glucuronide (Miricitrin)	Barbosa et al. (2006) Monagas et al. (2005)
12	P. biglobosa	12.9	273, 399	577 $[M - M]^-$	**433** $[(M + H) - 146]^+$ 415 $[(M + H) - H_2O]^+$	Quercetin-3-pentose-rhamnoside	Ferreres et al. (2004)

					413 [(M - H) - 164]$^-$ 293 [(M - H) - 164 - 293]$^-$		
13	P. biglobosa	16.1	274, 339	559 [M - H]$^-$	**415** [(M + H) - 146]$^+$ 271 [(M + H) - 146 - 144]$^+$ 395 [(M - H) - 164]$^-$ 269 [(M - H) - 164 -126]$^-$	Apigenin-7-O-dirhamnoside	Ferreres et al. (2007) Pereira et al. (2012)
14	Z. mauritiana	14.4	261, 348	579 [M - H]$^-$	**449** [(M + H) - 132]$^+$ 303 [(M + H) - 132 - 146]$^+$	Quercetin-3-O-rhamnose-pentoside	Martucci et al. (2014)
15	Z. mauritiana	14.9	269, 340	727 [M + H]$^+$ 449 [M + H]$^+$ 303 [M + H]$^+$ 725 [M - H]$^-$ 770 [(M - H) + 45]$^-$	**595** [(M + H) - 132]$^+$ 449 [(M + H) -132 - 146]$^+$ 303 [(M + H) - 132 - 146 - 146]$^+$ 579 [(M - H)] - 146]$^-$	Quercetin-3-O-rhamnose-O-pentoside, 7-rhamnoside	Martucci et al. (2014)

Base peak (100%) shown in bold

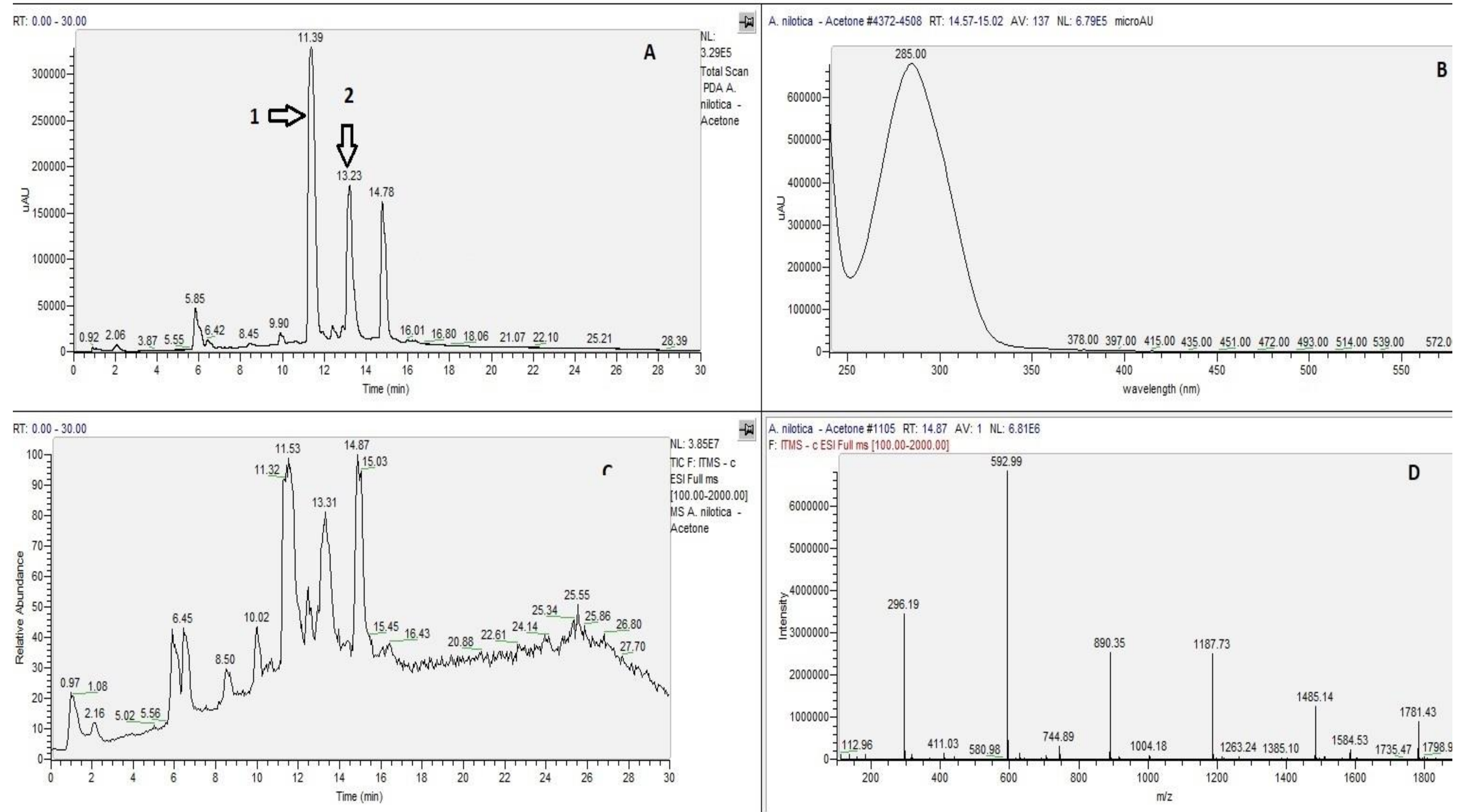


Figure 3.6: **A.** HPLC chromatogram of *A. nilotica* acetone extract detected by photodiode array and wavelength set at 240–400nm. **B**. UV absorption spectrum of peak 2. **C.** Negative PDA/ESI/MS chromatogram of *A. nilotica* acetone extract. **D**. Mass spectra in negative mode of peak 2

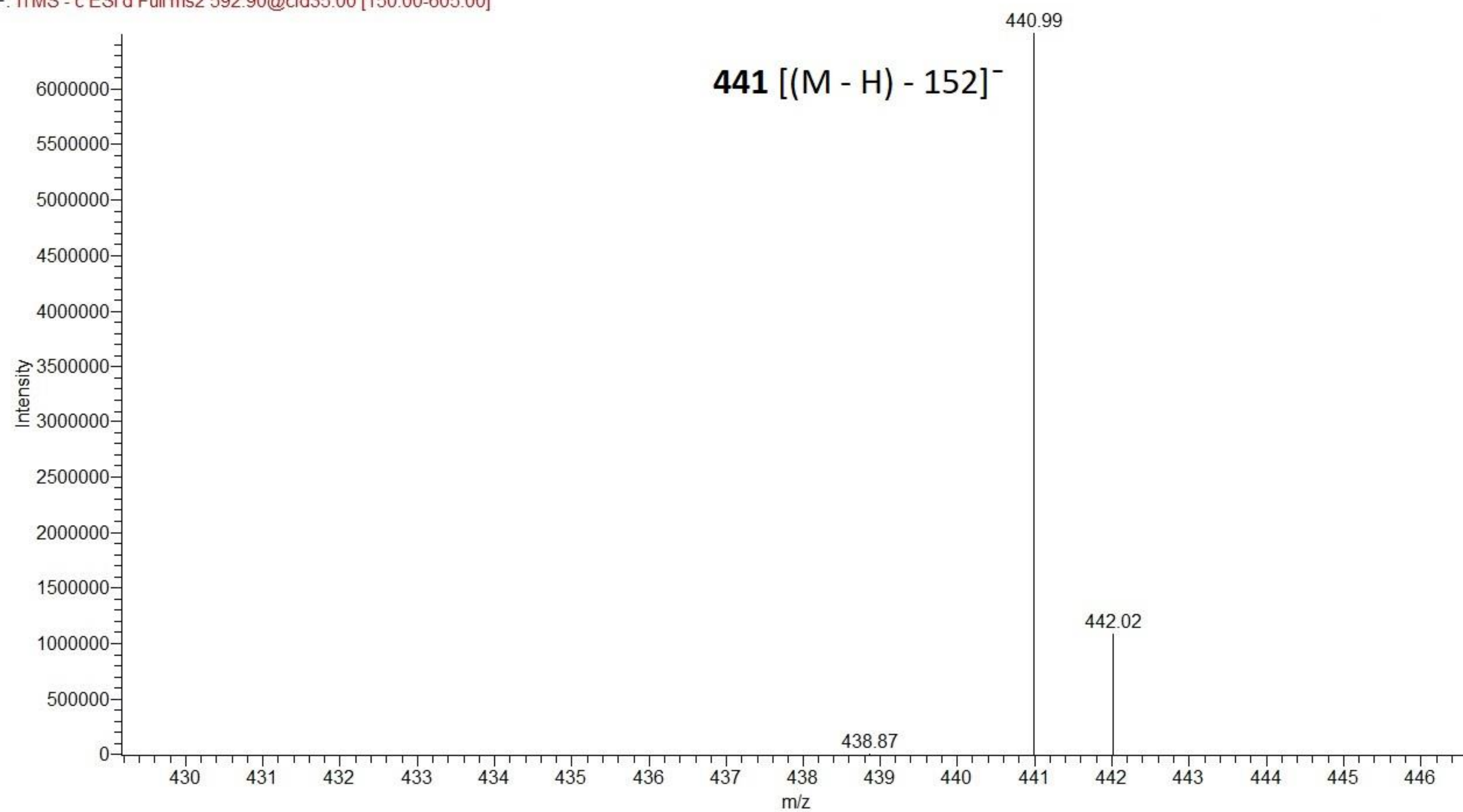


Figure 3.7: MS/MS spectrum of peak 2 in negative mode

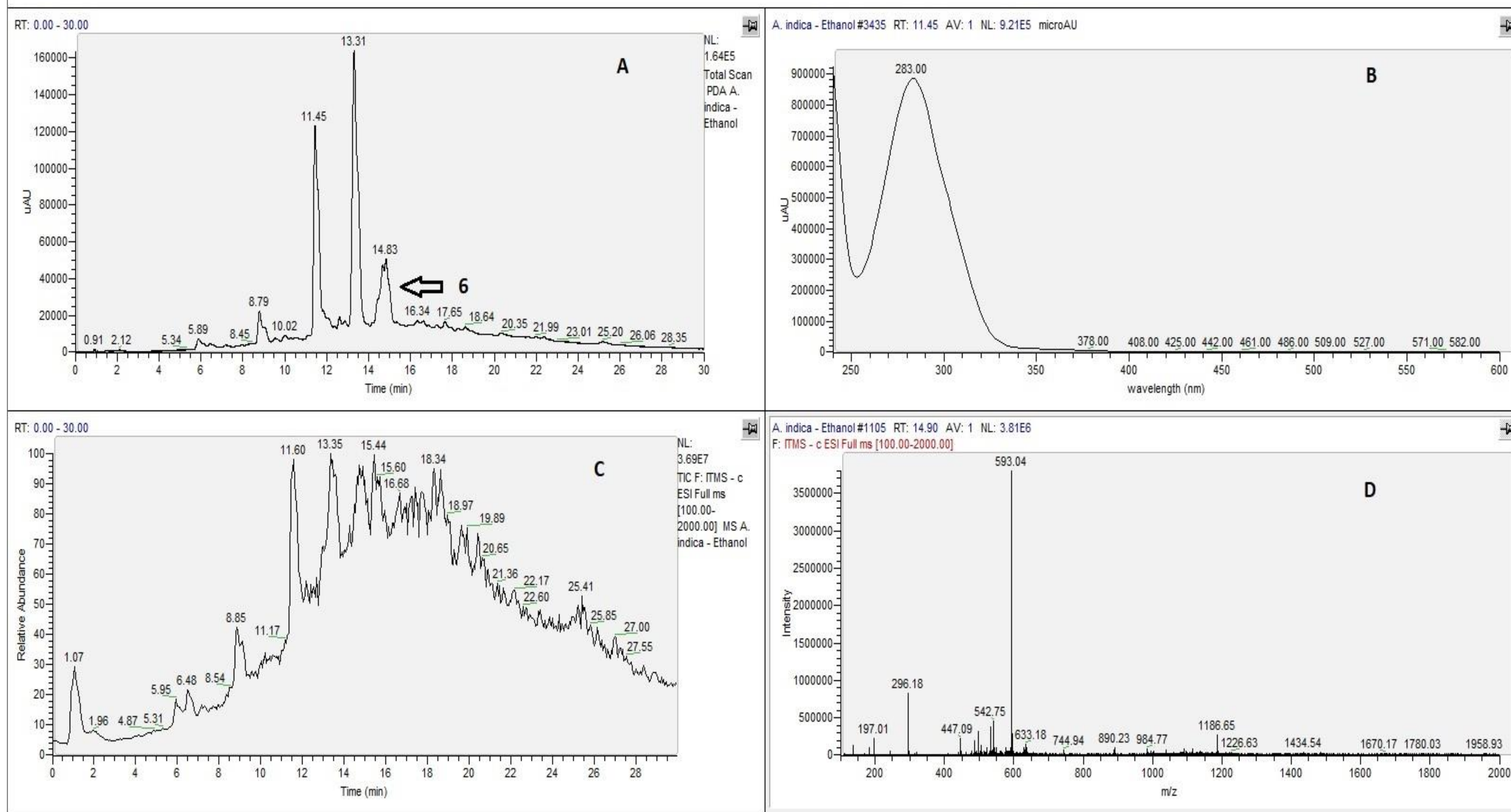


Figure 3.8: **A.** HPLC chromatogram of *A. indica* ethanol extract detected by photodiode array and wavelength set at 240–400nm. **B**. UV absorption spectrum of peak 6. **C.** Negative PDA/ESI/MS chromatogram of *A. indica* ethanol extract. **D**. Mass spectra in negative mode of peak 6

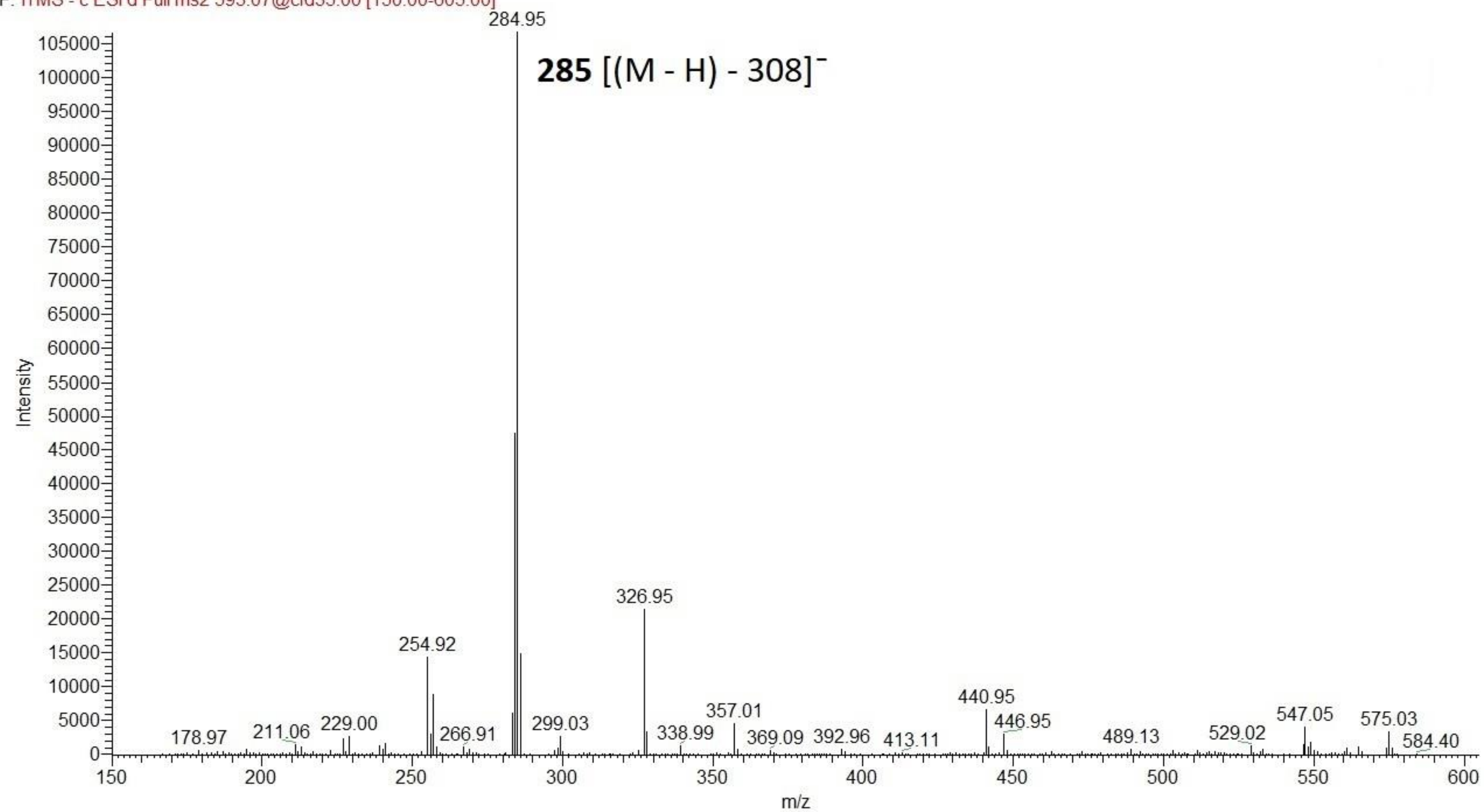


Figure 3.9: MS/MS spectrum of peak 6 in negative mode

Compound 3 was also detected in *A. nilotica* extracts. It had a with *m/z* 197 $[M - H]^-$ .MS^2 fragmentation yielded ions of *m/z* 169 $[(M – H) - 28]^-$ and *m/z* 125 $[(M – H) – 28 – 44]^-$. A similar fragmentation pattern has been described Wyrepkowski et al. (2014) who attributed the loss 28amu related to the loss of $CH_2=CH_2$. The m/z 125 fragment is typical for gallic acid and loss of 44amu has been attributed to the loss of the carboxylic function by Fischer et al., (2011) who described a similar fragmentation pattern. The molecule was therefore tentatively identified as ethyl gallate and has been previously detected in the leaves of *A. nilotica* (Sadiq et al., 2015). A dimer of *m/z* 395 $[2M - H]^-$ was also detected.

Compound 4 was detected in extracts of *A. indica* and *P. reticulatum*. It gave a molecular ion with *m/z* 463 $[M - H]^-$. Fragmentation of the molecular ion in negative ion mode ESI-MS/MS yielded MS^2 base ion *m/z* 301$[(M - H) - 162]^-$ assigned as quercetin (Vukics & Guttman, 2010). The loss of 162amu was attributed to the cleavage of a deoxyhexose moiety; the distinctive Y_i fragment suggests *O*-glycosylation (Vukics & Guttman, 2010). The compound was tentatively identified as quercetin 3-*O*-glucoside as reported by Ablajan and Tuoheti (2013).

Negative MS^2 fragmentation of **compound 5** with m*/z* 447 $[M - H]^-$ yielded a similar aglycone skeleton *m/z* 301 $[(M – H) – 146]^-$. The calculated loss was 146amu was consistent with the loss of a deoxyhexose unit(Vukics & Guttman, 2010). The molecule was tentatively identified as quercetin-3-*O*- rhamnoside. Similar fragmentation has also been described in literature (Ablajan & Tuoheti, 2013; Zhou et al., 2014). A dimer *m/z* 894 $[2M - H]^-$ and a trimer *m/z* 1341 $[3M - H]^-$ were also detected.

Compound 6 was detected at retention times 14.8min in the *A. indica* ethanolic extract ion and showed a molecular ion at *m/z* 593 $[M - H]^-$ (Figure 3.8). A dimer *m/z* 1186 $[2M - H]^-$ and a trimer 1780 $[3M - H]^-$ were also detected. Fragmentation of the molecule in negative ion mode ESI-MS/MS yielded MS^2 base ion *m/z* 285 $[(M - H) - 308]^-$ (Figure 3.9). The loss of 308amu suggesting the loss of rutinose (glucose(162) + rhamnose(146)) resulting in an aglycone skeleton identified as kaempferol (Vukics & Guttman, 2010). The molecule was tentatively identified as kaempferol-3-O-rutinoside (Kumar et al., 2017; Wang et al., 2008). This pattern of fragmentation has also been described in tea extracts by Del Rio et al. (2004). The presence of the flavonol has been previously reported in *A. indica* extracts (Chattopadhyay & Bandyopadhyay, 2005a).

Compound 7 was detected in *A. indica*. Mass spectrometry yielded a molecule at m*/z* 610 $[M - H]^-$. In the MS/MS spectra, after a 308amu loss (one rhamnose unit plus one glucose unit), the resultant aglycone skeleton (assigned as quercetin) (Vukics & Guttman, 2010) was observed *m/z* 302 $[(M – H) – (162 + 146)]^-$. Since the two sugars were lost together, it could be deduced that the sugar units were linked together and the sugar chain was located at the C3 position (Ferreres et al., 2004). MS/MS of the molecule also showed a minor ion at m/z465 $[(M - H) - 146]^-$ corresponding to a 146Da loss indicating a rhamnose residue at the terminal side of the sugar chain. Therefore, the compound was tentatively identified as quercetin-3-O-rutinoside (rutin) (Ablajan & Tuoheti, 2013; Cuyckens & Claeys, 2004; Zhou et al., 2014). A dimer of the molecule *m/z* 1219 $[2M - H]^-$ and a trimer *m/z* 1829 $[3M - H]^-$ were also detected. Quercetin-3-O-rutinoside has been previously reported in *A. indica* extracts by Chattopadhyay and Bandyopadhyay (2005b).

G. senegalensis, *L. acida* and *P. biglobosa* extracts all showed **compound 8.** It gave a molecular ion at *m/z* 463 $[M - H]^-$. Fragmentation of the molecule in negative ion mode yielded MS^2 base ion *m/z* 317 $[(M – H) - 146]^-$ assigned to the aglycone myricetin (Ferreres et al., 2004; Zhou et al., 2014). The molecule was tentatively identified as myricetin-3-O-rhamnoside from literature review and the MS^2 fragmentation pattern (Barbosa et al., 2006; Sawada et al., 2012). A dimer *m/z* 926 $[2M – H]^-$ and a trimer *m/z* 1390 $[3M - H]^-$ were also detected. The compound has been previously reported in *G. senegalensis* (Dirar et al., 2019).

Compounds 9, 10 and 11 were all detected in *L. acida* extracts. **Compound 9** gave a molecule at *m/z* 313 $[M - H]^-$. Fragmentation of the molecule in negative ion mode ESI-MS/MS yielded MS^2 base ion of *m/z* 298 $[(M - H) - CH3]^-$ after a calculated loss of 15amu.

Compound 10 was another molecule with a base peak of *m/z* 343 $[M - H]^-$ and a minor peak of *m/z* 313. This suggest that these two molecules have a related structure. Fragmentation of the latter molecule yielded product ions of *m/z* 327 $[(M - H) - CH_3]^-$ after a loss of 15amu. These two molecules were identified as methylated flavonoids. Methylated flavonoids are characterized by the loss of 15amu, resulting in a $[(M - H) - CH_3]^-$ (Justesen, 2001). It is possible to distinguish between methoxylated flavonoids with identical molecular mass, *e.g.* when screening plant extracts for flavonoid

composition. However, further (MS^3) fragmentation and comparison with standard compounds is necessary for the identification of unknown flavonoid aglycones (Justesen, 2001).

Fragmentation of **compound 11** precursor ion with *m/z* 493$[M - H]^-$ in negative MS^2 mode showed product ions at *m/z* 317 [(M - H) - 176] (assigned to the aglycone myricetin) and a calculated loss of 176amu consistent with cleavage of a glucuronide moiety (Kachlicki et al., 2016; Keski-Hynnilä et al., 1999). The molecule was therefore identified as myricetin-3-O-glucuronide (miricitrin) (Barbosa et al., 2006; Monagas et al., 2005). A dimer *m/z* 987 $[2M - H]^-$, trimer *m/z* 1481 $[3M - H]^-$ and quatrimer *m/z* 1976 $[3M - H]^-$ were also detected.

Compound 12 was detected in *P. biglobosa* a precursor ion at *m/z* 579 $[M + H]^+$ was detected at RT12.94 in ethanol and water and 12.97 in the acetone extracts. Fragmentation in positive mode the peaks showed product ions at *m/z* 433 $[(M + H) - 146]^+$ after a calculated loss of 146amu (rhamnose) (Ferreres et al., 2004). A minor peak of *m/z* 415 $[(M + H) - H_2O]^+$ indicates the loss of a water molecule (18amu). After negative fragmentation, the molecule *m/z* 577 $[M - H]^-$ lost a deoxyhexose Z_i fragment (164Da) resulting in a *m/z* 413 $[(M – H) - 164]^-$ product ion and then a $^{0,1}X$ pentose moiety (120Da) resulting in a *m/z* 293 $[(M - H) - 164 - 293]^-$ product ion. These stepwise losses suggest that the two linked sugar moieties attached to the same carbon on the precursor molecule. Had the molecule instead fragmented to yield Y_i deoxyhexose (146amu) and pentose (132amu) moieties, the resultant aglycone skeleton would have a *m/z* 301$[(M - H) - 146 - 132]^-$ suggesting the presence of quercetin (Ferreres et al., 2004; Vukics & Guttman, 2010). The molecule was tentatively identified as quercetin-3-pentose-rhamnoside.

Compound 13 was also detected in *P. biglobosa*. It showed precursor molecules at *m/z* 561 $[M + H]^+$; *m/z* 559 $[M - H]^-$. Positive ionization yielded a product ions with *m/z* 415 $[(M + H) - 146]^+$ and *m/z* 271 $[(M + H) - 146 - 144]^+$. The resultant aglycone was tentatively identified as apigenin (270amu) (Vukics & Guttman, 2010); and the parent molecule as apigenin-7-O-dirhamnoside. It was noted that the intermediate molecule between the possible aglycone deoxyhexose shows a value 2amu (146 - 144 = 2) lower

than that of deoxyhexose rhamnose. Apigenin derivatives containing a 144amu moiety have been previously described by Ferreres et al. (2007) and Pereira et al. (2012) although no structural feature was proposed. Negative MS^2 fragmentation yielded an ion of *m/z* 395 $[(M - H) - 164]^-$ after a loss of 164amu indicating cleavage of a Z_1 deoxyhexose moiety as the terminal sugar (Ferreres et al., 2007; Vukics & Guttman, 2010). There was a minor peak of *m/z* 269 $[(M - H) - 164 -126]^-$ after a loss of 126Da and also suggesting the presence of the deprotonated aglycone apigenin. The combined loss at the end of fragmentation (164amu + 126amu = 290Da) also shows the 2amu discrepancy of the expected total loss of two rhamnoside moieties (146amu x 2 = 292amu). Ferreres et al. (2007) named the molecule “unidentified 7-O-deoxyhexosyl-144 apigenin”.

Compounds 14 and 15 were detected in *Z. mauritiana* samples. **Compound 14** showed precursor ions at *m/z* 581 $[M + H]^+$ and *m/z* 579 $[M - H]^-$. MS^2 fragmentation in positive mode showed two product ions at *m/z* 449 $[(M + H) - 132]^+$ and *m/z* 303 $[(M + H) - 132 - 146]^+$. The loss of 132amu is consistent with cleavage of a Y_i fragment (terminal pentose) and 146amu with loss of Y_i deoxyhexose respectively (Ferreres et al., 2004). Therefore, molecule was tentatively identified as quercetin-3-O-rhamnose-pentoside (Martucci et al., 2014).

The peak representing **compound 15** showed three precursor ions in positive mode at *m/z* 727 $[M + H]^+$ *m/z* 449 [M + H]+ and 303 [M + H]+. The difference between these ions was 278amu (132 + 146) and 146amu respectively. MS^2 fragmentation of the first ion in positive mode at yielded three product ions at *m/z* 595 $[(M + H) - 132]^+$, *m/z* 449 $[(M + H) -132 - 146]^+$ and *m/z* 303 $[(M + H) - 132 - 146 - 146]^+$. These losses were attributed to the cleavage of terminal pentose unit(132), and two deoxyhexose (rhamnose) units (146 x 2) respectively (Vukics & Guttman, 2010). The initial tentative identification of this peak was Quercetin -3-O- dirhamnose-O-pentoside (Martucci et al., 2014). However, MS^2 in negative mode of the water extract at RT14.94 with *m/z* 725 $[M - H]^-$ indicated the presence of a terminal rhamnose (146amu) and an *m/z* 579 $([M - H]) - 146]^-$ residue (quercetin-3-O-hexose-pentoside). This suggests the molecule more likely to be quercetin-3-O-rhamnose-O-pentoside-7-rhamnoside. A carboxylic

group adjunct of the molecule with *m/z* 770 $[(M - H) + 45]^-$ was also detected in negative MS. A dimer of the molecule with *m/z* 1450 $[2M - H]^-$ was also detected.

3.4.3 *IN VITRO* FERMENTATION

There was an effect of plant species on gas production during *in vitro* fermentation of fodder tree leaves. *A. indica* showed the highest level of gas production corresponding to a higher rate and extent of *in vitro* degradation (Figure 3.10). *G. senegalensis* showed the lowest gas production; this correlated with the higher levels of lignin detected in this species (Table 3.5). There was an effect of ecological zone in the species *A. indica* and *P. biglobosa* (Table 3.8).

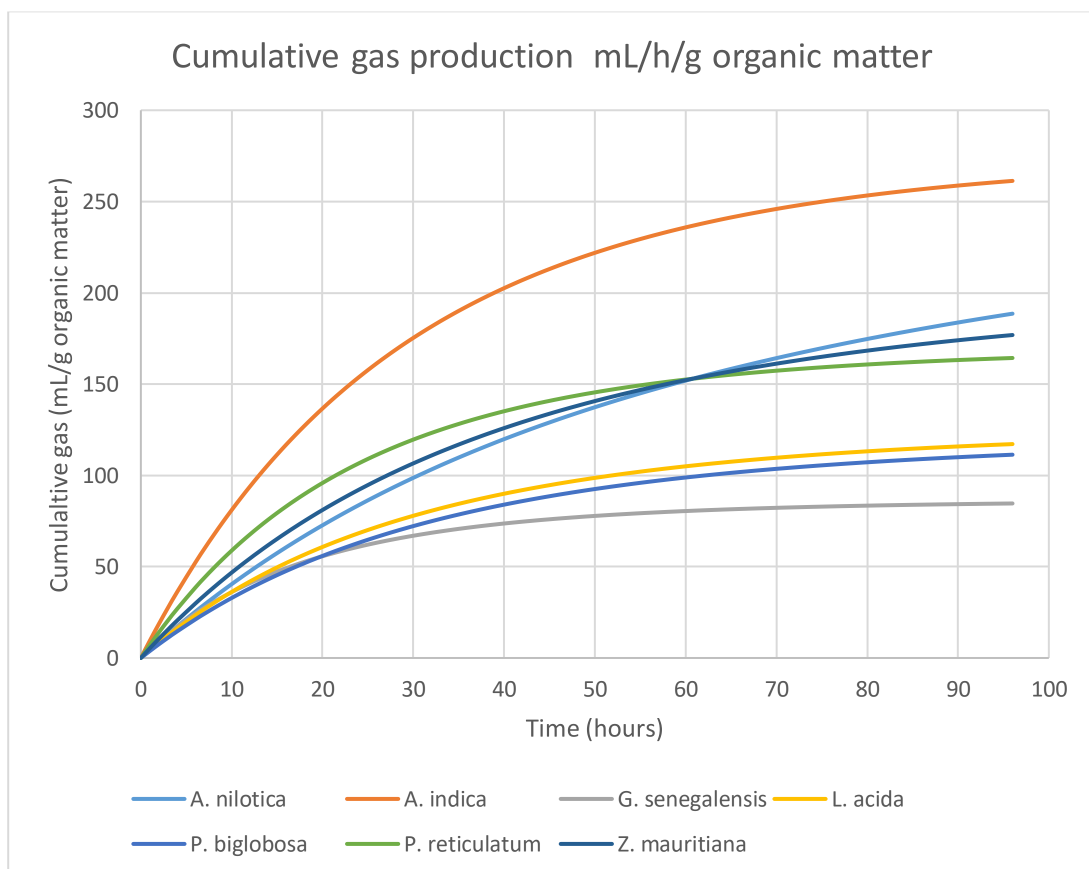


Figure 3.10: Cumulative gas production (mL/hour) from *in vitro* fermentation of leaves of *A. nilotica, A. indica, G. senegalensis, L. acida, P. biglobosa, P. reticulatum and Z. mauritiana* in rumen fluid for 96 hours showing a species effect.

Table 3.8: Volume of gas produced (a + b) and Rate of degradation (c)

	Extent of degradation (a+b, mL)				Rate of degradation (c, %/h)			
	Sahel	Sudan	N. Guinea	Mean	Sahel	Sudan	N. Guinea	Mean
A. nilotica	58.2	79.4	113.4	83.65^{d}	0.030	0.017	0.013	0.020^{a}
A. indica	93.7	48.1	82	74.58^{d}	0.031	0.047	0.035	0.038^{b}
G. senegalensis	31.4	22.8	20.4	24.88^{a}	0.065	0.056	0.044	0.055^{c}
L. acida	31.1	41.9	31.6	34.84^{ab}	0.045	0.028	0.035	0.036^{b}
P. biglobosa	40.4	39.7	22.6	34.26^{ab}	0.034	0.030	0.036	0.033^{ab}
P. reticulatum	44.7	49.3	51.9	48.63^{bc}	0.050	0.039	0.044	0.044^{bc}
Z. mauritiana	61.1	59.4	47.1	55.87^{c}	0.026	0.024	0.038	0.029^{ab}
s.e.d	Species			5.93				0.0051
	Zone			3.88				0.0033
	Species x Zone			10.26				0.0088
p value	Species			<0.001				<0.001
	Zone			0.567				0.164
	Species x Zone			<0.001				0.138

Means with superscripts depict significant difference

There was a significant effect of species on the production of total VFA. There was also a significant effect of ecological zone in the majority of species on the production of total VFA (Table 3.9 and Figure 3.13). Samples of *A. nilotica*, *G. senegalensis*, *L. acida* and *P. biglobosa* from the Sudan savannah zone showed the highest total VFA production compared to other zones. On the other hand, samples of *A. indica* and *P. reticulatum* from the Northern Guinea savannah had the highest total VFA production. In *Z. mauritiana*, the Sahel zone sample had the most VFA production.

Table 3.10 details the correlation between the different measurements carried out. There was a strong positive correlation between the measured levels of crude protein and the production of all VFA. The level of VFA production was also positively correlated with the extent of degradation (a + b, mL); the production of acetate and propionate was however negatively correlated with the rate of degradation (c, %/h). The production of acetate and propionate was also positively correlated to the levels of NDF.

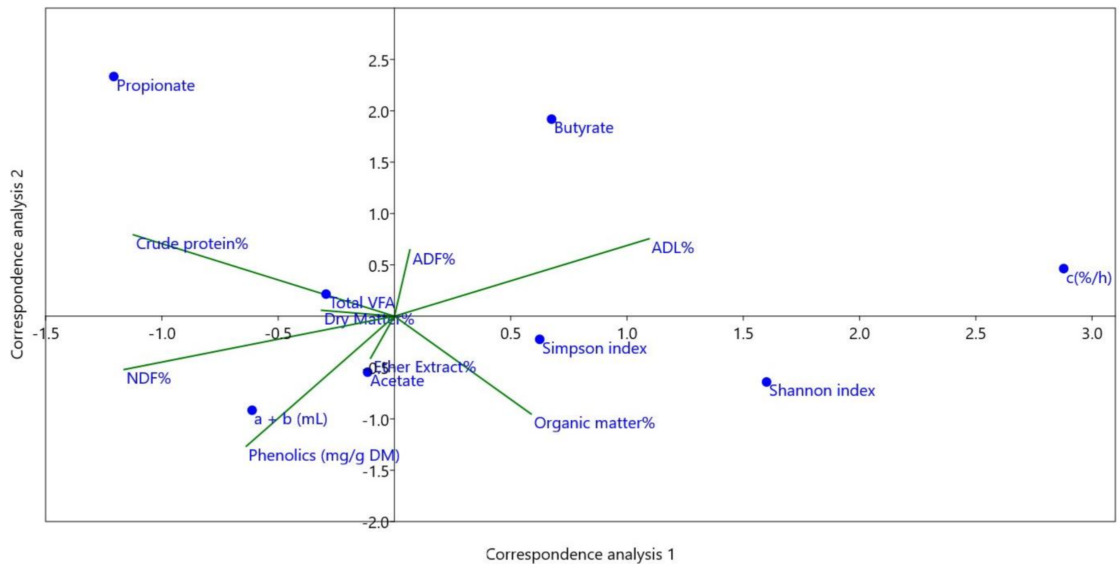


Figure 3.11: Canonical correspondence analysis illustrating the relationship between chemical composition of samples and rumen fermentation pattern *in vitro*. Plots show the direction of the gradient and those longer show a stronger correlation

Canonical correspondence analysis of the effect of chemical composition on the fermentation outcomes indicated a positive correlation between NDF% and the extent of degradation (a + b) (Figure 3.11 and Figure 3.12). There was also a positive correlation between ADL% and the rate of degradation (c, Figure 3.14).

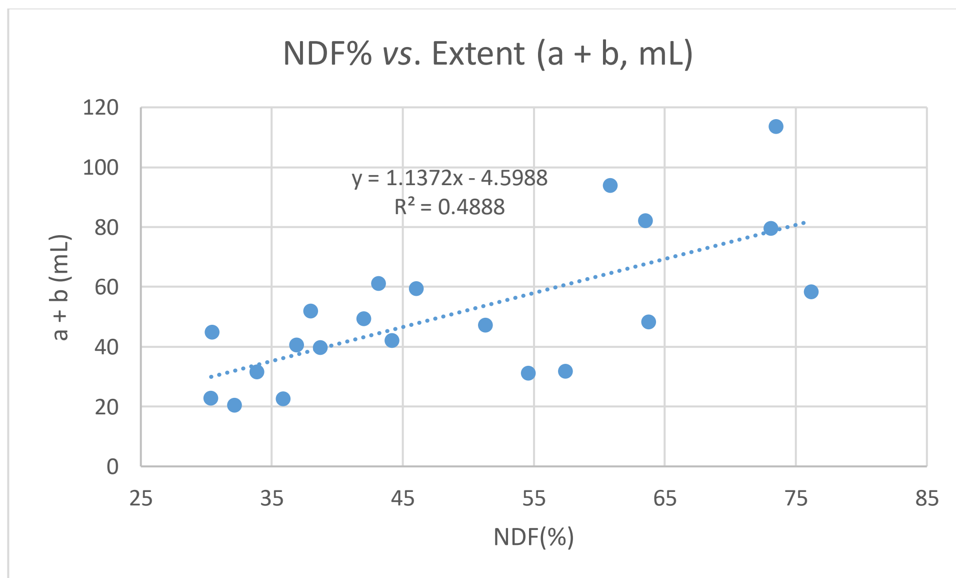


Figure 3.12: Relationship between NDF% and the extent of *in vitro* degradation (a + b, mL) of *A. nilotica, A. indica, G. senegalensis, L. acida, P. biglobosa, P. reticulatum* and *Z. mauritiana* from Sahel, Sudan and Northern Guinea savannah ecological zones in Nigeria

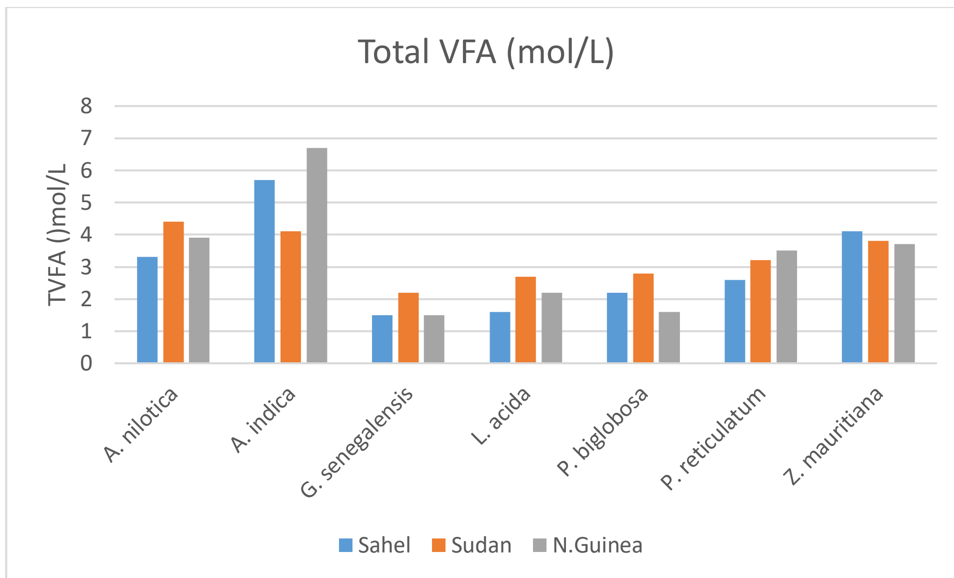


Figure 3.13: Total *in vitro* volatile fatty acid production (mol/L) of *A. nilotica*, *A. indica*, *G. senegalensis*, *L. acida*, *P. biglobosa*, *P. reticulatum* and *Z. mauritiana* from Sahel, Sudan and Northern Guinea savannah ecological zones in Nigeria

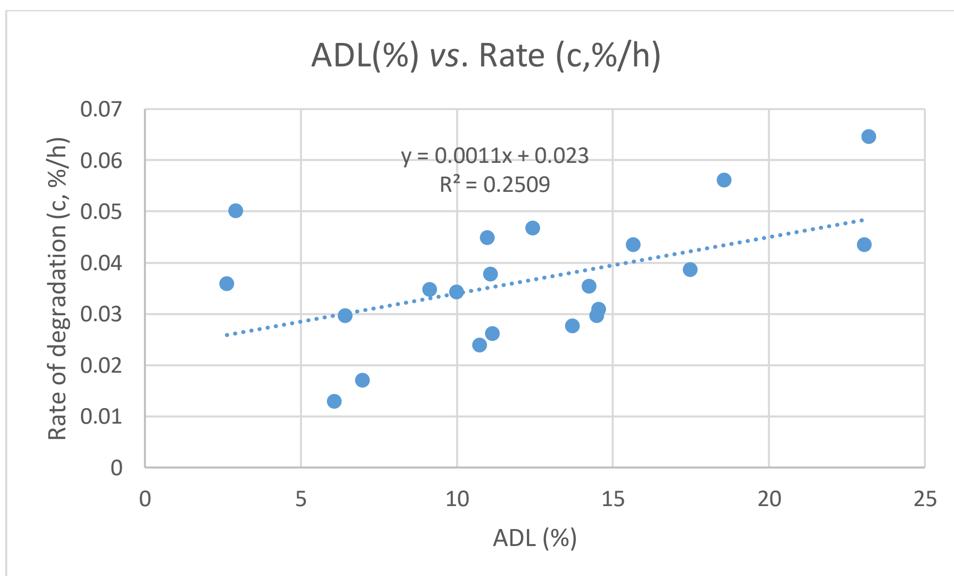


Figure 3.14: Relationship between ADL percentage and the rate of *in vitro* degradation (c) of *A. nilotica, A. indica, G. senegalensis, L. acida, P. biglobosa, P. reticulatum* and *Z. mauritiana* from Sahel, Sudan and Northern Guinea savannah ecological zones in Nigeria

Table 3.9: VFA production (mM) after *in vitro* incubation of *A. nilotica, A. indica, G. senegalensis, L. acida, P. biglobosa, P. reticulatum* and *Z. mauritiana* from Sahel, Sudan and Northern Guinea savannah ecological zones in Nigeria with rumen fluid

	Acetate				Propionate				Butyrate				Total VFA			
	Zone															
Species	Sahel	Sudan	Guinea	Mean	Sahel	Sudan	Guinea	Mean	Sahel	Sudan	Guinea	Mean	Sahel	Sudan	Guinea	Mean
A. nilotica	30.42	34.81	32.97	32.73^{cd}	2.61	6.29	4.58	4.5^{c}	0.50	2.09	1.26	1.28a^{b}	32.71	43.56	38.70	38.32^{c}
A. indica	36.68	24.12	39.33	33.38^{d}	13.73	13.66	16.87	14.8^{f}	3.85	2.20	5.75	3.93^{d}	57.36	40.51	66.92	54.93^{d}
G. senegalensis	12.8	17.41	12.56	14.26^{a}	1.48	2.34	1.39	1.74^{a}	1.09	2.76	1.70	1.85^{bc}	15.10	22.28	15.38	17.59^{a}
L. acida	14.43	22.08	17.74	18.08^{ab}	2.48	3.55	3.9	3.31^{b}	0.58	1.50	0.73	0.94^{a}	16.45	27.15	21.55	21.72^{a}
P. biglobosa	16.64	20.19	12.56	16.46^{a}	5.38	4.99	2.76	4.38^{bc}	0.89	1.70	0.80	1.13^{a}	22.71	27.70	15.70	22.04^{a}
P. reticulatum	18.55	22.73	25.01	22.09^{b}	5.59	7.03	7.57	6.73^{d}	1.09	1.48	1.69	1.42^{ab}	25.56	31.73	34.80	30.7^{b}
Z. mauritiana	28.98	28.58	27.39	28.31^{c}	9.65	8.49	8.25	8.8^{e}	2.37	2.06	2.09	2.17^{c}	41.15	38.14	36.89	38.73^{c}
s.e.d	Species			1.687				0.401				0.1988				2.173
	Zone			1.104				0.263				0.1302				1.423
	Species x Zone			2.922				0.695				0.3444				3.764
p value	Species			<0.001				<0.001				<0.001				<0.001
	Zone			0.303				0.011				<0.001				0.086
	Species x Zone			<0.001				<0.001				<0.001				<0.001

The superscripts depict significant difference

Table 3.10: Correlation matrix of chemical composition and *in vitro* fermentation kinetics of *A. nilotica, A. indica, G. senegalensis, L. acida, P. biglobosa, P. reticulatum* and *Z. mauritiana* from Sahel, Sudan and Northern Guinea savannah ecological zones in Nigeria

	DM	OM	Ash	NDF	ADF	ADL	CF	CP	a+b,mL	c,%/h	C1 VFA	C2 VFA	C3 VFA
DM	1.00												
OM	0.27	1.00											
Ash	-0.27	-1.00	1.00										
NDF	0.15	-0.26	0.26	1.00									
ADF	-0.16	-0.02	0.02	-0.51*	1.00								
ADL	0.02	0.01	-0.01	-0.38	0.01	1.00							
CF	-0.48*	-0.20	0.20	0.15	-0.33	-0.26	1.00						
CP	0.13	-0.42	0.42	0.27	0.27	-0.02	-0.00	1.00					
a+b, mL	0.15	-0.41	0.41	0.56*	-0.09	-0.18	0.13	0.61*	1.00				
c, %/h	0.22	0.29	-0.29	-0.49*	-0.02	0.51*	-0.51*	-0.35	-0.53*	1.00			
C1 VFA	0.27	-0.32	0.32	0.72*	-0.02	-0.22	-0.08	0.68*	0.86*	-0.50*	1.00		
C2 VFA	0.10	-0.29	0.29	0.67*	-0.02	-0.29	0.03	0.58*	0.75*	-0.46*	0.89*	1.00	
C3 VFA	0.23	-0.51*	0.51*	0.18	0.09	0.28	-0.14	0.76*	0.55*	-0.04	0.63*	0.58*	1.00
TVFA	0.27	-0.51*	0.51*	0.58*	0.02	-0.10	-0.11	0.78*	0.85*	-0.37	0.94*	0.83*	0.81*

*r values are statistically significant compared to the published critical value of 0.4329 at 19 degrees of freedom and p = 0.05

Table 3.11: Effect of fodder tree leaves on bacteria communities in *in vitro* systems.

	Treatments								SED	p value
	A. nilotica	*A. indica*	*G. senegalensis*	*L. acida*	*P. biglobosa*	*P. reticulatum*	*Z. mauritiana*	Blank		
Simpson index	0.977^{c}	0.957^{bc}	0.987^{b}	0.930^{b}	0.971^{c}	0.980^{c}	0.892^{a}	0.979^{bc}	0.038	<0.001
Shannon index	5.381^{cd}	5.280^{c}	5.962^{d}	4.469^{cd}	5.418^{cd}	5.570^{a}	3.758^{bcd}	5.677	0.717	<0.001

Means with different superscripts differ (p<0.05)

Table 3.12: Effect of leaves of *A. nilotica* (AN), *A. indica* (AI), *G. senegalensis* (GS), *L. acida*(LA), *P. biglobosa*(PB), *P. reticulatum*(PR) and *Z. mauritiana*(ZM) on *in vitro* relative abundance of bacteria phyla present at an average of more than 0.05% (false discovery rate for Benjamini–Hochberg: 0.25)

Phyla	Treatments								SED	Uncorrected p value	BH P-value
	AN	AI	GS	LA	PB	PR	ZM	Blank			
Bacteroidetes	0.448^{abcd}	0.489^{bde}	0.391^{a}	0.545^{be}	0.428^{abc}	0.399^{a}	0.623^{f}	0.399^{ab}	0.054	<0.001	0.009
Proteobacteria	0.032^{a}	0.063^{ab}	0.118^{cd}	0.029^{a}	0.084bc	0.071^{abc}	0.045^{ab}	0.242^{d}	0.099	<0.001	0.009
Unclassified	0.022^{b}	0.021^{b}	0.056^{c}	0.020^{b}	0.027^{b}	0.026^{b}	0.012^{a}	0.044^{bc}	0.036	<0.001	0.009
Firmicutes	0.298^{b}	0.300^{b}	0.311^{b}	0.167^{a}	0.303^{b}	0.353^{b}	0.166^{a}	0.252^{ab}	0.072	<0.001	0.009
Tenericutes	0.074^{e}	0.018^{b}	0.021^{bc}	0.051^{de}	0.016^{b}	0.034^{cd}	0.000^{a}	0.006^{ab}	0.050	<0.001	0.009
Candidatus Saccharibacteria	0.002^{c}	0.001^{bc}	0.003^{d}	0.001^{b}	0.001^{c}	0.001^{bc}	0.000^{a}	0.006^{e}	0.011	<0.001	0.009
Spirochaetes	0.080^{b}	0.065^{b}	0.060^{ab}	0.115^{c}	0.069^{b}	0.058^{ab}	0.113^{c}	0.026^{a}	0.044	<0.001	0.009
Actinobacteria	0.003^{c}	0.001^{b}	0.004^{d}	0.001^{ab}	0.002^{bc}	0.002^{bc}	0.001^{a}	0.008^{e}	0.012	<0.001	0.009
Fibrobacteres	0.002^{ab}	0.007^{abc}	0.003^{ab}	0.049^{e}	0.015^{acd}	0.036^{e}	0.015^{ad}	0.001^{a}	0.053	<0.001	0.009
Verrucomicrobia	0.004^{ef}	0.002^{cde}	0.002^{bcd}	0.001^{a}	0.001^{ab}	0.004^{f}	0.002^{bc}	0.005^{def}	0.013	<0.001	0.009
Synergistetes	0.001^{a}	0.002^{bcd}	0.004^{e}	0.001^{bc}	0.003^{d}	0.002^{cd}	0.001^{ab}	0.007^{e}	0.012	<0.001	0.009
Elusimicrobia	0.001^{ab}	0.001^{ab}	0.001^{bc}	0.000^{ab}	0.001^{c}	0.001^{abc}	0.000^{a}	0.002^{bc}	0.011	0.005	0.025
Cyanobacteria/Chloroplast	0.012^{e}	0.001^{abc}	0.003^{d}	0.002^{acd}	0.002^{ad}	0.000^{ab}	0.001^{abc}	0.000^{a}	0.021	<0.001	0.009

Means with different superscripts differ (p<0.05)

Table 3.13: Effect of leaves of *A. nilotica* (AN), *A. indica* (AI), *G. senegalensis* (GS), *L. acida*(LA), *P. biglobosa*(PB), *P. reticulatum*(PR) and *Z. mauritiana*(ZM) on *in vitro* relative abundance of bacteria genera present at an average of >0.05% (false discovery rate for Benjamini–Hochberg: 0.25).

Genera	Treatments								SED	Uncorrected p value	Benjamini-Hochberg p-value
	AN	AI	GS	LA	PB	PR	ZM	Blank			
Unclassified	0.477^{a}	0.622^{c}	0.547^{abc}	0.563^{abc}	0.535^{ab}	0.574^{abc}	0.610^{bc}	0.572^{abc}	0.046	<0.001	0.014
Acinetobacter	0.002^{a}	0.018^{a}	0.018^{a}	0.002^{a}	0.006^{a}	0.062^{bc}	0.020^{ab}	0.193^{c}	0.084	<0.001	0.014
Clostridium IV	0.003^{b}	0.005^{b}	0.011^{d}	0.004^{b}	0.007^{bc}	0.009^{cd}	0.001^{a}	0.011^{bcd}	0.018	<0.001	0.014
Anaeroplasma	0.069^{e}	0.017^{bc}	0.018^{bc}	0.050^{de}	0.013^{bc}	0.033^{cd}	0.000^{a}	0.000^{ab}	0.049	<0.001	0.014
Saccharofermentans	0.004^{c}	0.002^{abc}	0.003^{c}	0.001^{abc}	0.002^{abc}	0.002^{abc}	0.001^{a}	0.005^{bc}	0.014	<0.001	0.014
Saccharibacteria_genera_incertae_sedis	0.002^{bc}	0.001^{b}	0.003^{cd}	0.001^{ab}	0.001^{b}	0.001^{ab}	0.000^{a}	0.006^{d}	0.011	<0.001	0.014
Prevotella	0.066^{bd}	0.020^{a}	0.054^{bd}	0.028^{abc}	0.042^{bcd}	0.026^{abc}	0.025^{abc}	0.025^{ab}	0.044	<0.001	0.014
Lachnospiracea_incertae_sedis	0.011^{bcd}	0.012^{bcd}	0.008^{bcd}	0.004^{a}	0.008^{abc}	0.014^{bd}	0.008^{abc}	0.007^{ab}	0.020	<0.001	0.014
Butyrivibrio	0.035^{f}	0.018^{de}	0.004^{ab}	0.007^{abc}	0.013^{acd}	0.015^{ade}	0.020^{e}	0.004^{a}	0.022	<0.001	0.014
Paraprevotella	0.024^{ab}	0.030^{abc}	0.023^{ab}	0.058^{acd}	0.032^{abcd}	0.018^{ab}	0.059^{ad}	0.013^{a}	0.057	<0.001	0.014
Succiniclasticum	0.001^{bd}	0.000^{a}	0.001^{bd}	0.001^{abc}	0.001^{abc}	0.000^{a}	0.000^{a}	0.001^{ab}	0.007	<0.001	0.014
Anaerovorax	0.002^{a}	0.010^{b}	0.012^{b}	0.004^{a}	0.010^{b}	0.015^{b}	0.002^{a}	0.026^{b}	0.022	<0.001	0.014
Treponema	0.061^{bc}	0.034^{ab}	0.023^{a}	0.097^{c}	0.049^{b}	0.042^{ab}	0.091^{c}	0.004^{a}	0.050	<0.001	0.014
Coprobacter	0.003^{cd}	0.002^{ac}	0.004^{d}	0.001^{abc}	0.002^{cd}	0.001^{a}	0.001^{ab}	0.003^{abcd}	0.014	<0.001	0.014
Oscillibacter	0.002^{abcd}	0.002^{abcde}	0.005^{bf}	0.002^{abc}	0.005^{bdf}	0.006^{bf}	0.000^{a}	0.001^{ab}	0.022	<0.001	0.014
Christensenella	0.012^{bc}	0.015^{c}	0.019^{cde}	0.007^{ab}	0.015^{bc}	0.036^{d}	0.002^{a}	0.017^{abcd}	0.030	<0.001	0.014
Phocaeicola	0.001^{cde}	0.001^{bcd}	0.002^{e}	0.001^{bc}	0.002^{ce}	0.000^{ab}	0.000^{a}	0.001^{abcde}	0.009	<0.001	0.014
Bulleidia	0.011^{b}	0.000^{a}	0.001^{a}	0.000^{a}	0.001^{a}	0.000^{a}	0.000^{a}	0.000^{a}	0.017	<0.001	0.014
Ruminococcus	0.016^{bde}	0.003^{abcd}	0.001^{abc}	0.002^{abc}	0.003^{abcd}	0.000^{a}	0.025^{be}	0.000^{ab}	0.057	<0.001	0.014
Pseudobutyrivibrio	0.022^{b}	0.025^{b}	0.005^{a}	0.006^{a}	0.004^{a}	0.003^{a}	0.010^{a}	0.002^{a}	0.031	<0.001	0.014
Fibrobacter	0.002^{ab}	0.007^{abcd}	0.003^{abc}	0.049^{f}	0.015^{abde}	0.036^{aef}	0.015^{ade}	0.001^{a}	0.053	<0.001	0.014
Vampirovibrio	0.003^{acd}	0.002^{abc}	0.005^{d}	0.002^{abc}	0.003^{ac}	0.002^{abc}	0.001^{ab}	0.001^{a}	0.014	<0.001	0.014
Subdivision5_genera_incertae_sedis	0.004^{cd}	0.002^{bcd}	0.002^{abc}	0.001^{a}	0.001^{ab}	0.004^{d}	0.002^{ab}	0.005^{bcd}	0.013	<0.001	0.014
Sporobacter	0.010^{c}	0.009^{c}	0.010^{c}	0.004^{ab}	0.006^{bc}	0.007^{bc}	0.002^{a}	0.012^{abc}	0.023	<0.001	0.014
Coprococcus	0.009^{abc}	0.006^{abc}	0.003^{ab}	0.003^{ab}	0.006^{abc}	0.008^{abc}	0.015^{ac}	0.002^{a}	0.046	0.008	0.021
Sphaerochaeta	0.012^{a}	0.019^{abc}	0.031^{b}	0.012^{a}	0.014^{a}	0.011^{a}	0.013^{a}	0.017^{ab}	0.026	<0.001	0.014

Mogibacterium	0.000^{ab}	0.001^{bc}	0.001^{c}	0.000^{ab}	0.001^{bc}	0.001^{bc}	0.000^{a}	0.002^{a}	0.008	<0.001	0.014
Rikenella	0.007^{bde}	0.004^{abcd}	0.007^{be}	0.003^{a}	0.006^{bcde}	0.003^{abc}	0.003^{abc}	0.003^{ab}	0.017	<0.001	0.014
Anaerocella	0.004^{b}	0.001^{a}	0.001^{a}	0.001^{a}	0.001^{ab}	0.001^{ab}	0.001^{a}	0.001^{ab}	0.017	0.016	0.030
Schwartzia	0.001^{a}	0.004^{c}	0.002^{b}	0.004^{c}	0.002^{bc}	0.003^{bc}	0.002^{ab}	0.003^{abc}	0.012	<0.001	0.014
Fretibacterium	0.000^{a}	0.000^{ab}	0.001^{abc}	0.000^{abc}	0.001^{bc}	0.001^{c}	0.000^{ab}	0.002^{bc}	0.009	0.003	0.015
Syntrophococcus	0.000^{ab}	0.001^{bc}	0.001^{c}	0.000^{ab}	0.001^{c}	0.001^{bc}	0.000^{a}	0.001^{abc}	0.007	<0.001	0.014
Catabacter	0.001^{bcd}	0.001^{ab}	0.003^{ce}	0.001^{ab}	0.002^{cde}	0.002^{cde}	0.000^{a}	0.001^{abc}	0.011	<0.001	0.014
Streptococcus	0.002^{ab}	0.002^{a}	0.010^{b}	0.006^{ab}	0.002^{ab}	0.001^{a}	0.001^{a}	0.003^{ab}	0.046	0.009	0.021
Elusimicrobium	0.000^{ab}	0.000^{ab}	0.001^{ab}	0.000^{ab}	0.001^{b}	0.000^{ab}	0.000^{a}	0.002^{ab}	0.012	0.047	0.071
Solobacterium	0.001^{a}	0.001^{a}	0.000^{a}	0.000^{a}	0.000^{a}	0.001^{a}	0.000^{a}	0.001^{a}	0.007	0.306	0.306
Anaerosporobacter	0.000^{ab}	0.001^{ab}	0.000^{ab}	0.002^{b}	0.012^{c}	0.010^{c}	0.000^{a}	0.000^{ab}	0.029	<0.001	0.014
Anaerorhabdus	0.004^{cd}	0.003^{bc}	0.005^{d}	0.001^{ab}	0.002^{bc}	0.003^{bcd}	0.001^{a}	0.005^{abcd}	0.015	<0.001	0.014
Howardella	0.000^{ab}	0.001^{c}	0.001^{bc}	0.000^{a}	0.000^{ab}	0.000^{abc}	0.000^{a}	0.000^{abc}	0.013	<0.001	0.014
Mobilitalea	0.002^{abc}	0.002^{abc}	0.002^{abc}	0.001^{abc}	0.017^{d}	0.014^{bd}	0.000^{a}	0.000^{ab}	0.033	<0.001	0.014
Pseudomonas	0.004^{a}	0.000^{ab}	0.000^{ab}	0.000^{ab}	0.000^{ab}	0.000^{ab}	0.000^{ab}	0.000^{a}	0.030	0.002	0.015
Comamonas	0.001^{a}	0.001^{a}	0.006^{a}	0.000^{a}	0.004^{a}	0.000^{a}	0.000^{a}	0.002^{a}	0.072	0.15	0.173
Mucinivorans	0.005^{bcde}	0.006^{bde}	0.007^{bde}	0.002^{abc}	0.004^{abcd}	0.012^{be}	0.001^{a}	0.002^{ab}	0.022	<0.001	0.014
Pyramidobacter	0.000^{a}	0.001^{bc}	0.003^{d}	0.001^{abc}	0.001^{bc}	0.000^{a}	0.001^{ab}	0.003^{c}	0.010	<0.001	0.014
Roseburia	0.010^{ad}	0.006^{cd}	0.006^{bcd}	0.003^{abc}	0.002^{a}	0.001^{a}	0.003^{ab}	0.000^{a}	0.022	<0.001	0.014
Campylobacter	0.000^{a}	0.003^{a}	0.002^{a}	0.000^{a}	0.003^{a}	0.000^{a}	0.002^{a}	0.000^{a}	0.054	0.293	0.306
Streptophyta	0.012^{e}	0.001^{abc}	0.003^{ad}	0.002^{abcd}	0.002^{acd}	0.000^{ab}	0.001^{abcd}	0.000^{a}	0.021	<0.001	0.014
Acholeplasma	0.001^{ab}	0.000^{ab}	0.001^{ab}	0.000^{ab}	0.001^{b}	0.000^{ab}	0.000^{a}	0.002^{ab}	0.026	0.01	0.021
Escherichia/Shigella	0.001^{ab}	0.000^{ab}	0.006^{b}	0.000^{ab}	0.002^{ab}	0.000^{a}	0.000^{ab}	0.000^{ab}	0.056	0.031	0.052
Citrobacter	0.003^{ab}	0.002^{ab}	0.010^{b}	0.001^{ab}	0.011^{b}	0.000^{a}	0.004^{ab}	0.000^{ab}	0.062	0.009	0.021
Proteiniclasticum	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}	0.001^{a}	0.000^{a}	0.000^{a}	0.001^{a}	0.024	0.098	0.123
Pluralibacter	0.002^{ab}	0.003^{ab}	0.009^{ab}	0.002^{ab}	0.015^{b}	0.000^{a}	0.004^{ab}	0.000^{ab}	0.080	0.06	0.082

Means with different superscripts differ ($p<0.05$)

3.5 BACTERIAL POPULATION

PERMANOVA showed a strong effect of the tree species on the bacterial population (p=0.001) (Figure 3.15). However, no effect of ecological zone was observed (P=0.629). Pairwise comparison showed that the structure of the bacterial community differed between *P. reticulatum* and *Z. mauritiana*, *G. senegalensis* and *Z. mauritiana*.

Principal Coordinate Analysis illustrated the separation between species; the primary axes accounted for 51.9% of the variation (Figure 3.15). In order to study the chemical composition factors which correlated with the structure of the bacterial community, a Canonical Correspondence Analysis (CCA) was performed. This analysis also showed separations in the ordination plot. Additionally, the separation was correlated with the various chemical characteristics of the fodder tree leaves. Total phenolics showed a strong effect on the ordination of *A. nilotica* while NDF, ADL and ADF correlated with the distribution of *L. acida* and *Z. mauritiana*, *G. senegalensis* and *P. reticulatum* respectively (Figure 3.16).

There was an effect of the fodder-tree species on both the Shannon and the Simpson indices ($p<0.001$). *G. senegalensis* showed the highest level of diversity on both indices while *Z. mauritiana* showed the least (Table 3.11). Differences in bacterial abundances were seen at the phylum level (Table 3.12 and Figure 3.18, $p<0.05$) and at the genus level (Table 3.13 and Figure 3.19; $p<0.05$). The bacterial genera that showed the biggest effect of treatment were *Bulleidia*, *Pseudomonas*, *Anaerosporobacter* and *Escherichia*/*Shigella* (Figure 3.17).

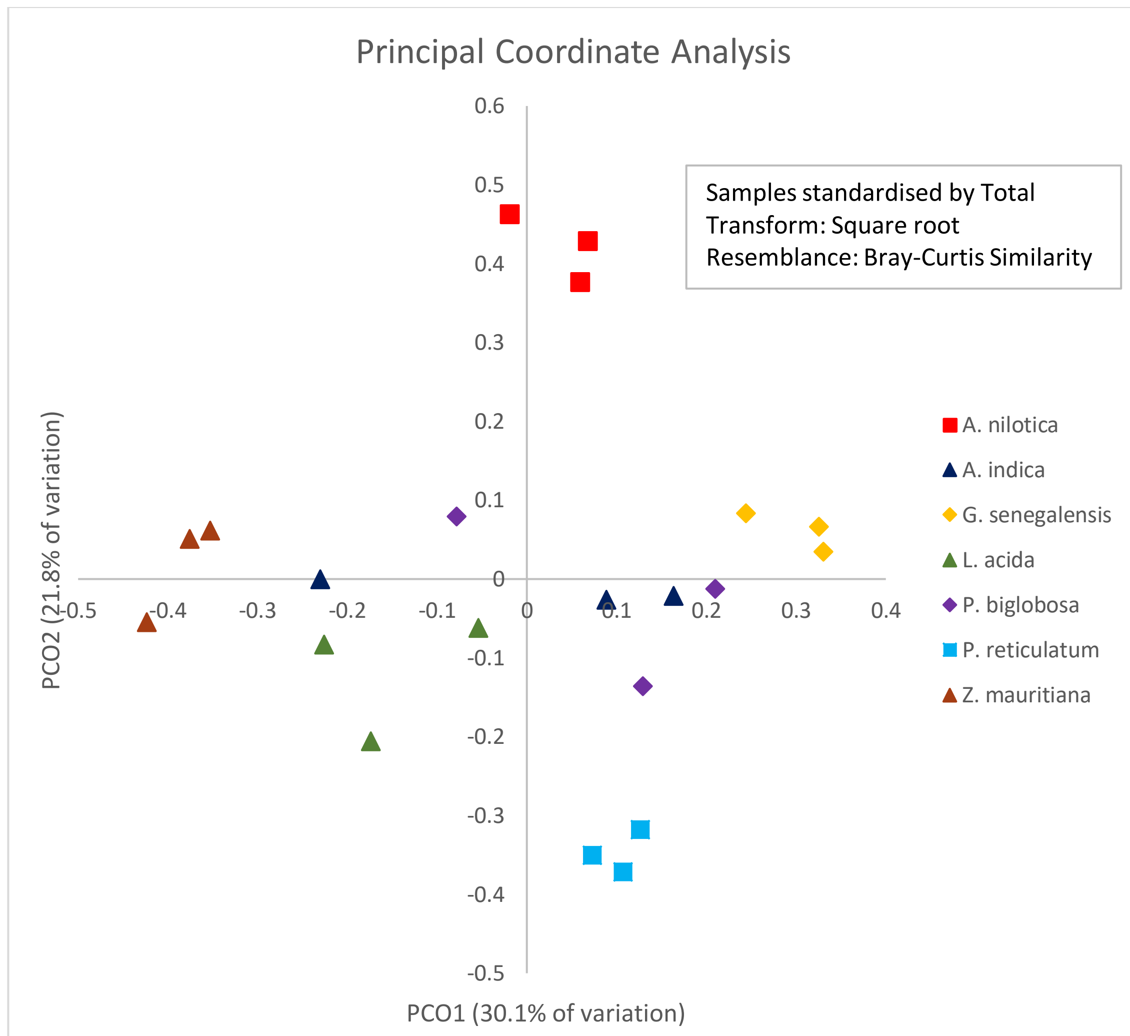


Figure 3.15: Principal Coordinate Analysis illustrating the separation between species PCO analysis of the *in vitro* effect of *A. nilotica*, *A. indica*, *G. senegalensis*, *L. acida*, *P. biglobosa*, *P. reticulatum* and *Z. mauritiana* leaves from three ecological zones in Nigeria on sheep rumen bacteria analysed using Ion Torrent 16S rRNA gene sequencing. Samples standardised by total and transformed by square root. Resemblance: Bray-Curtis Similarity.

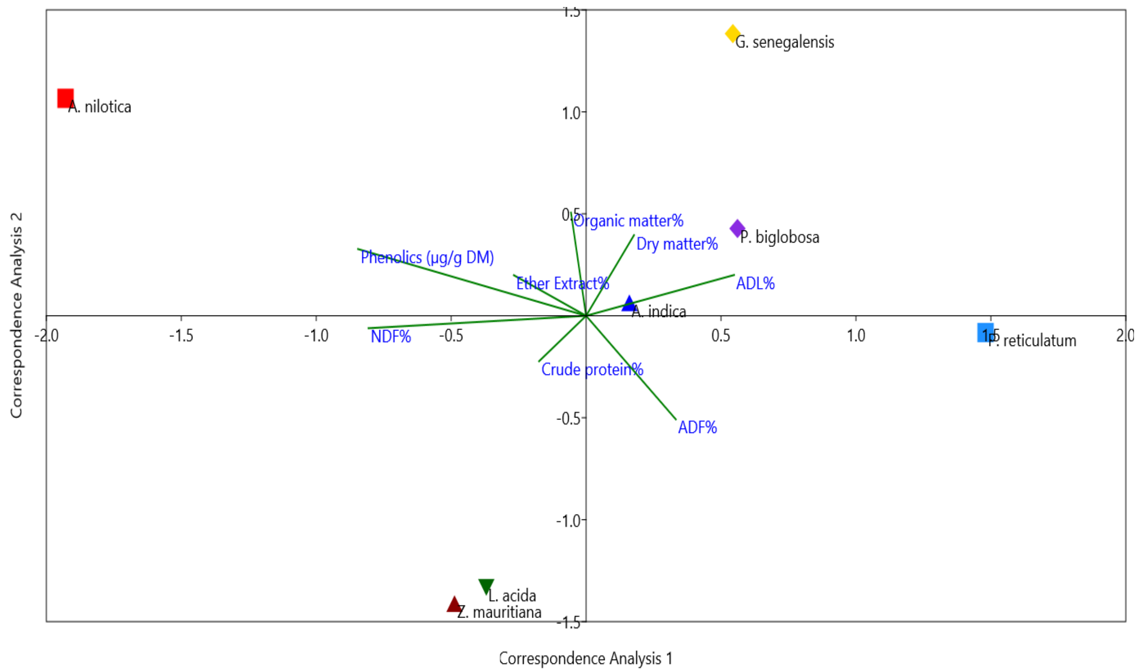


Figure 3.16: Canonical correspondence analysis illustrating the relationship between the structure of the bacterial community and the chemical composition of samples. Plots show the direction of the gradient and those longer show a stronger correlation (P < 0.05).

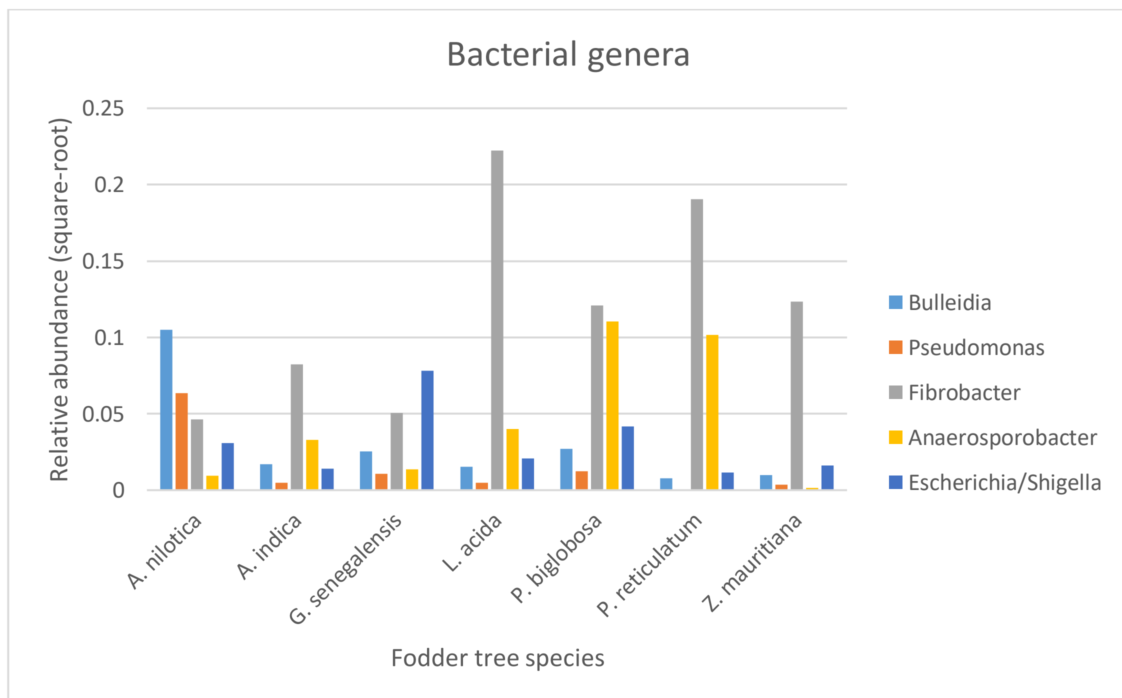


Figure 3.17: The effect of *A. nilotica, A. indica*, *G. senegalensis*, *L. acida*, *P. biglobosa*, *P. reticulatum* and *Z. mauritiana* leaves in the *in vitro* abundance of five bacterial genera in sheep rumen analysed using Ion Torrent 16S rRNA gene sequencing.

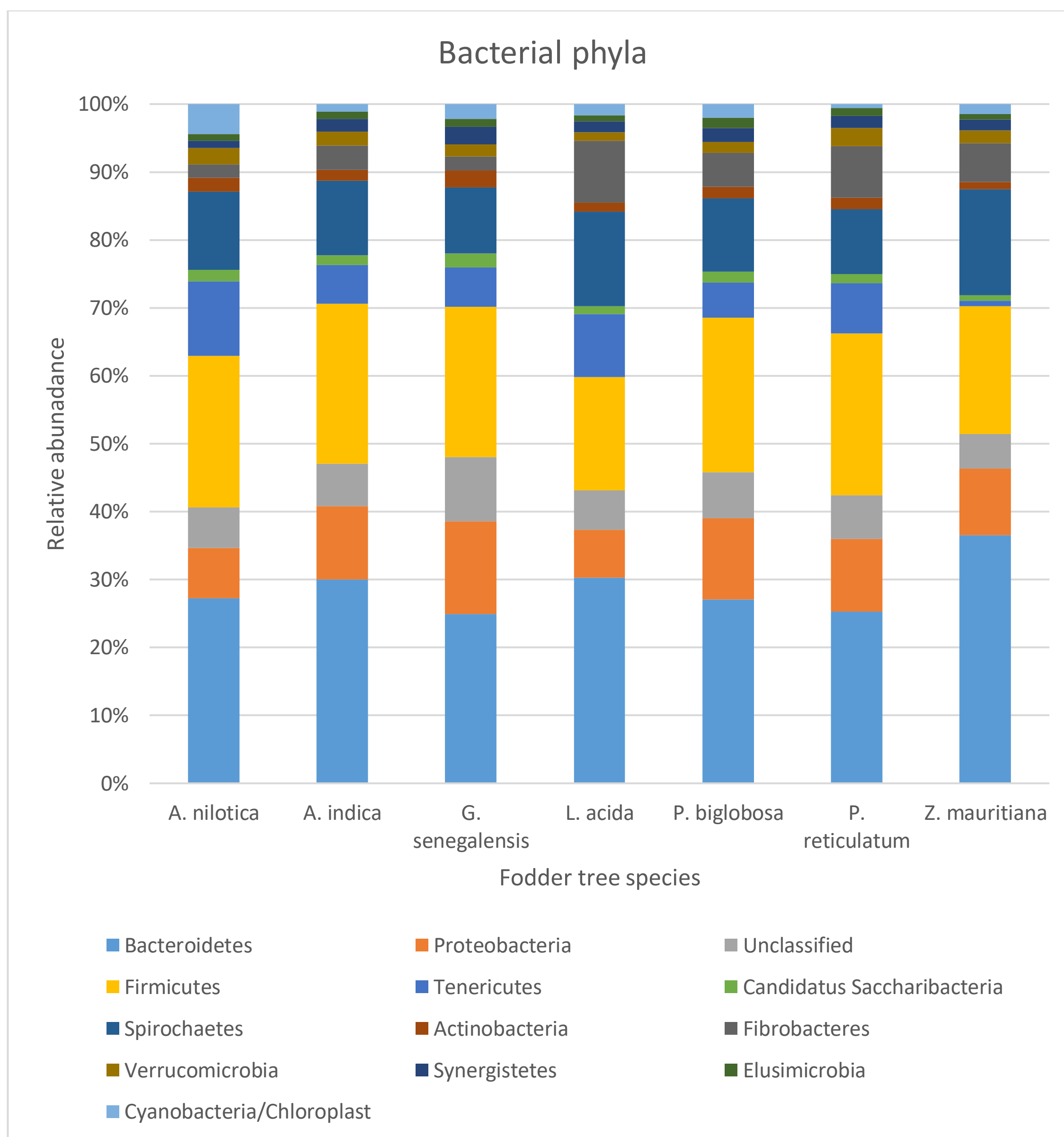


Figure 3.18: Stacked histograms illustrating phylum-level bacterial composition present at an average of more than 0.05%.Ion Torrent 16S rRNA gene sequence reads were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II

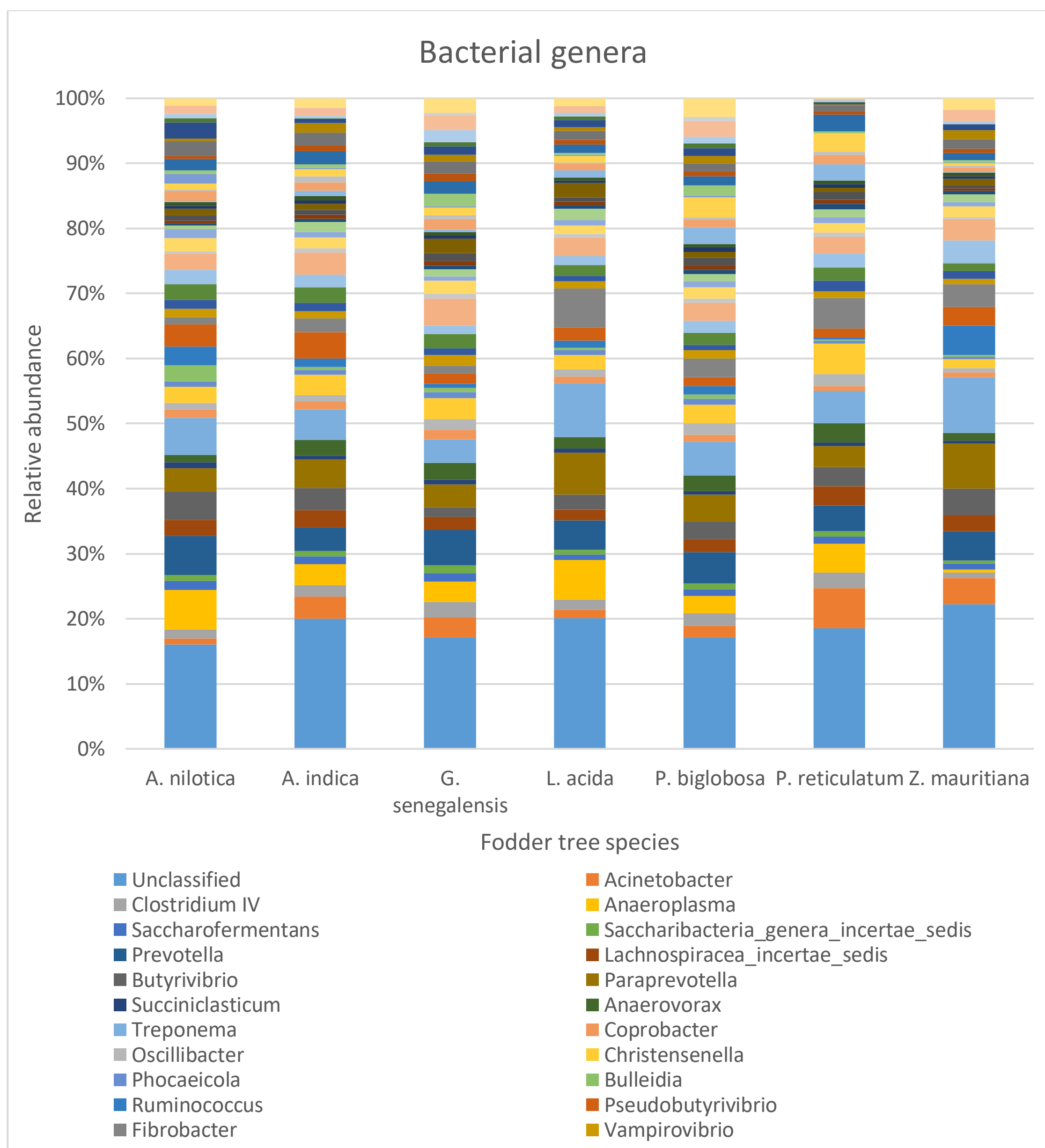


Figure 3.19: Stacked histograms illustrating genus-level bacterial composition present at an average of more than 0.05%. Ion Torrent 16S rRNA gene sequence reads were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II. Data transformed by square rooting.

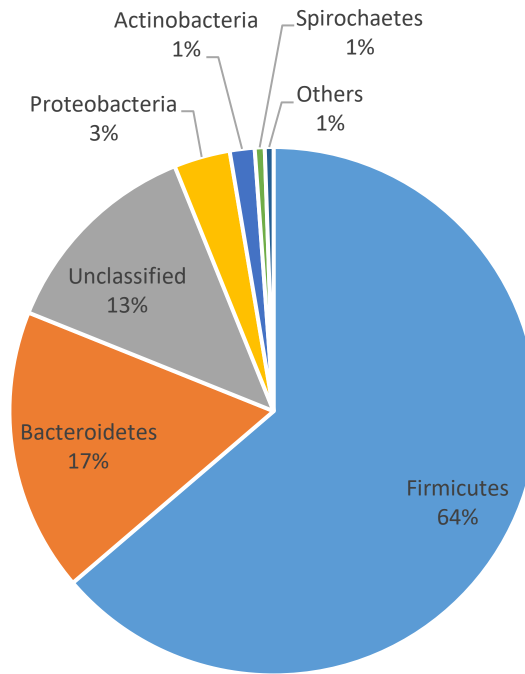


Figure 3.20: Pie illustrating phylum-level taxonomy of unclassified bacterial OTUs. Ion Torrent 16S rRNA gene sequence reads were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II.

3.6 DISCUSSION

The values obtained for the chemical composition and *in vitro* DMD in the present study are consistent with the range of values obtained for tree leaves in the subtropical and tropical conditions in other countries (Bakshi & Wadhwa, 2004; Ndlovu & Nherera, 1997). Although there were large differences in chemical composition among the tree species, all had CP contents of >10%, except *Lannea* species from the Northern Guinea savannah. This suggests that tree leaves have potential as CP supplements to the existing low-quality forage-based diets of livestock in the arid and semiarid regions of SSA. The low-quality fibrous feeds in these regions are often deficient in CP, particularly during the dry seasons. For example, the local range grasses contain less than 7% CP and crop residues contain less than 4% CP (Anele et al., 2009). These levels are too low to meet minimum nitrogen requirement of 8% DM necessary for optimal rumen microorganisms function (Annison & Bryden, 1998). There have been previously reported effect of climatic condition on the chemical composition of leaves of fodder trees in Nigeria (Carew et al., 1980; Larbi et al., 1996) which may result in differences in cell-wall lignification. In this experiment, an effect of ecological zone was seen in CP, CF and OM contents, but not in the fibre fractions.

Thousands of plant secondary metabolites (PSM) from numerous structural classes exist in nature. Their presence and concentration in a given plant are influenced by genetics, phenology, and a host of biotic and abiotic environmental factors (Estell, 2010). Their concentrations vary temporally and spatially among and within species, and their proportions relative to other compounds (both primary and secondary) are in constant flux. In this study, significant differences in the structure and concentration was found between the various species investigated (Table 3.7). Further differences were also seen in the concentration of the metabolites between samples of the same species harvested in different ecological zones (Table 3.6). An ecological effect on the structure and concentration of PSM has been reported previously. McKey et al. (1978) reported that mature leaves of trees growing on acid white-sand soils of rainforests in the Douala-Edea Reserve, Cameroon contained approximately twice the concentration of phenolic compounds found in similar rainforest vegetation on lateritic soils of the

Kibale Forest, Uganda. The consequence was that compared to Colobus monkeys in Uganda, the Black Colobus monkeys of Cameroon avoided ingesting leaves of almost all abundant tree species in the area, opting instead to eat leaves of less ubiquitous vegetation (deciduous trees and second-growth vines) and seeds. The authors hypothesised that plants growing on low-nutrient soils produced relatively high concentrations of PSM as a deterrent to herbivores and pathogens (McKey et al., 1978). Olive trees (*Olea europaea*) growing in their natural habitats in the Mediterranean region (Tunisia, Malta and Montenegro) contained higher concentrations of total phenols and flavonoids; the metabolites also had higher biological activity compared to samples those from cultivated plants (Stanković et al., 2017). In this case, the authors speculated that these active substances played an important role in the adaptation of plants to the stress caused by arid conditions of the natural habitat and thus the higher levels. The large differences in both the type and concentrations of many PSMs across physical and biotic environments can be attributed to local adaptation or genotypic sorting and selection across habitats and are rarely predicted by the carbon-nutrient balance hypothesis (Hamilton et al., 2001; Moore et al., 2014).

The type and concentration of PSM plays a pivotal role in the palatability of the plant by herbivores. Owen-Smith et al., (1987) reported that the preference and intake of woody browse species was negatively related to phenolic concentrations in impala (*Aepyceros melampus*) and greater kudu (*Tragelaphus strepsiceros*), both African antelope. Similar observations have been reported in sheep and goats (Degen et al., 2002; Papachristou et al., 2003; Provenza & Malechek, 1984). High concentrations (>55g/kg DM) of condensed tannins generally have been shown to reduce voluntary feed intake and digestibility, and depress rates of body and wool growth in grazing ruminants (Min et al., 2003).

In this study, condensed tannins were identified in *A. nilotica* samples at levels ranging from 55g/kg to 94g/kg. Rubanza et al. (2003) also reported high tannin compositions in *A. nilotica* growing in Tanzania. The authors observed that the presence and concentration of tannins depressed both forage OM degradability and digestibility *in vitro*. Barman and Rai (2008) also observed the reduction of *in vitro* gas production and CP digestibility with increased levels of tannins from *A. nilotica* pods (Barman &

Rai, 2008). In this case, ruminal microbes of goat were capable of withstanding up to 4% tannin before the deleterious effects set in (Barman & Rai, 2008). *In vivo* digestibility of *A. nilotica* has also been shown to be negatively affected by high levels of tannin (Alam et al., 2006). Apart from phenolic compounds, the presence and levels of saponins and alkaloids also affect palatability of browse species in sheep and goats (Salem et al., 2006).

Chemical analysis alone is not sufficient to predict the utility of a tree species as a source of fodder. The nutritional value of fodder is determined by the dry matter intake and the ability of a feedstuff to provide the nutrients required by the animal for maintenance, growth and reproduction. Feed intake in ruminants consuming fibrous forage is primarily determined by the level of rumen fill, which in turn is directly related to the rate of digestion and passage of fibrous particles from the rumen (Van Soest, 1994, p. 349). Gas production (GP) techniques can predict rumen OM degradation by providing kinetic information. However, optimal use of GP data to predict microbial efficiency and VFA production is best achieved when the possibilities and limitations of *in vitro* environments are recognised. The mechanisms governing microbial efficiency and VFA molar proportions *in vitro* are not necessarily transferrable to *in vivo* situations and results need to be carefully interpreted. The profile of VFA in GP is only a reflection of the dynamic VFA profile *in vivo* where the products of fermentation are constantly being absorbed and new material from the host and the environment is continuously added into the fermentation chamber. Researchers have to consider the fractional passage rate of ingesta from the rumen when making estimations of OM degradation, microbial efficiency and amount of VFA produced. The predictive accuracy of GP can be improved by combining it with mechanistic modelling but cannot be relied on to directly predict the nutrient supply to the animal (Dijkstra et al., 2005). In this study, there was a strong positive correlation between gas production and VFA concentrations (Table 3.10) suggesting that both gas GP and VFA production could function as a reliable measure of *in vitro* digestibility.

The levels of total VFA produced during *in vitro* fermentations varied between fodder tree species (Table 3.9). Such variations have been described before. Medjekal et al. (2018) observed this when they incubated ten browse species of Algerian arid and

semi-arid areas namely *Arthrocnemum macrostachyum*, *Atriplex canescens* , *Artemisia herba-alba*, *Astragalus gombo*, *Calobota saharae* ,*Spartidium saharae*, *Genista saharae*, *Hedysarum coronarium*, *Medicago sativa, Ononis natrix*, *Hordeum vulgare* and *Stipa tenacissima in vitro* rumen in sheep rumen fluid. *A. gombo* resulted in the highest and *S. tenacissima* in the lowest VFA production. Gas production was highest for *M. sativa* and lowest for *S. tenacissima* (Medjekal et al., 2018).

The rumen bacterial community is dominated by three phyla, Firmicutes, Bacteroidetes and Proteobacteria (Jami & Mizrahi, 2012). These were also the dominant phyla in this experiment regardless of treatment. However, the different fodder-tree species were shown to affect the diversity and the composition of rumen bacterial *in vitro* (Table 3.11, Table 3.12 and Table 3.13). Diet is the main factor affecting the composition of the rumen microbial community (Henderson et al., 2015). It has been previously suggested that feeding forages with plant secondary plant metabolites (PSM) may potentially manipulate digestive function in the rumen by altering the composition of the microbial community (Leng et al., 1992). Whether this effect is positive or negative can only be discovered *via* experimentation due to the sheer number of fodder trees and shrubs used across the world and the differences in the secondary metabolites they produce.

Any effect on rumen bacteria alters not only extent of digestion, but also fermentation patterns and end products available for metabolism (Estell, 2010). Hartley and Akin (1989) reported that phenolic acids (including p-coumaric acid) could be toxic to ruminal cellulolytic bacteria. Terpenes can decrease *in vitro* digestibility in ruminants due to negative microbial effects (Oh et al., 1967, Schwartz et al., 1980a). Broudiscou et al. (2007) reported that terpenes generally reduced fermentation *in vitro*. Tannins may reduce fibre digestion by complexing with lignocellulose and preventing microbial digestion or by directly inhibiting cellulolytic microorganisms or both (McSweeney et al., 2001). There was a marked decrease in the population of rumen cellulolytic bacteria including *F. succinogenes* and *Ruminococcus* spp. in animals fed a diet containing 30% Calliandra (McSweeney et al., 1998). A similar effect of *Fibrobacter* was observed in this study (Figure 3.17), but not on *Ruminococcus*. Further, the presence of tannins in *A. nilotica* was positively correlated with the relative abundance of the genera *Pseudomonas* and *Bulleidia* (Figure 3.17). Salami et al. (2018) also

reported higher abundance of *Bulleidia* in the presence of condensed tannins in *Acacia negra*. *Pseudomonas* has been shown to produce enzymes that degrade tannins and utilize them for energy (Aboagye & Beauchemin, 2019). In the rumen, microbes that utilize tannins degrade them into their subunits that are subsequently converted through the dihydrophloroglucinol and the 3-hydroxy-5-oxohexanoate pathways to acetate and butyrate to generate energy (Aboagye & Beauchemin, 2019; Bhat, Singh, & Sharma, 1998).

Belanche, Jones, et al. (2016) reported that 5% inclusion of tannin-rich brown seaweed in the diet did not seem to compromise rumen microbial function. While it is possible for rumen microbes to tolerate tannins, the mechanism enabling this has not been elucidated. McSweeney et al.(2001) described adaptive strategies based on the secretion of extracellular polysaccharide that separate the microbial cell wall from active tannins, and formation of a thick glycocalyx with high binding affinity to tannin.

Some effects of PMS on the rumen microbes may be beneficial to ruminants and to production. Inclusion of tannin-rich brown seaweed as feed ingredient was shown to decrease the concentration of *E. coli*, an important faecal contaminator of carcasses and a source of foodborne disease in humans (Belanche, Jones, et al., 2016). In the current study, all the fodder trees tested showed low levels of *Escherichia/Shigella* with the exception of *G. senegalensis* (Figure 3.17). Although this tree species showed total phenol levels comparable and even higher than some of the other species (Table 3.6), only one flavonoid molecule, myricetin-3-O-rhamnoside, was identified (Table 3.7). While myricetin-3-O-rhamnoside activity against *E. coli* has been previously reported (Madikizela, Aderogba, & Van Staden, 2013), the low activity could be due to the lack of an additive effect of having more than one flavonoid molecule acting against the bacteria. Saponins have been reported to increase microbial nitrogen flow from the rumen and decrease methane production *via* defaunation of ruminal protozoa (Abreu et al., 2004; Newbold et al., 1997).

The study of *in vitro* and *in vivo* effects of flavonoids on rumen fermentation and microbial composition has been carried out on individual flavonoids, plant extracts consisting of several flavonoids and blends of plant extracts high in flavonoids in combination with other classes of secondary plant metabolites (Olagaray & Bradford, 2019). Oskoueian, Abdullah, and Oskoueian (2013) evaluated the *in vitro* effect of

flavone, myricetin, naringin, catechin, rutin, quercetin, and kaempferol on the rumen microbial activity. The phenolic compounds were included at the concentration of 4.5% DM the substrate composed of a 60:40 mixture of guinea grass and concentrate. Individual flavonoids quercetin and naringin showed no effect on DMD relative to control. However, DMD was reduced by 5–7% for flavone, myricetin, catechin, rutin, and kaempferol. The authors attributed the reduced DMD and decreased total VFA concentration by flavone, myricetin, and kaempferol to the strong antibacterial effects of these individual flavonoids. Naringin, catechin, rutin, and quercetin all produced total VFA concentrations comparable to control. The utilisation of catechin and rutin as an alternate carbon source for fermentation by the microbes may have been the reason for the lack of expected decrease in VFA concentration despite the reduction in DMD reported in these two compounds (Oskoueian et al., 2013).

The effect of flavonoids on fermentation and microbial population has been shown to be dose dependent (Olagaray & Bradford, 2019). The inclusion of quercetin at lower concentrations (200–250 mg/L) had no effect on VFA concentrations; however, VFA concentrations were increased by 12% with at higher levels of inclusion (500mg/L) (Berger et al., 2015; Lourenço et al., 2008). *In vitro* incubation of 50:50 orchard grass hay + barley substrate with *Solidago vigaurea* extract containing 16.2% DM rutin was reported to show a total VFA concentrations 11% higher than control (Broudiscou, Papon, & Broudiscou, 2000). Similar results were obtained by incubating a rice straw, corn meal and soybean meal (6:3:1) substrate in the presence of a 40mg *Portulaca oleracea* extract containing kaempferol, apigenin, myricetin, quercetin, and luteolin. In this case a 13% increase in total VFA production was reported (Wang et al., 2013).

Flavonoids have been shown to significantly affect the rumen microbial population. Oskoueian et al. (2013) reported that flavone, myricetin, and kaempferol decreased populations of total bacteria, fungi, protozoa, and methanogens compared to control; catechin decreased the total population of protozoa compared to control. Although naringin and quercetin did not affect total bacterial or fungal populations, populations of total protozoa and methanogens were decreased. Flavone, myricetin, catechin, rutin, and kaempferol all decreased DMD.

The effect of flavonoids on rumen bacteria has been attributed to differences in cell membrane structure of gram-positive and negative bacteria. These differences make

flavonoids selective against gram-positive bacteria in the rumen (Olagaray & Bradford, 2019). Flavonoids interact with the nucleophilic centres in peptidoglycan. Gram-positive bacteria have a larger proportion of peptidoglycan in the cell wall compared to their negative counterparts, thus the higher sensitivity (Babii et al., 2016; Babii et al., 2018). This selectivity was demonstrated by Kim et al. (2015) where incubation of rumen fluid with flavonoid-rich plant extracts decreased populations of *Ruminococcus albus* and *Ruminococcus flavefaciens* both Gram positive bacteria. Conversely, the diversity of the gram-negative *Fibrobacter succinogenes* was shown to increase. Since gram-negative bacteria produce more propionate, the reduction in gram-positive bacteria leads to the decrease in acetate-to-propionate ratio seen with flavonoids (Olagaray & Bradford, 2019; Oskoueian et al., 2013; Wang et al., 2013). The effect on VFA production and ratios in this experiment was however shown to be influenced more by the CP content of the substrate than the phenolic content (Figure 3.11); the effect was particularly strong for propionate production (Figure 3.13).

The positive effects of flavonoid-rich plant extracts on methane emission and methanogens population *in vitro* as well as *in vivo* have been examined (Kim et al., 2015). Oskoueian et al. (2013) reported that flavonoids decreased methane production compared to control; the extent of inhibitory action was higher in myricetin, followed by kaempferol, flavone, quercetin, naringin and rutin; and was lowest in catechin (Oskoueian et al., 2013). Extracts of *Equisetum arvense* and *Salvia officinalis* rich in isoquercetin and luteolin-7-glucoside respectively were both shown to decreased methane by 14 and 8%, respectively (Broudiscou et al., 2000). Similar effects were reported for *P. oleracea* and monensin. In this case, methane production as proportion of total gas production was decreased from 21% to 9 and 12%, respectively. This decrease was accompanied by large population decreases for methanogens and protozoa (Wang et al., 2013). Different flavonoids reduced The methanogen population when incubated in inoculum from cattle fed high forage, high concentrate, and a 60:40 forage + concentrate mixture (Oskoueian et al., 2013; Wang et al., 2013). *In vitro* screening therefore suggests the individual flavonoids myricetin, flavone, and kaempferol produce potent antimicrobial effects with negative effects on fermentation; catechin and rutin have little effect, and quercetin and naringin exhibit

positive effects on fermentation (Olagaray & Bradford, 2019). Differences in antimicrobial effects can be attributed to structures of each (Broudiscou et al., 2000).

In vitro results provide valuable preliminary insights on flavonoid interactions with rumen microbes and fermentation. However, it is important to consider dosage and type of rumen inoculum used when interpreting results and designing *in vivo* experiments. In general, relative flavonoid doses used in many *in vitro* studies are much higher than those used or feasible in *in vivo* studies (Olagaray & Bradford, 2019). Additionally, the *in vitro* effects of flavonoids are confounded by the formulation of the test-compound used ranging from individual flavonoids, plant crude extracts to whole plant (Oskoueian et al., 2013).

Compared to *in vitro* studies the effects of flavonoids on fermentation and methane production are much less pronounced during *in vivo* studies; in addition, the results are also influenced by diet (Olagaray & Bradford, 2019). Cui et al. (2015) reported that cows supplemented with 3.0mg/kg rutin in the diet had a lower rumen pH, ammonia nitrogen concentration, number and protein content of rumen protozoa and blood urea nitrogen. On the other hand, the concentration of total volatile fatty acid, microbial crude protein and serum lysozyme content were higher compared to the control group (Cui et al., 2015). Supplementation of sheep with flavonoid-rich mulberry leaf extract at the rate of 2g/head/day decreased ruminal populations of protozoans and methanogens and increased the populations of *Fibrobacter succinogenes*. The result was improved the digestibility of organic matter and reduced methane output by inhibiting the populations of microbes involved in methanogenesis (Ma et al., 2017).The authors also reported an increase in VFA concentration but no change in acetate to propionate ratio. The increase in VFA concentrations was attributed to either improved digestibility of organic matter or degradation of flavonoids (Ma et al., 2017).

Contradictory outcomes regarding the positive effect of dietary flavonoids on digestibility have also been published. Silva et al. (2014) found that supplementation with Propolis extract (containing flavonoids, phenolic acids, esters, phenolic aldehydes and ketones) did not alter the digestibility of dry matter, organic matter, crude protein, neutral detergent fibre, or acid detergent fibre in feedlot lambs. Stelzer et al. (2009) also reported no change in digestibility in lactating cows supplemented with

Propolis ethanolic extract. Indeed, Prado et al. (2010) reported a reduction in DM digestibility in growing cattle on a roughage-based diets supplemented with Propolis extract. It is possible, therefore, that the greater total VFA concentrations reported with supplementation of various flavonoids and extracts can be attributed to the supplement acting as an alternative carbon source for ruminal microbes rather than from improved digestibility (Oskoueian et al., 2013). It must also be considered that flavonoids may alter absorption or passage rates thus contributing to observed changes in VFA concentrations (Olagaray & Bradford, 2019).

In regards to methanogenesis, the previously mentioned study by Ma et al. (2017) showed a decrease in the populations of methanogen and protozoan in response to mulberry leaf flavonoids. The decrease coincided with a 12% decrease in methane emissions. Similarly, supplementation of dairy cows with tannin-rich grape marc resulted in a to the 17–20% drop in methane production (Moate et al., 2014). Conversely, rutin supplementation (100mg/kg for 2 weeks) did not alter methane emissions in dairy cows (Stoldt et al., 2016). In this case, the lack of effect was attributed an insufficient period of exposure of methanogens to supress them and to lower dosage of the supplement compared to the levels deployed in *in vitro* studies.

Rumen microbes can adapt to PSM (Estell, 2010). After an adaptation period, goat were shown to proportionally increase intake of plants with high oxalic acid content (Duncan & Young, 2000). Rumen microbes can metabolise PSM resulting in compounds that are different than those originally consumed (Estell, 2010). Because of rumen microbial activity, ruminants are often exposed to a different array of secondary compounds than originally consumed. In case of ingested monoterpenes and sesquiterpenes, the extent of ruminal degradation was shown to be highly variable (Broudiscou, Cornu, & Rouzeau, 2007). Thus, for this class of compounds, the profile consumed *versus* that present for absorption may be quite different.

Simple plant phenolics undergo varying degrees of transformation and degradation by rumen microorganisms (Martin, 1982). Phenolic glycosides such as rutin and naringin and flavonoids (e.g. quercitrin) are metabolised in the rumen by hydrolysis of the glycoside and cleavage of the heterocyclic ring (Lowry & Kennedy, 1996). The products of flavonoid degradation in the rumen include acetate, butyrate, dihydroxyphenolics, monohydroxyphenolics, and phloroglucinol. The dihydroxyphenolics and

monohydroxyphenolics are probably released from the B ring while phloroglucinol would derive from the A ring of the flavonoid (McSweeney et al., 2001). Although the flavonoid ring systems of the common plant flavonoids are readily degraded in the rumen, there is no evidence of cleavage of the heterocyclic ring system of flavan-3-ols (e.g. catechin, epicatechin) that are the monomeric units of condensed tannins. In fact, catechin is inhibitory to rumen fermentation (Lowry & Kennedy, 1996). Depolymerisation of condensed tannins by cleavage of carbon–carbon bonds has not been demonstrated under anaerobic conditions and may not occur in the rumen (McSweeney et al., 2001).

Microbes may protect ruminants from toxins if metabolism results in degradation rendering them less toxic *via* pre-gastric fermentation (Duncan & Young, 2000). Conversely, microbial PSM metabolism can increase toxicity. For example, hydrolysis of cyanoglucosides and glucosinolates to sugars and aglycones releases cyanide and thiocyanates, respectively (Kakes, 1991; Majak, 2001). Metabolism of gallic acid by rumen microbes releases pyrogallol which is hepatotoxic and nephrotoxic (Murdiati, McSweeney, & Lowry, 1992).

In regards to management of livestock exposed to PMS, additives such as PEG, charcoal, and alkaline treatments (*e.g.*, $CaOH_2$) may prove useful for helping ruminants cope with PSM (reduce absorption and/or increase excretion) and enhance use of non-preferred shrubs (Estell, 2010; Mueller-Harvey, 2006). Sheep fed free-choice PEG regulated its intake in response to tannin intake and spent more time in locations where PEG was present when consuming high-tannin diets (Provenza et al., 2000; Villalba & Provenza, 2002). Altered browsing behaviour (increased number/decreased length of feeding bouts) was restored when PEG was fed to small ruminants consuming high-tannin shrubs (Perevolotsky et al., 2006).

Alterations in rumen fermentation can increase overall animal efficiency by increasing energy digestibility and reducing methane production. Methane production accounts for 2–12% of gross energy intake losses in ruminants and is a potent greenhouse gas (Olagaray & Bradford, 2019). Some flavonoids have selective antibacterial effects against gram-positive bacteria (Cushnie & Lamb, 2005); reduction of Gram-positive bacteria increases the production of propionate relative to acetate thus improving energy metabolism. Both of these manipulations can contribute to increased efficiency

of the ruminant animal (Olagaray & Bradford, 2019). Bactericidal activity has been attributed to several mechanisms including cytoplasmic membrane damage, inhibition of nucleic acid synthesis, inhibition of energy metabolism, inhibition of cell wall synthesis and inhibition of cell membrane synthesis (Cushnie & Lamb, 2011).

Gas production is a reflection of generation of VFAs. The other nutritionally important fermentation product is microbial biomass. Both these products are linked by ATP production. This can impose an inverse relationship upon VFA and microbial biomass yield (Leng, 1993). This relationship applies also for gas production *in vitro* and microbial biomass yield when both variables were related to a unit of substrate fermented (Blümmel, Makkar, & Becker, 1997). In ruminant nutrition, it is recommended to select substrates for high microbial biomass production (Leng, 1993; Van Soest, 1994, p. 476). In line with this, Blümmel et al. (1997) suggested selection of roughages for high substrate degradability, but proportionally low gas production.

To enable this selection, *in vitro* gas production may be complemented with residue determination when evaluating of the nutritive value of feeds (Blümmel & Bullerdieck, 1997). However, this approach may result in inaccurate results in substrates containing significant levels of plant polyphenols. This is due to leaching of polyphenols from the feed during fermentation, contributing to the dry matter loss but without contributing to the gas (Getachew et al., 1998). Therefore, in polyphenol-containing feedstuffs, gas measurements should be combined with microbial mass determination using qPCR (Makkar, Blümmel, & Becker, 1998).

3.7 CONCLUSIONS

The results showed a large variation in the chemical components of leaves among the tree species and an effect of ecological zone between samples of the same species. Except for one sample (*Lannea* sp.), leaves from all tree species had CP content of >10% demonstrating that leaves from the majority of tree species can be used as low-cost CP supplement. However, the large variations also mean that the fodder trees cannot be applied universally to ruminant diets; each of the species would have a separate inclusion rate. This problem is compounded by the differences in chemical composition seen in the same species of fodder growing in the difference ecological zones. The demonstrated differences in the microbial community resulting from the different feed species experiment warrants further investigation on whether this is a positive or negative effect in terms of improving the efficiency of fermentation in the rumen.

CHAPTER 4: COMPARISON OF RUMEN MICROBIAL PROFILES IN SHEEP RETRIEVED BY ION TORRENT AND OXFORD NANOPORE SEQUENCING PLATFORMS

4.1 INTRODUCTION

The rumen ecosystem is one of the most complex microbial ecosystems know to microbiologists. It has an intricate web of interaction between the microorganisms, the host's physiology and the material ingested (Ouwehand & Vaughan, 2006, p. 387). Great efforts have been made to understand the composition of the rumen microbial community and how it changes in response to a variety of selective pressures (Huws et al., 2018; Huws et al., 2014).

Scientists began to study the rumen ecology in the 19th century as part of the burgeoning field of microbiology. There were early attempts to grow cellulolytic bacteria from the rumen which involved adding rumen fluid to a meat-extract medium; although these attempts were not successful in growing cellulolytic bacteria, fermentation acids were detected, which indicated the fermentation process occurred in the rumen (Tappeiner, 1884). Efforts to understand the relationship between rumen fluid, bacteria and fibre digestion continued into the 20th century with modest success. Eventually in 1944 Robert Hungate was able to successfully grow cellulose-digesting rumen bacteria through a combination of anaerobic techniques and reducing agents (Hungate, 1944). The modifications included having ruminal fluid as an essential nutritional supplement in the media, creating CO_2 rich atmosphere and introducing buffers to simulate the effect of saliva in the rumen. Since then, this approach has been modified further to isolate various strains of bacteria in the rumen. Additionally, rumen bacteria were exposed to other sugar substrates (other than cellulose) to observe their responses; such substrates include lactate, pectin, cellobiose, trehalose, sorbitol, salicin and xylose. In some instance, strains of bacteria were enriched by the choice of substrate and *vice versa* (Russell, Strobel, & Chen, 1988).

While a substantial body of knowledge on the biology of the principal cellulolytic bacteria has been generated using culturing techniques, only 3.6% of the operational taxonomic units (OTUs) found by sequencing have representative isolates (Zehavi, Probst, & Mizrahi, 2018). Additionally, while microscopic examination of rumen

contents shows that the rumen contains a rich diversity of microbes (Creevey et al., 2014), few microorganisms have sufficiently distinctive morphology to be recognized by microscopy (Ward, Weller, & Bateson, 1990). Currently, only 117 bacterial species (excluding different strains) of rumen origin are available from international culture collections (Zehavi et al., 2018). The limitations to isolating rumen microbes in pure culture could be due to several factors. Some bacteria may be changing their metabolic status once they are removed from their preferred niche in the rumen and thus do not enter a multiplication state when put on artificial media (Zehavi et al., 2018). It could also be due to the inability of microbiologists to reproduce the exact *in vivo* environment for the microbes that are much more fastidious or have complex interactions within the ecosystem. Therefore culture-based approaches do not provide a representative picture of the ecosystem in regards to its members and their interactions (Tajima et al., 1999). This has compelled scientist to shift their approach to using molecular techniques to further the research (Creevey et al., 2014).

The application of cultivation-independent methods such as metagenomics has greatly enhanced the understanding of the structure and function of the rumen microbial ecosystem (Ribeiro et al., 2016). These cultivation-independent studies suggest that many of the microbes of the rumen are uncharacterized (Hess et al., 2011). Initially, these techniques were complex and expensive, but have progressively become easier and cheaper (Muers, 2011); thus precipitating a great expansion in the understanding of not only rumen microbes but also microbial ecosystems in general.

4.2 MOLECULAR TECHNIQUES

The majority of molecular techniques used to study microbial ecosystems involve the identification and differentiation of a variety of biomarkers such as cell wall components, proteins, lipids, DNA and RNA. Differences in the small subunit (16S) ribosomal RNA between microbes is among the mostly widely used biomarker. When scientists adopted this approach, they quickly discovered that there is a much wider diversity of microbes in various ecosystems than it was previously believed and raised questions of how well the Earth's biota and its biochemical potential was understood (Ward et al., 1990).

Improvement of genomic technologies has revolutionised the study of microbial ecosystems by permitting high-throughput techniques such as DNA metabarcoding. DNA metabarcoding refers to high-throughput multispecies identification using the total DNA extracted from an environmental sample (Taberlet et al., 2012). DNA metabarcoding is based on the use of primers specific coupled with next-generation sequencing (NGS) (Valentini et al., 2016).

NEXT GENERATION SEQUENCING (NGS)

The first NGS platform to enter the field was pyrosequencing. It was developed by Ronaghi et al. (1998) and was commercialised in form of the GS-FLX pyrosequencer which initially could generate 100bp read lengths which were later improved to up to 500bp. Illumina sequencing is the other pioneering NGS technology. The method was patented by Canard and Safarti (1994) and is based on reversible dye-terminators that enable the identification of single bases as they are introduced into DNA strands. It is comparatively cheaper than pyrosequencing since it only uses DNA polymerase enzyme as opposed to multiple enzymes used in pyrosequencing (Pettersson, Lundeberg, & Ahmadian, 2009). Additionally, it has a 99% read accuracy compared to 454 pyrosequencing (98.93%). The Ion Torrent personal genome machine (PGM) was launched in February 2010 (Rusk, 2011). It is based on the detection of hydrogen ions that are released during the polymerization of DNA *i.e.* "sequencing by synthesis".

The rapid drop in cost and increase in scale of DNA sequencing is often compared to the trend seen in the semiconductor industry in the second half of the twentieth century, which was described by Moore's law (Muers, 2011). Consequently, the

platforms which were dubbed “next generation sequencing” have been relegated to being called “second generation sequencing” due to the rise of a third generation (Van Dijk et al., 2018). Second-generation sequencing platforms produce short reads a few hundred base-pairs long. As a result, although they can be applied for population-scale analyses of single nucleotide and other small variants, the short reads mean that they are limited when the structural variations are larger. The limitation led to the development of new single-molecule sequencing technologies that can produce longer read lengths (>10,000bp up to 100,000bp or more) (Lee et al., 2016). Many third-generation sequencing platforms are still in development and are yet to overcome limitations such as high error rates. Consequently, despite its limitations, NGS remains the standard approach many laboratories (Van Dijk et al., 2018). However, both first and second-generation platforms still provide a valuable and reliable tool for studying the rumen microbial ecosystem. Comparisons between first and second generation sequencing platforms have showed a strong correlation between the results when used to study the bacterial diversity in the rumen (de la Fuente et al., 2014). However care must be taken when comparing sequencing platforms to ensure that studies being compared are consistent in DNA extraction methods, PCR protocols, and bioinformatics pipelines (Indugu et al., 2016).

THIRD GENERATION SEQUENCING (TGS)

Currently, there are three commercially available TGS platforms. These are the Pacific Biosciences (PacBio) Single Molecule Real Time (SMRT) sequencing, the Illumina Tru-seq Synthetic Long-Read technology and the Oxford Nanopore Technologies (ONT) sequencing platforms. All three can produce long reads averaging between 5,000bp to 15,000bp, with some reads exceeding 100,000bp. The platforms use single-molecule sequencing or clonal amplification and sequencing of long molecules.

PacBio SMRT technology was commercially launched in 2010. It sequences DNA using sequencing-by-synthesis, and optically monitors fluorescently tagged nucleotides as they are incorporated into individual template molecules (Roberts, Carneiro, & Schatz, 2013). The PacBio RS II model produces read lengths of up to 100,000bp with the greatest throughput (approximately 8GB per day) of the currently available long-read technologies and is poised to increase the output seven-fold with the recent

introduction of their PacBio Sequel instrument. The main disadvantage of PacBio sequencing is the significantly higher cost compared to second-generation plantforms. While this has limited its application for analysing large numbers of genomes (including metagenomes), numerous projects have successfully used PacBio sequencing to produce high quality genomes of microbes, fungi, plant and vertebrate species (Berlin et al., 2015).

Illumina TruSeq Synthetic Long Reads was initially marketed as Moleculo protocol when it was introduced in 2012 (Kuleshov et al., 2014). Using this approach, ~10kbp molecules of DNA are clonally amplified and barcoded before sequencing with a short-read instrument; long reads are then synthetically created from the short-read sequences. The synthetic long reads are very accurate with an approximately 0.1% error and can be used for phasing analyses and assembly without error correction. However, the available read lengths generated by the platform are shorter than the other TGS approaches because TruSeq relies on long-range amplification and the reads are synthetically generated. It is also prone to termination and biases in any region where the Illumina chemistry is biased, such as regions with high GC content or tandem repeats. Further, since 900x to 1500x or more short read coverage may be required to assemble 30x coverage of synthetic long reads, generating sufficient coverage for *de novo* genome assembly can be even more costly than PacBio sequencing. Regardless, the platform has been employed widely for assembling complex genomes (Kuleshov et al., 2014).

The most recent TGS technology was released by Oxford Nanopore Technologies in 2014 (Loman & Watson, 2015). The Oxford Nanopore MinION is a handheld device that sequences DNA by electronically measuring the minute disruptions to electric current as DNA molecules pass through a nanopore (Berlin et al., 2015). Measuring only 10 x 3 x 2 cm and weighing just 90g, the MinION is the smallest sequencing device currently available. It plugs directly into a standard USB3 port on a computer with low hardware requirement and simple configuration.

Nanopore DNA sequencing is performed by adding the sample to the flow cell. When DNA molecules pass through or near the nanopore, there is a change in the magnitude of the current in the nanopore, which is measured by a sensor. The data streams are passed to the application-specific integrated circuit (ASIC) and MinKNOW (the

software that runs on the host computer) generates the signal-level data. The MinION currently generates read lengths similar to those produced by PacBio. However, the reads still have a lower comparative accuracy and throughput (Loman, Quick, & Simpson, 2015).

The MinION however has the advantage of being comparatively smaller and cheaper enabling its use in very remote locations. For instance, the MinION was used to monitor the Ebola outbreaks in the field in West Africa (Quick et al., 2016), and for metagenomic characterization of microbiota in a High Arctic glacier(Edwards et al., 2016). Further, DNA was for the first time ever successfully sequenced in space using the MinION on the International Space Station (ISS) (Castro-Wallace et al., 2017).

The MinION sequences single DNA molecules and enables very long reads to be obtained, compared to most second-generation sequencing platforms (Kerkhof et al., 2017). Consequently, this platform can generate nearly full-length rRNA operon sequence data yielding species resolution when used for rRNA gene characterisation (Benítez-Páez, Portune, & Sanz, 2016). While researchers around the world have begun apply the MinION to determine near a full-length sequence of 16S rRNA gene amplicons (Kerkhof et al., 2017), the ability this platform to analyse the gut microbiota composition at the species level has not been fully demonstrated in comparison with that of short-read sequencing technologies (Shin et al., 2016). Most full-length 16S rRNA gene studies currently published tested mock communities to demonstrate proof-of-concept rather than using the MinION as the stand-alone platform to detect experimental perturbation (*e.g*., light, temperature, nutrient variation) on microbial communities (Kerkhof et al., 2017). The MinION could provide a nearly full-length sequence of 16S rRNA gene amplicons and generate species-level resolution a 20-member model community using representative DNA from different bacterial phyla with accuracies ranging from 80 to 94% (Benítez-Páez et al., 2016; Brown et al., 2017). Mitsuhashi et al. (2017) analysed a 20-member mock community and reported that the results were comparable to 16S rDNA sequencing results using an Ion Torrent PGM sequencer for time, effectiveness and accuracy.

A study by Shin et al. (2016) who sequenced the microbial community in mouse faecal material was is among the few studies to study a complex environmental sample. The authors reported identifying more bacterial species using the MinION data compared

with the MiSeq, and could demonstrated robust phylogenetic resolution of species. Kerkhof et al. (2017) used the MinION to analyse full-length 16S amplicons from two different complex microbial communities mixed in known concentrations (farm soil and a grey-water bioreactor). They reported that the MinION had the ability to provide rRNA operon sequence data of sufficient quality for characterizing the microbiota of complex environmental samples and that the results that were reproducible, quantitative, and consistent (Kerkhof et al., 2017). So far, no studies have been published analysis of full-length 16S amplicons of rumen bacteria with the MinION.

4.3 AIMS AND OBJECTIVES

The lack of sufficient understanding of the rumen microbiome is one of the major knowledge gaps hindering effective enhancement and modification of rumen function (Huws et al., 2018; Kingston-Smith, Davies, et al., 2013). Recently, the use of next generation sequencing platforms has greatly enhanced our knowledge of rumen microbes, their genes and enzymes (Indugu et al., 2016). While it is accepted that platforms requiring significant investment are the “gold standard” for delivering sequence data (Van Dijk et al., 2018), it is possible that results from newer, less expensive technologies may be acceptable for some applications. Here, a comparison was made of the relative suitability of 16S sequencing on two platforms, Ion Torrent PGM and The Oxford Nanopore MinION.

4.4 EXPERIMENTAL DESIGN, MATERIALS AND METHODS

This chapter was a continuation of the investigation detailed in Chapter 3. The microbial DNA extracted and for that experiment was retrieved from -80^oC storage and sequenced using the MinION. Samples were selected to represent the microbial populations present during fermentation of four different fodder tree leaves species *i.e. Acacia nilotica*, *Guiera senegalensis*, *Piliostigma reticulatum* and *Ziziphus mauritiana* (Chapter 3: Section 3.3.4), specifically those that had exhibited a strong effect of the tree species on microbial population after Ion Torrent sequencing. *In vitro* incubation (Section 2.1.4), DNA extraction, library preparation, sequencing and bioinformatics for both Ion Torrent (Section 2.1.6) and Nanopore sequencing (Section

2.1.7) are detailed in Chapter 2. Ion Torrent sequencing results for the selected samples obtained in Chapter 3 were also retrieved for comparison.

After sequencing and bioinformatics, the relative bacterial taxa abundance data were subjected to square root transformation. Principal coordinate analysis (PCO) ordination on a Bray-Curtis distance matrix was done to identify patterns in the data. The tree species effects on transformed data were analysed based on their Bray-Curtis distance metric within the function unweighted pair group method with arithmetic mean (UPGMA). One-way PERMANOVA was carried out using default settings with unrestricted permutations. All tests were carried out using the PAST v3 statistical package (Hammer et al., 2001).

4.5 RESULTS AND DISCUSSION

The 16S rRNA gene is an important genetic marker for the characterization of microbial community structure by 16S rRNA gene amplicon sequencing with conserved primers (Schmalenberger, Schwieger, & Tebbe, 2001). Bacterial 16S rRNA genes contain nine "hypervariable regions" (V1 – V9) that demonstrate considerable sequence diversity among different bacteria (Van de Peer, Chapelle, & De Wachter, 1996). Hypervariable regions are flanked by conserved stretches in most bacteria, enabling PCR amplification of target sequences using universal primers. Species-specific sequences within a given hypervariable region constitute useful targets for diagnostic assays and other scientific investigations. No single region can differentiate among all bacteria (Chakravorty et al., 2007). Until recently, most studies of microbiota have relied upon second-generation sequencing platforms that examine 1-2 of the hypervariable regions of the 16S gene. The most common choices for host-associated microbiota are V4 or V1-V2 regions, which present different taxonomic coverage and resolution depending on the taxa (Cuscó et al., 2018). The V1–V2 amplicon has a total sequencing template length of approximately 437 base pairs (Watts et al., 2017).

While second-generation sequencing platforms produce high quality short read length sequences, it can be difficult to obtain taxonomic assignment of the sequences down to the species level (Cuscó et al., 2018). The introduction of third-generation

sequencing technologies with ultra-long read capabilities has made it possible to obtain species level and even sub-species level taxonomic identification. Nanopore technology is capable of sequencing ultra-long reads enabling the 16S gene to be sequenced in its entirety (Benítez-Páez et al., 2016). The increase in read length means that different multi-hypervariable regions can be selected for amplification using universal and barcoded primers. The most common primer pairs used in molecular microbial ecology research for constructing clone libraries of the full-length 16S rRNA gene sequence are 27F and 1492R (Frank et al., 2008; Mao et al., 2012). Amplicons generated from this pair have expected size of ∼1400bp (dos Santos et al., 2019).

For this study, MinION was tested on whether it could be used to rapidly sequence bacterial ribosomal operons from rumen samples. To validate the approach, samples were selected from an earlier experiment which had demonstrated a treatment effect of fodder tree species on sheep rumen bacteria *in vitro* (Chapter 3). For Nanopore sequencing, PCR amplification of 16S rRNA genes was conducted using the SQK-RAB204 16S Barcoding Kit containing the 27F/1492R primer set (Kai et al., 2018). The primers contain barcodes and 5' tags, which facilitate the ligase-free attachment of Rapid Sequencing Adapters. The kit contains twelve barcoded primer pairs. For Ion Torrent sequencing, Amplification of the V1–V2 hypervariable regions of the 16S rRNA gene was carried out using bacterial primers (27F and 357R) followed by Ion Torrent adaptors (Morales et al., 2018). In both cases, severe problems were encountered when carrying out the PCR reactions. The samples used were selected from the experiment detailed on Chapter 3; the DNA was extracted after various fodder tree species were incubated *in vitro* in rumen fluid. The resultant DNA had significant levels of contamination with plant polyphenol. When DNA purity is measured using Nanodrop these compounds absorb around 230nm resulting in a lower 260/230 optical density (OD) ratio (Desjardins & Conklin, 2010) than the recommended cut-off of 1.8 (Sambrook & Russell, 2001, p. A8.20). When in oxidized form, polyphenolics and tannins covalently bind with DNA and making it resistant to restriction enzymes (Katterman & Shattuck, 1983). Kingston-Smith et al. (2003) reported a similar problem while extracting DNA from red clover (*Trifolium pratense*). The efforts to overcome this contamination for Ion Torrent sequencing is detailed in Chapter 2 (Section 2.1.6). Despite these efforts, the DNA still did not meet the recommended purity for

Nanopore library preparation (OD 260/280 of 1.8 and OD 260/230 of 2.0-2.2) and as a result did not amplify when PCR was attempted. Stewart et al. (2019) reported similar problems when using MinION for rumen microbiome DNA while following standard recommended protocols. They reported that while clean up with methods such as AMPure XP beads were sufficient to obtain optical density ratios within the recommended range, DNA from these methods typically led to poor or failed sequencing runs. The authors were able to overcome the problem using RNase treatment and phenol–chloroform purification (Stewart et al., 2019).

To overcome the problem of contamination in this study, the amplification of 16S RNA gene was performed in two steps. The first PCR was carried out with universal primers 27F/1492R. The second (nested) PCR was done with barcoded primers 27F/1492R primers from the SQK-RAB204 barcoding kit; and with the product of a first PCR serving as the template. Nested PCRs have been used previously with Nanopore sequencing for full-length 16S rRNA gene microbiota profiling (Cuscó et al., 2018) and 18S rRNA gene identification of human *Plasmodium* species (Imai et al., 2017).

The two sequencing platforms generated the expected length of sequences with Ion Torrent at ∼350bp and Nanopore MinION at ∼1500bp (Figure 4.1). Ion Torrent sequencing generated 30,000 to 200,000 reads per barcode. The MinION produced between 8,000 and 24,000 reads per barcode; a majority of which were >1400bp (Table 4.1). During processing, the Nanopore reads were size-filtered to only those reads between 1300 and 1600bp. Long-read sequences generated using MinION sequencing can compensate for low numbers of reads for bacterial classification (Taylor et al., 2019).

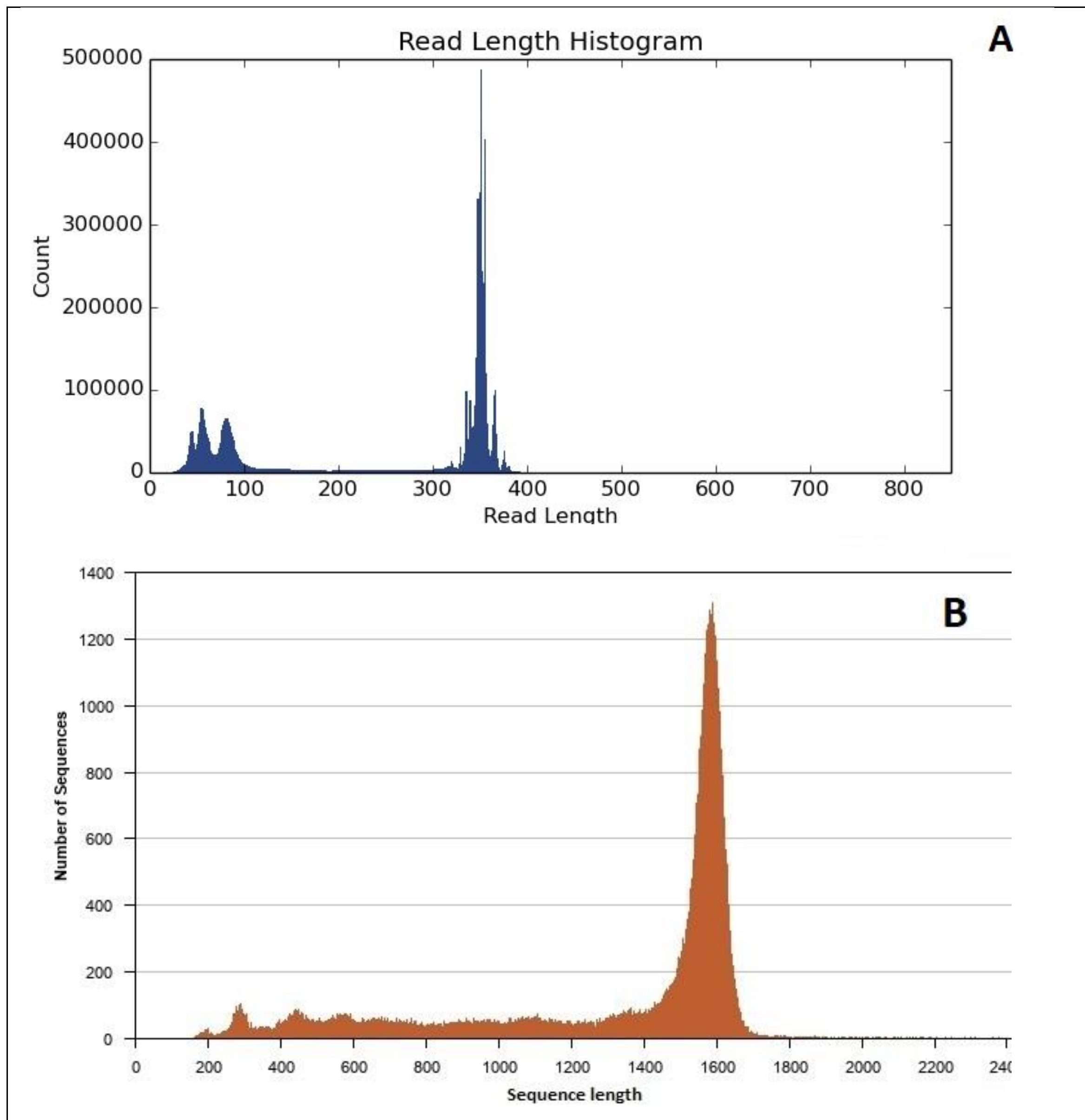


Figure 4.1: Run statistics and read/sequence length (base-pairs) histogram for library metagenome sequenced using (A) Ion Torrent PGM and (B) Oxford Nanopore MinION

Table 4.1: Summary of run statistics for library metagenome sequenced using Oxford Nanopore MinION

Substrate	**Barcode**	**No. of Sequences**	**Sequence length (base pairs)**	
			Minimum	**Maximum**
A. nilotica	Barcode 1	10,469	143	3,503
	Barcode 2	8,441	119	4,123
	Barcode 3	11,974	166	3,428
G. senegalensis	Barcode 4	18,337	184	4,520
	Barcode 5	15,050	171	4,637
	Barcode 6	13,751	186	3,588
P. reticulatum	Barcode 7	11,416	180	4,102
	Barcode 8	11,948	166	4,735
	Barcode 9	14,545	176	4,791
Z. mauritiana	Barcode 10	19,048	126	3,685
	Barcode 11	21,209	134	4,738
	Barcode 12	24,326	160	4,614

An essential first step in analysing targeted sequencing data sets is the demarcation of basic units of diversity ideally corresponding to true microbial lineages present in the sample (Schmidt, Matias Rodrigues, & von Mering, 2015).

The inability to determine complete 16S rDNA sequences using first and second-generation sequencing has led to the development of multiple algorithms designed to theoretically discern the microbial species present in samples according to the level of similarly between sequences *i.e.* Operational Taxonomic Units (OTUs) (Benítez-Páez et al., 2016). OTU-based approaches retain a significant degree of uncertainty especially in regard to the number and abundances of theoretical species. While OTUs are ecologically consistent in general, there are significant differences between clustering methods (Schmidt, Rodrigues, & Von Mering, 2014). Clustering methods are also susceptible even to slight threshold variations (Schmidt et al., 2015). In addition, commonly accepted denoising and clustering methods used in different combinations produce significantly different outcomes (Koskinen et al., 2015). Reproducibility in microbial ecology research depends on the reliability of OTU-based 16S ribosomal subunit RNA (rRNA) analyses. Closed-reference methods produce completely stable OTUs; however, sequences that do not match a pre-existing reference sequence collection are discarded (He et al., 2015). In addition to unstable OTUs many public database accessions may contain sequences from species other than the species of question; these include symbionts, parasites, pathogens, and sequencing linkers/primers/adapters that can lead to false discovery rates (Brown et al., 2017).

The generation of OTUs from complex rRNA gene data sets is a conceptually straightforward task and is usually computationally efficient. As a result, bioinformatic pipelines for analysis of data second generation of massive and parallel sequencing methods have been standardized, mostly using QIIME and MOTHUR (Pitta et al., 2018). A major constraint for metagenomic applications to the rumen microbiome is the complexity of bioinformatics required to analyse the massive amounts of sequencing data. Short reads obtained from sequencing runs are often assembled to form contigs for predictive annotations (Kerkhof et al., 2017). The software used for this (*e.g.,* QIIME and MOTHUR), although free available, are ran using command lines. As a result, they are not friendly to users without training in the proper UNIX syntax and Python scripts. The large computational and data storage requirements limit the use of these technologies to organizations that maintain or can access such computing facilities (Pitta et al., 2018). The introduction of single-molecule sequencing platforms has opened the possibility of obtaining ultra-long reads (Calus, Ijaz, & Pinto, 2018). Long reads sequencing of the 16S rRNA gene is a promising approach to provide high-resolution analysis of microbial communities at the species level (Shin et al., 2016). Long reads can circumvent the disadvantages of short-read platforms, as long as a sufficient proportion of the genome for each component organism is available in the sequencing library and there are minimal errors in the sequences and the reference database (Brown et al., 2017).

In this study, the Nanopore sequences were quality-filtered to a mean quality score of using the Filtlong, a tool designed to filter long noisy sequencing data (Wick, 2019). Filtlong has been used to analyse Nanopore data previously (Liechti et al., 2019; Nakagawa, Tsuchiya, & Takahashi, 2019). Passed reads were then mapped to the to the RefSeq 16S database with Minimap2 a general-purpose alignment program for mapping long sequences against a large reference database (Li, 2018). Ion Torrent reads were clustered into >2300 OTUs compared to 292 species from the Nanopore data. Although Nanopore-based sequencing produces fewer reads compared to Ion Torrent, the MinION DNA sequencer full-length 16S rRNA sequencing provided higher taxonomic resolution (Figure 4.3, Figure 4.4) and microbial diversity analysis (Figure 4.5).

Taxonomic identification is fundamental to all microbiology studies. The power of sequencing-based community analysis relies on there being sequenced type strains deposited in culture; otherwise results are many “unclassified”. A lot of effort is required to collect and maintain the most up-to-date taxonomic information; as a result, only a few public 16S reference databases are available for microbial identification (Park & Won, 2018). The various reference databases originating from microbial isolates are still insufficient, limiting our ability to interpret and understand existing and new data. Therefore, there is an urgent need to improve the reference databases and to isolate yet-to-be-cultured microorganisms (Zehavi et al., 2018). Efforts such as The Hungate1000 project endeavour to increase the microbial culture collection of rumen origin. Before this project began, only 15 rumen microbial genomes were available to the scientific community. The project generated an additional reference set of 410 genome sequences of microbes found in the rumen of sheep and cattle representing approximately 75% of the genus-level bacterial and archaeal taxa present in the rumen (Seshadri et al., 2018).

The dominant bacterial phyla in the rumen are Firmicutes, Bacteroidetes and Proteobacteria, accounting for 57.8%, 26.7% and 6.9% (M. Kim et al., 2011). In this study, the sequences from both platforms were also dominated by Firmicutes, Bacteroidetes and Proteobacteria, although their relative abundance differed depending on the sequencing platform used (Figure 4.2). The Firmicutes to Bacteroides ratio was 0.6:1 in Ion Torrent results and 2.2:1 from Nanopore results. The difference could be due to a number of factors. Bahl, Bergström, and Licht (2012) reported that freezing of human faecal samples increased the Firmicutes to Bacteroidetes ratio. The authors suggested that shift could be caused by a better extraction or stability of PCR amplifiable DNA from of gram-positive (Firmicutes) compared gram-negative bacteria (Bacteroidetes) following freeze storage. They attributed this to differences in the cellular composition between the two (Bahl et al., 2012). The relative ratio in abundance of Bacteroidetes and Firmicutes OTUs from rumen samples was also affected by the addition of cryoprotectants when freezing, sample treatment and the type of analysed DNA (Fliegerova et al., 2014). The DNA used in this experiment had been in storage at -80˚C for several months between the two runs without any cryoprotectants; this may have contributed to the shift in the

Firmicutes to Bacteroidetes ratio. On the other hand, Firmicutes has been shown to be the dominant phylum in sheep rumen (Tanca et al., 2017; Wang et al., 2017) and the MinION may have detected a more accurate phylogenetic composition than Ion Torrent. The use of different primer pairs could also have contributed to the shift in the ratio. Although considered universal, the primers will target different ranges of bacteria and will thus show differences in the fractions of the bacterial community (Fredriksson, Hermansson, & Wilén, 2013).

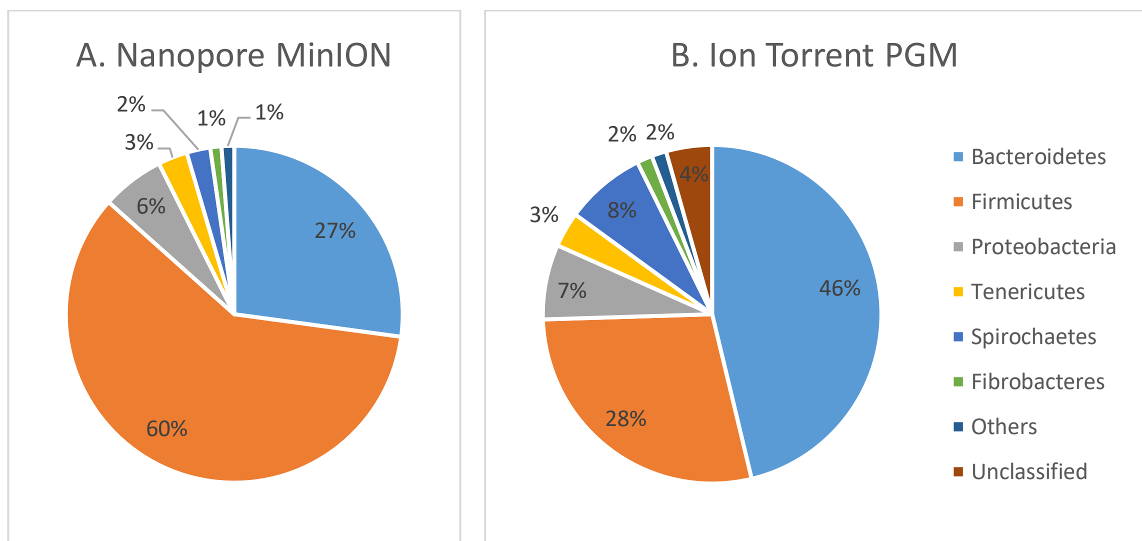


Figure 4.2: Pie chart showing the average distribution of the phyla (>0.05%) across all ruminal samples sequence using the (A) Oxford Nanopore MinION and the (B) Ion Torrent PGM. Samples standardised by total.

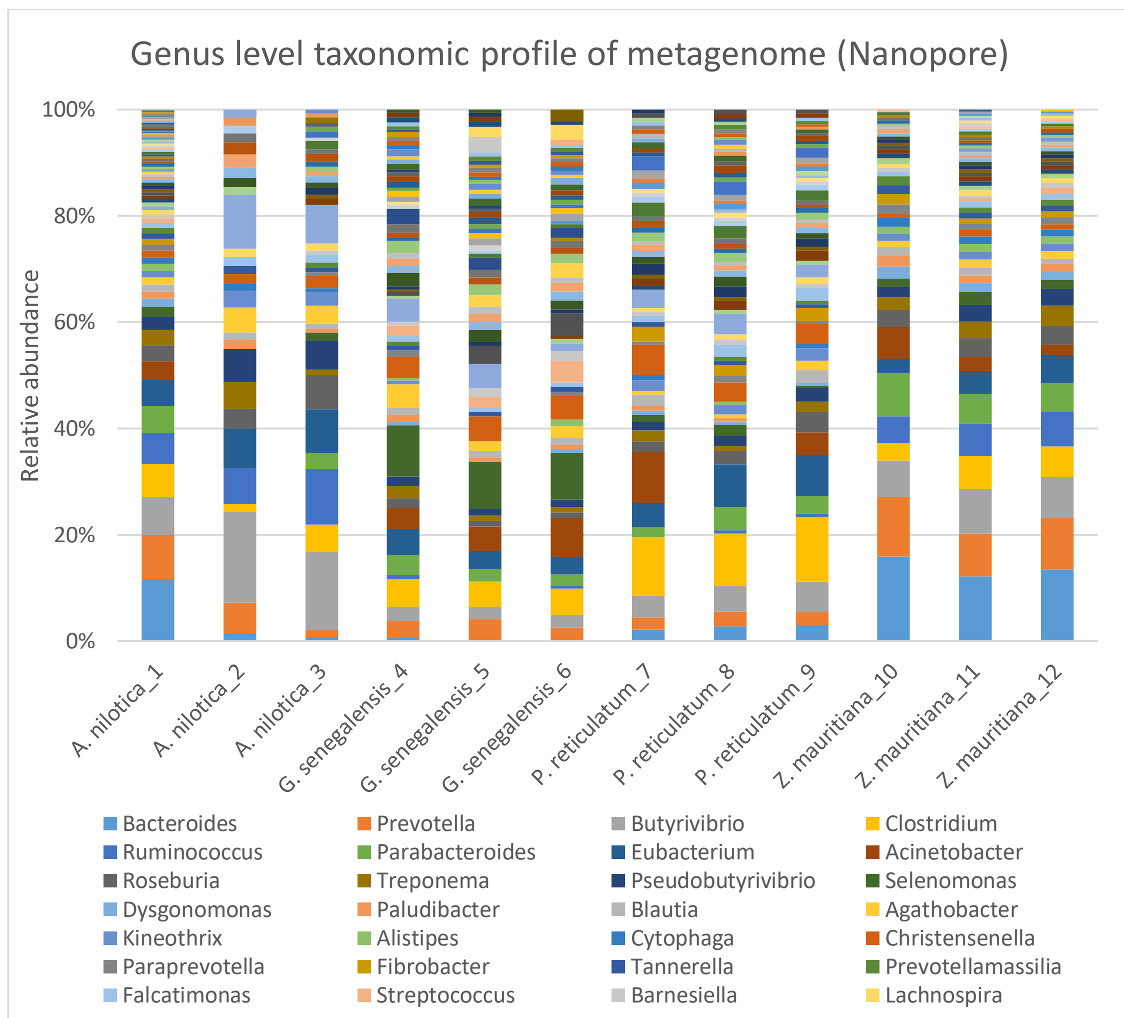


Figure 4.3: Stacked histograms illustrating genus-level bacterial composition present at an average of more than 0.05% showing the *in vitro* effect of *A. nilotica*, *G. senegalensis*, *P. reticulatum* and *Z. mauritiana* leaves on sheep rumen bacteria analysed using Oxford Nanopore MinION 16S rRNA gene sequencing. Reads were mapped with Minimap2 against RefSeq 16S database. Samples standardised by total and transformed by square root.

The Ion Torrent sequences showed a large number (>2300) of OTUs compared to the MinION (292). However, the majority of the OTUs from the former were unclassified (Figure 4.4). As a result, the MinION sequences showed a larger number of identified bacteria genera in the samples (Figure 4.3). This difference was also reflected in the diversity of the microbial population with MinION detecting a higher diversity that the Ion Torrent as both Shannon and Simpson indices (Figure 4.5).

The MinION was able to detect the treatment effect of the sample. The experiment on detailed in Chapter 3 showed that there was a significant effect of the fodder tree species on rumen bacteria *in vitro*. PCO analysis of the data selected from the Ion Torrent sequencing showed a separation between treatments with the primary axes accounting for 79.9% of total variation (Figure 4.6B, Figure 4.7B). One-way

PERMANOVA showed a significant difference between the treatment (p=0.0001). The treatment effect was also seen with MinION; PCO analysis also showed a separation between treatment with the primary axes accounting for 77.1% of the variation (Figure 4.6A, Figure 4.7A). One-way PERMANOVA also showed a significant difference between the treatment albeit to a lower extent (p=0.0012). The resolution of the treatment groups clustering was lower with the MinION than in the Ion Torrent, particularly for *A. nilotica* samples.

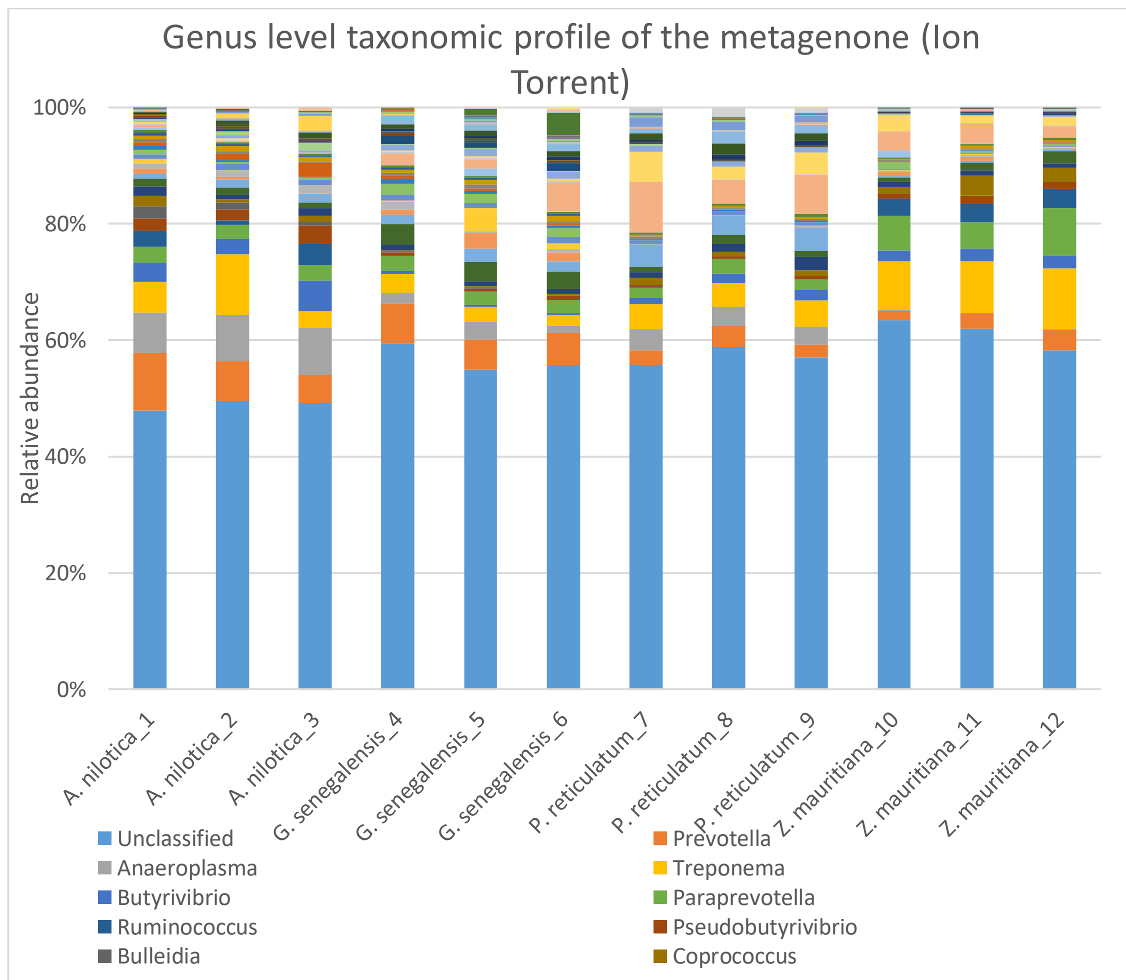


Figure 4.4: Stacked histograms illustrating genus-level bacterial composition present at an average of more than 0.05% showing the *in vitro* effect of *A. nilotica*, *G. senegalensis*, *P. reticulatum* and *Z. mauritiana* leaves on sheep rumen bacteria analysed using Ion Torrent PGM 16S rRNA gene sequencing. Reads were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II. Samples standardised by total and transformed by square root

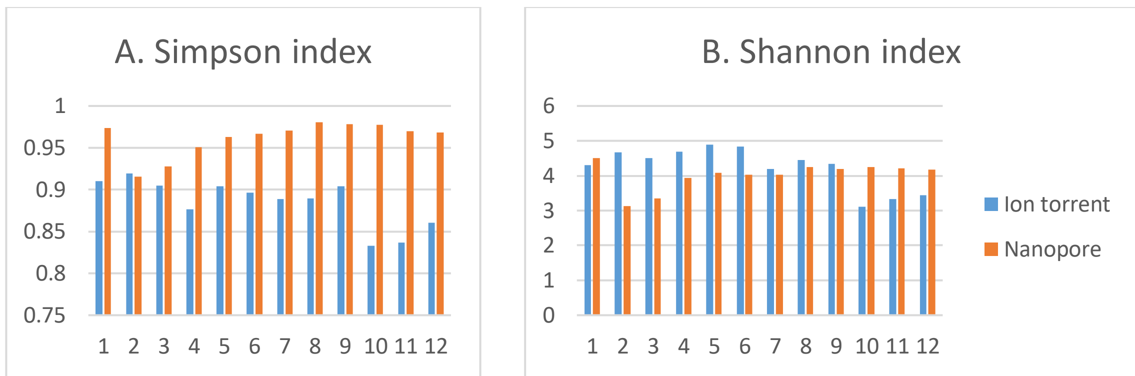


Figure 4.5: Simpson (A) and Shannon (B) diversity indices of sheep rumen bacteria incubated with three replicates of leaves from each of *A. nilotica*, *G. senegalensis*, *P. reticulatum* and *Z. mauritiana* analysed using Oxford Nanopore MinION and Ion Torrent PGM 16S rRNA gene sequencing.

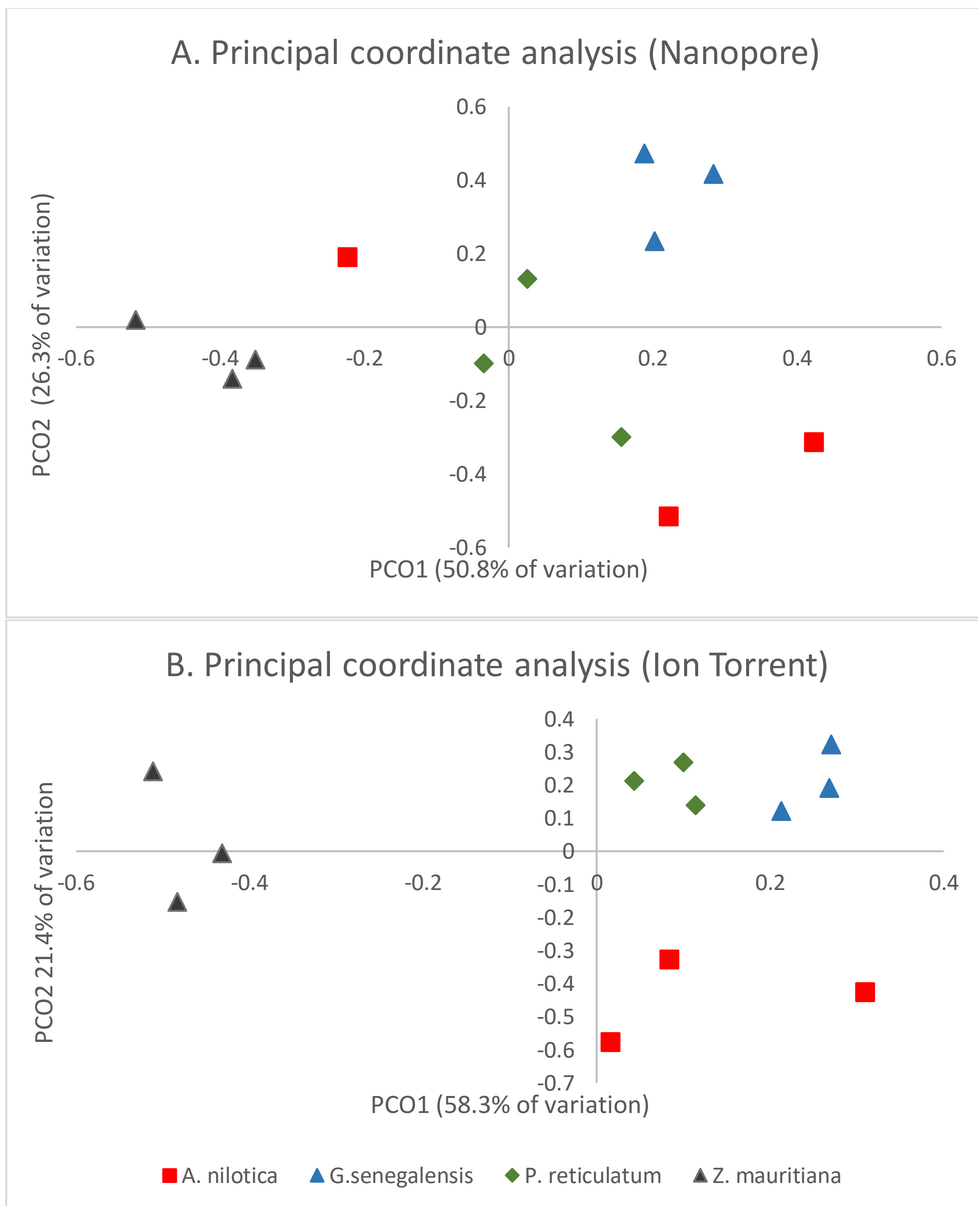


Figure 4.6: PCO analysis of the *in vitro* effect of fermentation of *A. nilotica, G. senegalensis*, *P. reticulatum* and *Z. mauritiana* leaves by sheep rumen bacteria analysed using (A) Oxford Nanopore MinION and (B) Ion Torrent 16S rRNA gene sequencing. Samples standardised by total and transformed by square root. Resemblance: Bray-Curtis Similarity.

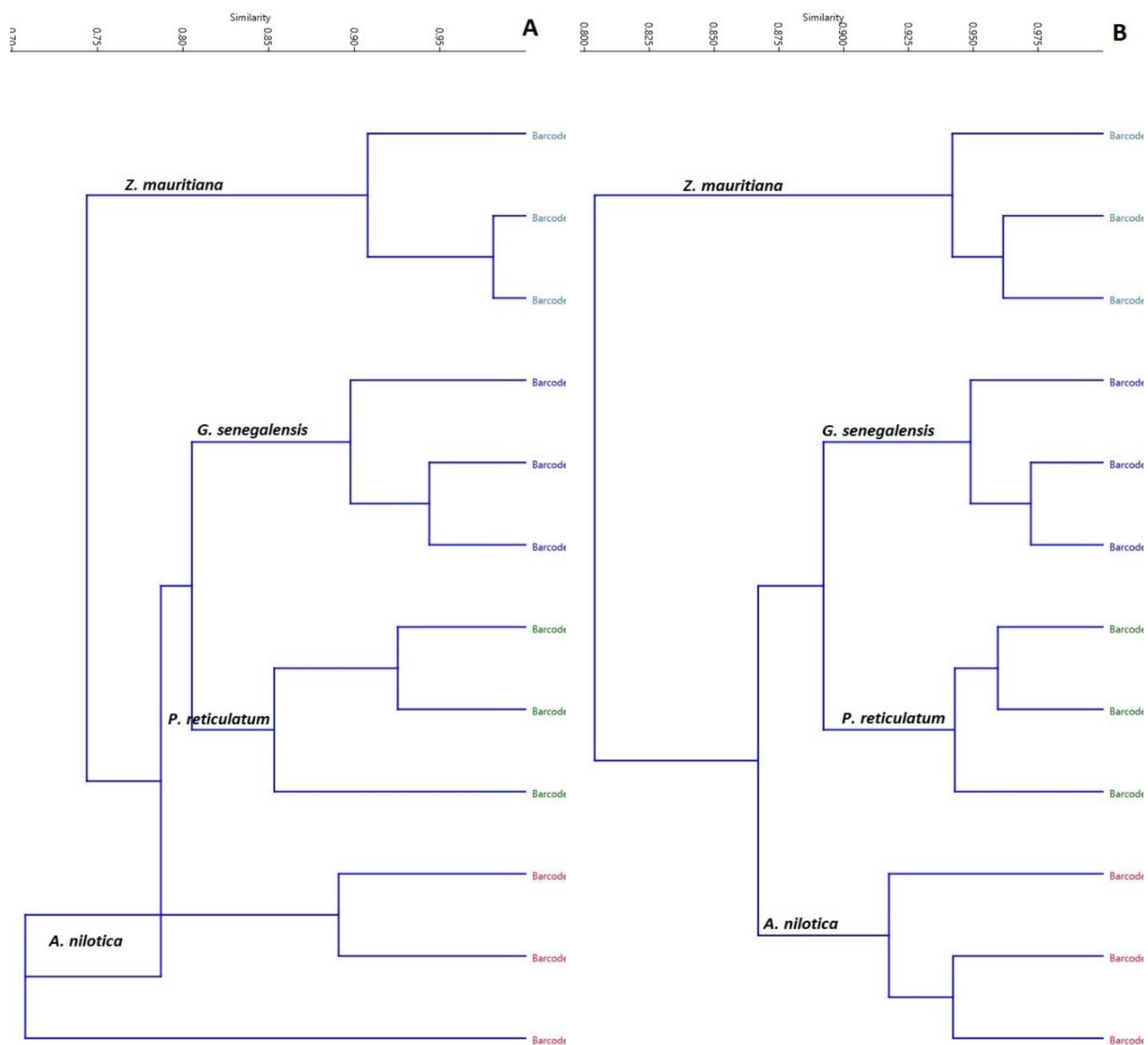


Figure 4.7: Cluster analysis of the *in vitro* effect of *A. nilotica*, *G. senegalensis*, *P. reticulatum* and *Z. mauritiana* leaves on sheep rumen bacteria analyses using (**A**) Oxford Nanopore MinION and (**B**) Ion Torrent PGM 16S rRNA gene sequencing. Dendrogram is based on the UPGMA clustering of the Bray-Curtis distances. Samples standardised by total and transformed square root; minor genera discarded (<0.05%).

4.6 CONCLUSION

In terms of the study of rumen microbial communities, results obtained using 16S rDNA amplicon sequencing through the MinION device are promising. The MinION was able to reconstruct nearly full-length16S rDNA sequences for 292 different species analysed from the sheep rumen. It permits taxonomic identification at the species level through 16S rDNA sequence comparisons, and a relative quantification to determine the species abundance. This type of analysis will likely become more accurate over time as Nanopore chemistry is improved in future releases, with the

resultant increase in throughput, required to detect the hundreds of species present in complex rumen microbial ecosystem. Accordingly, sequence studies of the entire 16S rDNA molecule could allow OTU-based analysis to be bypassed completely, thus making it feasible to obtain a direct inventory of bacterial species and relative abundance, as well as to determine the key players at the species level in different microbial communities of interest.

Therefore, the MinION is a cost-effective alternative for profiling the microbiota that is small and portable and can be used in the field or on autonomous platforms. As previously mentioned, the MinION has been used in very remote locations such as the Arctic (Edwards et al., 2016) and in space (Castro-Wallace et al., 2017). The portability, low cost and real-time DNA sequencing capability also allowed the MinION to sequence whole plant virus genomes to identify the begomoviruses causing the devastating cassava mosaic disease ravaging smallholder farmers' crops in Tanzania, Uganda and Kenya (Boykin et al., 2018). Within the ruminant production setting, there is potential for identification of members of a microbiota community *in situ*. Sequencing of rumen microbes on the site of production (*e.g*. on the farm) would allow farmers to monitor the state of their livestock in real-time thus allowing for immediate interventions. It would minimise sample degradation and loss during shipping (Boykin et al., 2018); and would eliminate sample storage steps that may cause loss of important species used as biomarkers (*e.g*., *Bacteroidetes*) due to long-term freezing of samples (Bahl et al., 2012).

Further, automatic devices (*e.g*. VolTRAX, Oxford Nanopore Technologies) and improved library construction methods will be used as the multiplex, fast, and high-throughput methods for preparing the Nanopore sequencing library in the future. The results presented here shows the MinION platform to be a reliable methodology to study the diversity of microbial communities in the rumen.

CHAPTER 5: RICE STRAW AS RUMINANT FEED

5.1 INTRODUCTION

Rice is the World's largest cereal crop after wheat and its demand is still growing (Bruinsma, 2017). About 3 billion people, half the world's population, depend on rice for their survival (Muhunyu, 2012).The production and consumption of rice is of particular importance in East and Southeast Asia where ninety percent of the production occurs (Van Soest, 2006). However, the greatest potential for expansion of production is in Sub-Saharan Africa where many governments have prioritised increased rice production and consumption as an important component of national food security, economic growth, and poverty alleviation (Balasubramanian et al., 2007). In SSA, production of rice increased from 8.6 to 21.6 million tons between 1980 and 2006. The increase in output is attributed to land expansion, increase in both population and incomes and due to changing of consumer preference in favour of rice in urban centres (Balasubramanian et al., 2007).

Globally, about 150 million hectares is estimated to be under rice cultivation with an annual production of 500 million metric tons (Atera, Onyancha, & Majiwa, 2018). The area under rice represents 29% of the total output of grain crops worldwide with Africa accounting for about 10 to 13%. Currently, rice is grown in over 75% of the 54 African countries and its territories, with a total population of nearly 800 million people depending on rice for their food and livelihoods (Atera et al., 2018).

Kenya covers 583,000km^2, of which 17% is arable and 83% is arid or semi-arid. Agriculture contributes 45% of total government revenues and provides 75% of industrial raw materials. More importantly, it contributes to national food security, because 80% of Kenyans live in rural areas and derive their livelihoods from crop and livestock production, forestry, and the exploitation of other natural resources (Muhunyu, 2012). Rice has been an important staple food for urban dwellers for a long time but is increasingly being consumed by the rural population (Balasubramanian et al., 2007). Currently, rice consumption outstrips production in Kenya. Annual consumption is about 400,000t and production is 80,000t. The difference is met through imports (Muhunyu, 2012). The annual consumption rate of rice is increasing by 12% a year, as compared with 4% for wheat and 1% for maize. In

response to the growing need for support, the Kenyan government developed the National Rice Development Strategy (NRDS) for the period 2008-2018, with the objective of promoting rice production in Kenya. Most rice in Kenya is grown under irrigation, mainly in four irrigation schemes managed by the National Irrigation Board (NIB): Mwea in Central Kenya, Ahero and West Kano in Nyanza, and Bunyala in Western Kenya. The rice grown in these schemes accounts for about 95% of total domestic production. The remaining 5% is grown under rain-fed conditions in coastal counties such as Kwale, Kilifi, and Tana River, and in some parts of Western Kenya (Onyango, 2014). The total area under rice production has ranged between 14,000 and 20,000 ha in the past two decades, with increases in production generally mirroring increases in the area under cultivation (Onyango, 2014). There is a high potential for expansion of irrigable area in Kenya and about 400,000 ha are suitable for rice production. Expansion of rain-fed rice production along the coast and in Western Kenya also presents great potential that has yet to be exploited (Ouma-Onyango, 2014).

Crop residues have been described as “Agriculture’s largest harvest” representing more than half of the world’s agricultural phytomass. This phytomass is composed of cereal and legume straws; in tops, stalks, leaves, and shoots of tuber, oil, sugar, and vegetable crops and in prunings and litter of fruit and nut trees. 60% of these residues were produced in low income countries; 45% specifically originate from the tropics (Smil, 1999). Rice production is a major source of crop residues. The annual global production of dry rice is about 526 million tons per year (Kim & Dale, 2004). Typical values for straw: grain ratios for rice vary from 0.7 to 1.5, which means that for every ton of rice grain, 700 to 1500 kg of rice straw is produced (Table 5.1) (Bakker et al., 2013).

Table 5.1: Rice production in different regions of the world and estimated of rice straw production. Data based on FAO grain production data of 2009. Data are ranked by rice yield/year (Bakker et al., 2013)

	Production	
Region	kton Rice/year	kton straw/year
World	684,595	727,400
Eastern Asia	216,630	230,200
Southern Asia	202,889	215,600
South-Eastern Asia	197,777	210,100
South America	25,568	27,200
Western Africa	10,392	11,000
Northern America	9,972	10,600
Eastern Africa	6,701	7,100
Northern Africa	5,593	6,000
European Union	3,152	3,350
Southern Europe	2,906	3,100
Caribbean	1,246	1,300
Central America	1,228	1,300
Eastern Europe	1,183	1,250
Central Asia	696	740
Central Africa	663	700
Western Asia	138	150
Oceania	82	90
Southern Africa	3	3

In Southeast Asia, open burning of rice straw is viewed by farmers as the cheapest and fastest way to clear land, remove residues, control weeds and release nutrients for the next crop cycle (Gadde et al., 2009). To ensure the food security and to meet the export demand, rice production has been intensified generating of a huge volume of rice straw annually and hence the practice of open burning. Open burning is the low temperature combustion of the vegetation with the release of large quantities of products of incomplete combustion, such as particulate matter with black carbon and organic carbon components, carbon monoxide and volatile organic compounds; certain amounts of nitrogen oxides and sulphur oxides are also released alongside key greenhouse gases like methane, nitrous oxide, and carbon dioxide. In addition to the release of these toxic air pollutants, open burning results in the waste of huge amounts of agro-residue biomass (Gadde et al., 2009; Oanh et al., 2018). Ninety percent of cereals rice produced is used for human consumption with little left to supplement animal feeds. Therefore, in parts of Southeast Asia, virtually all-arable land is in crop production and animals are dependent on straw, garbage, by-products and other wastes (Van Soest, 2006). In Kenya, rice straw previously used to be burned

in one part of the farm and the ash spread all across on the paddy. However, this has changed because of increased demand for the straw as animal feed (Figure 5.1). After the harvest, the rice straw is collected in a central location and fed to livestock or baled for sale (Muhunyu, 2012).

5.1.1 RICE STRAW AS RUMINANT FEED

In tropical zones around the world, ruminants depend on year-round grazing on natural pastures or the animals are fed with cut grass and crop residues (Sarnklong et al., 2010). In Africa and other developing countries, feeds and feeding comprise 60-70% of total production costs; livestock production is increasingly constrained by rising feed stuff prices and global inflation causing feed scarcity and the high cost of feeds (Lukuyu et al., 2011). Most of these areas also face seasonal dry periods in which the availability of pasture decreases. The quality of the pasture also decreases due to a reduction in the content of digestible energy and nitrogen. In areas where rice straw is abundantly available, farmers offer rice straw as the main roughage source to their animals (Sarnklong et al., 2010).

Figure 5.1: Rice straw fed to Zebu cows directly after harvesting from the rice paddies in the background. Rice straw is usually fed untreated and without supplements. Photo by author (Mwea, Kenya, December 2018).

Feeding only rice straw does not provide enough nutrients to the ruminants to maintain high production levels due to the low nutritive value of this highly lignified

material. The high level of lignification and silicification, the slow and limited ruminal degradation of the carbohydrates and the low content of nitrogen are the main deficiencies of rice straw, affecting its value as feed for ruminants (Van Soest, 2006). Consequently, as rice straw is poorly fermented, it has low rates of disappearance in the rumen and low rates of passage through the rumen, reducing feed intake (Sarnklong et al., 2010). However, compared to other cereal straws, rice straw is lower in lignin (Table 5.2**,** Van Soest (2006). Additionally, rice straw contains comparatively more leaf than stem (Agbagla-Dohnani et al., 2003). Leaf to stem ratio is an important factor in determining quality, diet selection and forage (Ferri & Molas, 2013). A higher proportion of leaf is positively associated with nutritive value, longer grazing time and forage intake (Chacon & Stobbs, 1976).

Table 5.2: Composition and digestibility of various crop residues (Van Soest, 2006)

	SiO_2 (g/kg)	Lignin (g/kg)	NDF (g/kg)	ADF (g/kg)	Digestibility
Rice straw	130	52	820	531	0.45
Barley straw	20	110	800	590	0.49
Oat straw	20–50	140	700	470	0.48
Wheat straw	10–50	85–140	828	540	0.44
Maize silage		40	460	260	0.72

NDF: Neutral Detergent Fibre ADF: Acid Detergent Fibre

5.1.2 SILICA IN RICE STRAW

Silicon is the second most abundant element on the Earth’s crust after oxygen; it mainly occurs in form of silica dioxide (silica) and silicates (Luyckx et al., 2017). Soluble silicon in the soil mainly occurs in the form of ortho-silicic acid (Mengel et al., 2001). Silicon is taken up by the roots when the solution pH is below 9 in the form of silicic acid [$Si(OH)_4$], an uncharged monomeric molecule, (Ma & Yamaji, 2006). Following uptake, silicon is translocated to the shoot *via* the xylem in the form of silicic acid (Mengel et al., 2001). In the shoot, silicic acid is concentrated through transpiration and is polymerized converting it to colloidal silicic acid and finally to silica gel with increasing silicic acid concentration. In rice plants, >90% of total silicon in the shoot is present in the form of silica gel (Ma & Yamaji, 2006).

Many plants actively take up silicon and incorporate it into their structure in form of silica at levels that can be up to 10% in some grasses (Hartley & DeGabriel, 2016). Silicon is a nutrient element for plants and is likely to have more than one function in

plant metabolism. The roots of rice absorb several times more silicon than common nutritional minerals such as nitrogen, phosphorus and potassium (Isa et al., 2010). Silicon deficiency in rice results in soft and droopy leaves thus increasing mutual shading, reduced photosynthetic activity and lowers grain yields (Yamamoto et al., 2012). Deficient plants show increased susceptibility to fungal diseases such as blast (caused by *Pyricularia oryzae*) or brown spot (caused by *Bipolaris oryzae*) (Ashtiani et al., 2012; Dallagnol et al., 2011). Severe silicon deficiency reduces the number of panicles and the number of filled spikelets per panicle (Tamai & Ma, 2008). Silicon-deficient plants are also particularly susceptible to lodging (Isa et al., 2010). Hence it is not surprising that silicon has been shown to enhance the resistance of crops to insect herbivores; for instance, the application of soluble silicon to wheat was shown to decreased damage by aphids (Goussain, Prado, & Moraes, 2005). Large responses in rice yield and resistance to fungal diseases were shown where there was adequate silicon nutrition (Meharg & Meharg, 2015). Additionally, a high correlation exists between straw silica content and yield of grain where silica was present in the straw at up to about 12% (Mengel et al., 2001).

As a structural element, silica complements lignin and strengthens and rigidifies cell walls (Ghasemi et al., 2013). Silica is effectively distributed to specific tissues (such as the leaf sheath and midrib) that are involved in rigidity of the rice plant, rather than being passively deposited in any growing tissues (Isa et al., 2010). Yamamoto et al. (2012) demonstrated an increase in secondary cell wall components (cellulose, hemicellulose and lignin) in rice leaves to compensate for silicon deficiency.

The active accumulation of silicon by rice (a C3 plant) may enable them to compete with the more photosynthetically efficient C4 plants in tropical ecosystems (Van Soest, 2006). Active transport of silicon costs one ATP atom per silicon atom compared to 24 ATP atoms required for the synthesis of an equivalent amount of lignin (Raven, 1983). This extra amount of silicon, however, is associated with an increase in ash content and a decrease in the degradability of rice straw (Ghasemi et al., 2013).

The high leaf content of modern rice straw varieties should promote the utilisation of rice straw as a ruminant feed. However, scanning electron microscopy has located major silica deposits in rice straw epidermal layers with a preferential deposition of silica at the epidermis surface and not in the inner tissues (Agbagla-Dohnani et al.,

2003). Additionally, there's preferential deposition of silica in leaves compared to stems (Table 5.3; Ghasemi et al. (2013). This may be linked to the transpiration rate which is greater in leaves than in stems (Agbagla-Dohnani et al., 2003). As a result, while the leaf had a lower neutral detergent fibre (NDF) than the stem, it has a higher total and insoluble ash (Vadiveloo, 2000). Therefore, unlike wheat straw or corn stover, the stem of rice straw is more rapidly degraded than the leaf (J. Wang et al., 2007).

Table 5.3: Silica content in rice straw of three varieties (Ghasemi et al., 2013)

	Silica content (g/kg)		
Variety	Leaf Blade	Leaf Sheath	Stem
Firozan	93	65	26
Sazandegi	112	77	30
Zayanderood	86	62	29

In terms of feed quality, silica limits digestibility by forming a physical barrier; a silicified waxy cuticular layer in leaf blades of rice plants forms a barrier to digestion to unsilicified tissue underneath (Van Soest, 2006). This effect is more pronounced in the leaves than in the stem; Agbagla-Dohnani et al. (2003) observed a greater *in vitro* degradation in stems than in leaves. Additionally, stem epidermis was more prone to colonisation in low-silica straw, which had no silica layer, than in high-silica straw, which had a thick outer silica layer. It has been suggested that this silica layer impeded the access of micro-organisms to the underlying sclerenchyma and lignified parenchyma. Unlike lignin which protects cell wall carbohydrates through bonding and sets an ultimate limit to digestion, silica appears to operate by incrustation (Van Soest, 2006).

Due to the importance of silica in rice growth and yield, any interventions to improve rumen degradation of rice straw would have to be applied post-harvest.

5.2 AIMS AND OBJECTIVES

As mentioned earlier, the accumulation of silica on the surface of rice straw has been proposed as the primary barrier to rumen degradation. However, no studies have been carried out to characterise the bacterial colonisation of untreated rice straw.

Previous work has shown that the dynamics colonisation of perennial ryegrass by rumen bacteria holds the key understanding and possibly improving the breakdown of forage in the rumen. Those studies have revealed that colonisation is biphasic with distinct 0-2h phase and a 4h and onwards phase (Huws et al., 2016; Huws et al., 2013). This pattern of colonisation has been attributed to the structure of the substrate surface determining the plant-microbe interaction (Huws et al., 2014). Further investigations suggested that nutrient utilization in the rumen can potentially be improved either by altering the cellulolytic capability of rumen bacteria or by altering the cell wall component of fodder (Mayorga et al., 2016).

This study therefore proposed to carry out a series of experiments to determine:

1. The effect of silica content of rice straw on the *in vitro* degradation by rumen microbes.
2. The *in vitro* colonisation dynamics of rice straw by rumen microbes *via* 16S rRNA metabarcoding.

5.3 EXPERIMENTAL DESIGN

5.3.1 SELECTION OF PLANT MATERIAL

Plant material was kindly donated by Prof Sue Hartley at University of York. Samples originated from the BBSRC-GCRF project titled, “Developing rice resources for resilience to climate change and mitigation of carbon emissions”. The goal of the project was to identify existing rice varieties with low silica or highly digestible straw to investigate and demonstrate the advantages of using straw with better quality for applications as animal feed and for biofuel production. The samples were sent to Aberystwyth University (IBERS) for to investigate the effect of silica content on degradation by rumen microbes.

The separated leaves were milled and analysed for organic matter, silicon and fibre as detailed in Sections 2.2.2 and 2.2.3. A sample of hay was included for comparison (Table 5.4).

Statistical analyses were done on Genstat 19th edition (VSN International Ltd., Hemel Hempstead, UK). Analysis of chemical composition was done using one-way ANOVA, with chemical composition as a dependent variable and straw variety as the fixed factor. Multiple comparisons of the means (Fisher's protected LSD, p=0.05) was done as the *post hoc* test.

Table 5.4: Treatments

Treatment	Identity	Treatment	Identity
1	L8-1	11	L103-2
2	L8-2	12	L103-3
3	L8-3	13	L117-1
4	L12-1	14	L117-2
5	L12-2	15	L117-3
6	L12-3	16	L123-1
7	L94-1	17	L123-2
8	L94-2	18	L123-3
9	L94-3	19	Hay
10	L103-1		

5.3.2 *IN VITRO* DEGRADATION

In order to determine the degradability of the rice straw leaves in the rumen, gas production over 96h was determined using the method of Theodorou et al. (1994) which relies on an inverse relationship between gas accumulation and degradation of the biomass (Section 2.2.5). Eighteen samples were incubated with pooled rumen fluid from four cannulated cows in triplicate (6 x 3) making a total of 54 bottles plus 3 blanks. A sample of hay was included for comparative purposes. At the end of the incubation, the separated supernatant was analysed for volatile fatty acids as described in Section 2.1.5. Statistical analysis of rate and extent of degradation, and VFA production was done using one-way ANOVA and Fishers protected LSD (p=0.05) as the *post hoc* test.

5.3.3 TEMPORAL DYNAMICS OF COLONISATION

Accession L94-2 was selected for further analysis based on the median values for silica content and gas production (see Section 5.4). As described fully in Section 2.2.6, chopped samples of straw leaf was placed in fifteen replicates and incubated with pooled rumen fluid from four cannulated cows. The residue was harvested at 1, 2, 4, 6, 8 and 12 hours (6 x 3 = 18 bottles). The diversity of attached bacteria was assessed

using Ion Torrent PGM sequencing of the V1–V2 region the of 16S rRNA gene (Section 2.2.7).

Following bioinformatic processing, the relative bacterial taxa abundance data were subjected to square root transformation. Principal coordinate analysis (PCO) ordination on a Bray-Curtis distance matrix was done to identify patterns in the data. Permutation multivariate analysis of variance (PERMANOVA) was used to determine overall significant differences in community data. The effects of hour of harvest on transformed data were analysed based on their Bray-Curtis distance metric within the function unweighted pair group method with arithmetic mean (UPGMA). PERMANOVA was carried out using default settings with unrestricted permutations. All tests were carried out using the PAST v3 statistical package (Hammer et al., 2001).

Absolute concentrations of DNA from total bacteria was determined using qPCR and serial dilutions of specific DNA standards as detailed in Section 2.2.8.

5.4 RESULTS

The silica content of the dried rice leaves was measured to confirm if content reflected the content previously determined in stems. There was a significant difference between the silica content in rice straw varieties and as compared with hay (Table 5.5). This confirmed that rice straw contains a higher silica content compared to other fodder such as hay; and was the reason of including the hay in the experiment for comparative purposes. There was also a positive correlation (R^2=0.5004) between the silica content of the samples and the measured ash content (Figure 5.2). There were significant differences between the samples in measurements of NDF, ADF and ADL fibre components. There was a positive correlation (R^2=0.4197) between the ADL% and the silica content (Figure 5.3). The lignin content was lower than that reported previously for rice straw (Table 5.2, Van Soest (2006), and the positive correlation with the silica content was unexpected.

Table 5.5: Chemical composition of rice straw varieties in comparison to hay (%)

	Minerals		Fibre		
	Si %	Ash %	NDF%	ADF%	ADL%
L8_1	5.59^{bcde}	17.54^{fgh}	73.84^{bcde}	42.37^{bc}	6.69^{ab}
L8_2	6.36^{e}	18.86^{h}	75.11^{cdef}	44.52^{cde}	8.08^{abcde}
L8_3	6.12^{ef}	17.17^{fgh}	76.67^{ef}	46.45^{defg}	7.54^{abcd}
L12_1	6.27^{f}	16.06^{def}	76.48^{ef}	46.20^{defg}	9.34^{defg}
L12_2	5.67^{cde}	15.24^{cd}	76.02^{def}	45.53^{def}	8.23^{abcdef}
L12_3	6.03^{def}	15.29^{cde}	77.02^{ef}	46.80^{defg}	8.83^{bcdef}
L94_1	5.55^{bcd}	15.11^{cd}	76.87^{ef}	46.14^{defg}	7.00^{abc}
L94_2	6.30^{f}	17.07^{efg}	77.66^{f}	48.83^{g}	9.06^{cdefg}
L94_3	6.25^{f}	15.3^{cde}	77.66^{f}	47.59^{efg}	8.51^{bcdef}
L103_1	5.88^{def}	15.24^{cd}	76.97^{ef}	45.69^{defg}	9.62^{defgh}
L103_2	6.24^{f}	15.12^{cd}	77.12^{ef}	47.50^{efg}	10.39^{fgh}
L103_3	5.35^{bc}	13.73^{bc}	76.56^{ef}	45.89^{defg}	10.12^{efgh}
L117_1	5.91^{def}	16^{def}	71.50^{b}	40.71^{b}	7.74^{abcd}
L117_2	7.59^{g}	17.58^{fgh}	72.71^{bcd}	47.13^{defg}	11.78^{h}
L117_3	7.23^{g}	18.08^{gh}	72.21^{bc}	44.13^{cd}	11.09^{gh}
L123_1	5.09^{b}	12.23^{b}	77.69^{f}	45.30^{cdef}	8.06^{abcde}
L123_2	5.84^{cdef}	14.88^{cd}	77.25^{ef}	47.75^{fg}	9.29^{defg}
L123_3	5.62^{cde}	13.85^{cd}	77.15^{ef}	46.45^{defg}	8.93^{cdefg}
Hay	1.51^{a}	6.21^{a}	66.01^{a}	35.31^{a}	6.16^{a}
SED	0.1409	0.4435	0.9490	0.8400	0.593
P value	<0.001	<0.001	<0.001	<0.001	<0.001

Means with different superscripts differ ($p<0.05$)

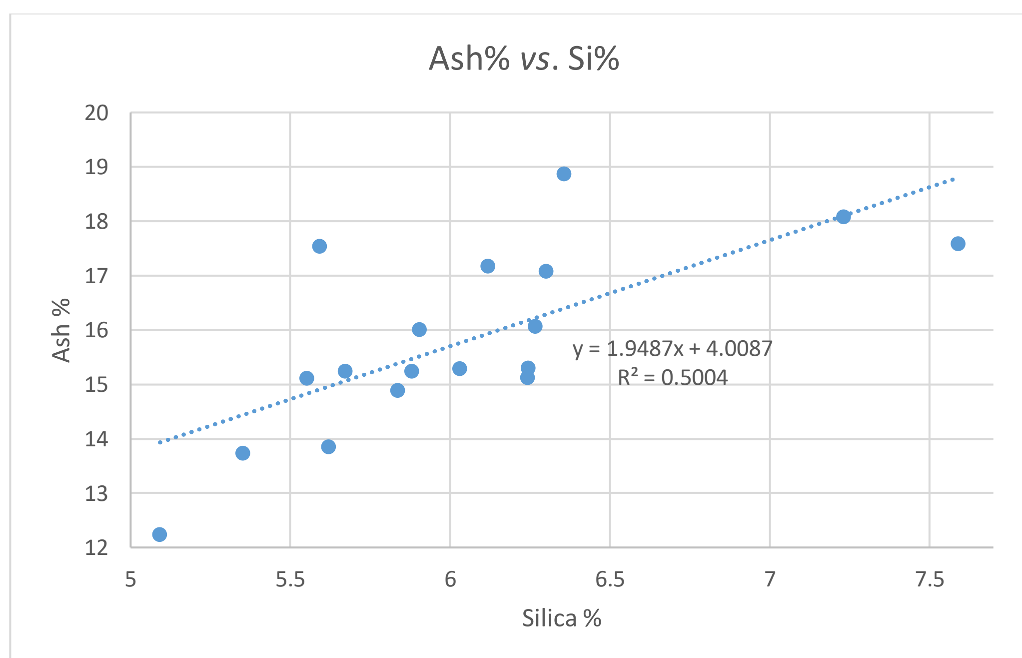


Figure 5.2: Correlation between ash and silica content (%) (R^2=0.5004) of rice straw and hay samples

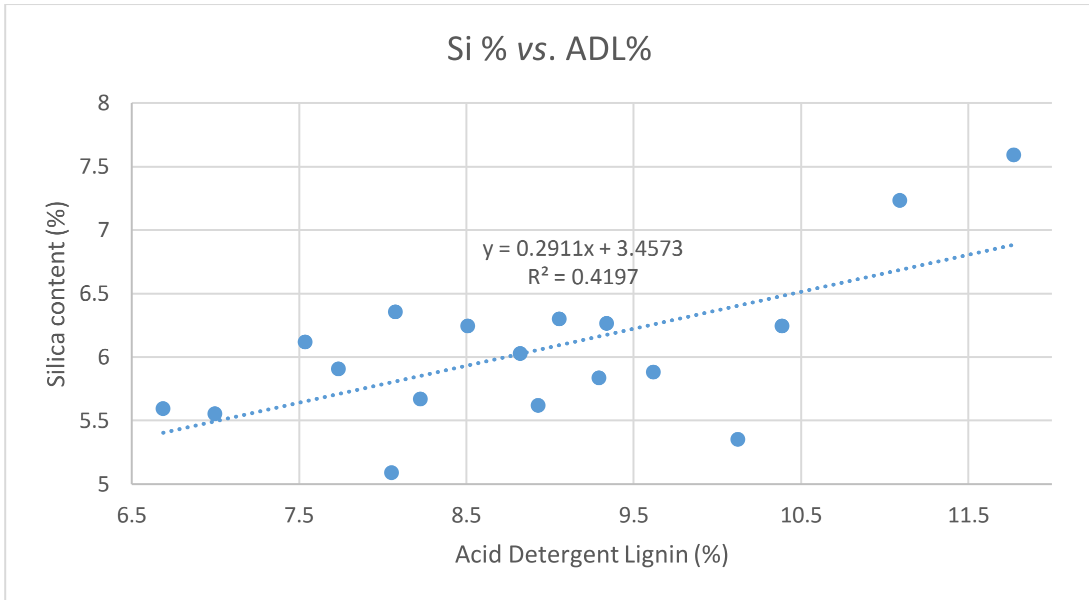


Figure 5.3: Correlation between silica content (%) and acid detergent lignin (ADL%) (R^2=0.4197) of rice straw samples

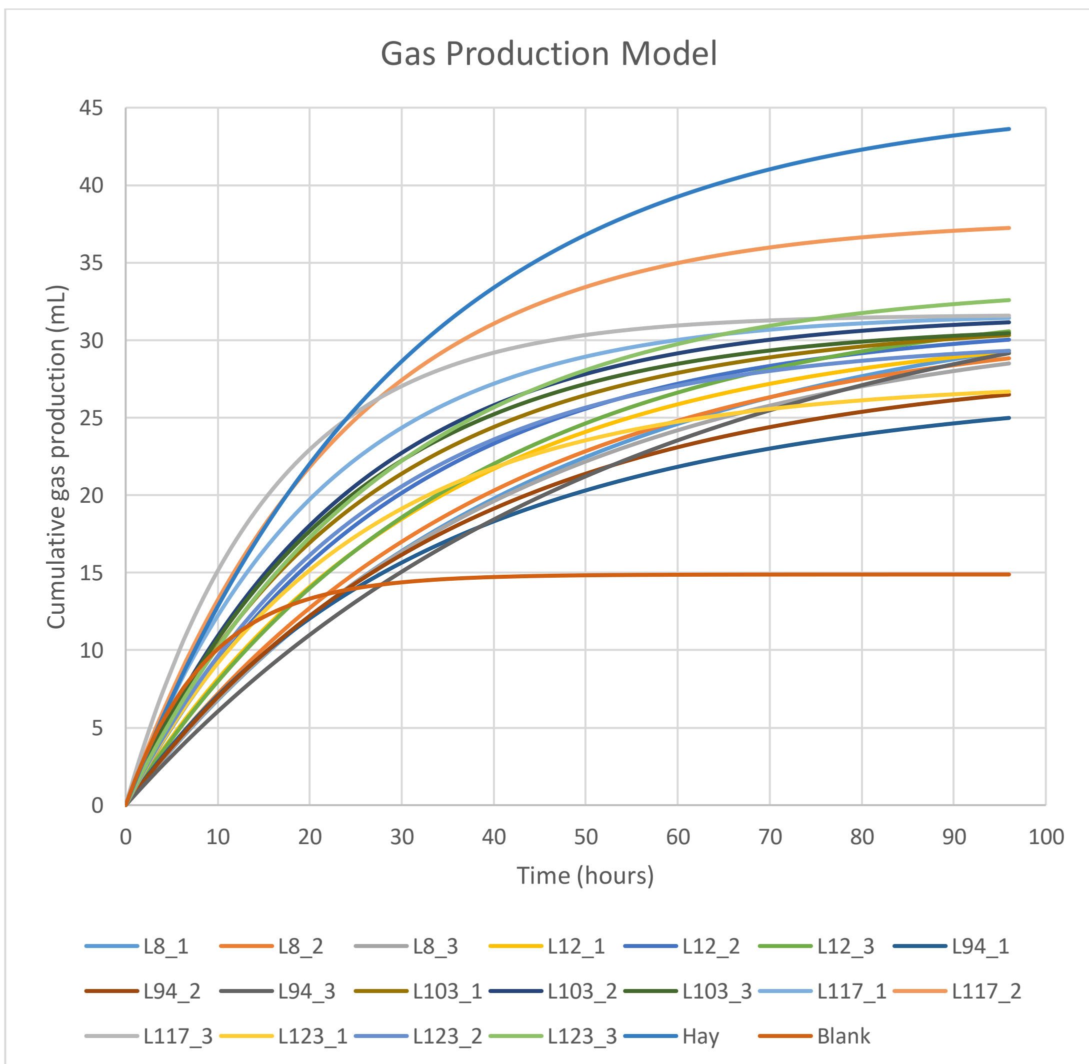


Figure 5.4: Kinetics of the accumulated gas production profiles (plots of Y from the model)

Gas production was used to evaluate the degradation of rice straws and hay by a rumen population. There was a clear difference between the gas production profile of hay compared to rice straw samples; and between some of the straw samples (Figure 5.4, Table 5.6). There was a positive correlation (R^2=0.3006) between the volume of gas produced (a + b (mL), representing the extent of *in vitro* degradation) and the silica content in the rice straw samples (Figure 5.5). This runs contrary to the reported and expected negative effect of silica content on the degradation of rice straw in the rumen. The accuracy of the *in vitro* gas production measurements was confirmed by the strong positive correlation (R^2=0.8267) between the volume of gas produced (a + b, mL) and the total VFA (mM/L) measured at the end of the fermentation (Figure 5.6). It was observed that the blank bottles (without the substrate) had a higher rate of gas production during the exponential phase of the curve compared to those with the treatment substrate (Figure 5.4). The presence of feed particles suspended in the rumen fluid can lead to an overestimation of the actual gas production due to the preferential fermentation of these particles rather than the relatively indigestible straw substrate (Maccarana et al., 2016). Reduction of the rumen fluid to buffer ratio from the 1:2 used in this experiment may offer a more accurate estimation of *in vitro* digestibility.

Table 5.6: Volume of gas produced (a + b) and Rate of degradation (c)

		Extent of degradation (a+b, mL)	Rate of degradation (c, %/h)
L8_1		33.44[bc]	0.02[ab]
L8_2		31.45[b]	0.03[ab]
L8_3		31.84[bc]	0.02[ab]
L12_1		31.16[b]	0.03[ab]
L12_2		31.39[b]	0.04[ab]
L12_3		32.96[bc]	0.03[ab]
L94_1		27.33[ab]	0.03[ab]
L94_2		28.6a[ab]	0.03[ab]
L94_3		36.1[bc]	0.02[a]
L103_1		31.34[b]	0.04[abc]
L103_2		31.74[b]	0.04[abc]
L103_3		31.05[b]	0.04[abc]
L117_1		31.74[b]	0.05[bc]
L117_2		37.85[bc]	0.04[abc]
L117_3		31.66[b]	0.07[c]
L123_1		27.44[ab]	0.04[abc]
L123_2		30.08[b]	0.04[ab]
L123_3		33.68[bc]	0.04[ab]
Hay		45.55[c]	0.03[ab]
Blank		14.88[a]	0.11[d]
s.e.d	excluding hay	3.730	0.0071
	including hay	3.646	0.0069
p value	excluding hay	0.002	<0.001
	including hay	<0.001	<0.001

"a" and "b" parameters explain the potential of fermentation ("a + b" reflects the maximum potential of fermentation), and "c" parameter explained the speed/slope of the curve of fermentation. Means with different superscripts differ ($p<0.05$). Comparisons of the means were made between the treatments to both include the exclude the hay sample

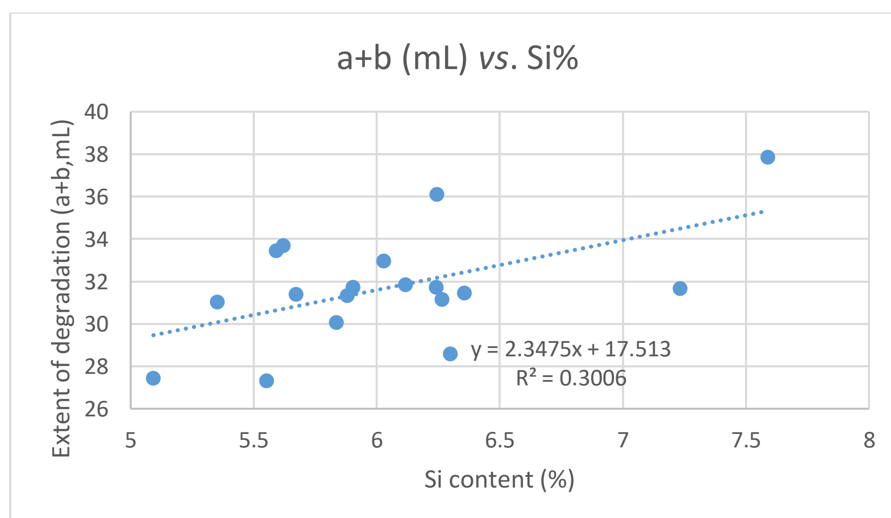


Figure 5.5: Correlation between extent of degradation (a+b, mL) and Silica content (%) (R^2=0.3006) in straw samples only

Table 5.7: Volatile acid concentration (mM/L) and pH measured at the end of the incubation

		Volatile fatty acids (mM/L)							pH
		Acetic	Propionic	Butyric	Valeric	Caproic	Heptanoic	Total	
L8_1		0.71^{a}	0.36^{a}	0.31^{abc}	0.16^{ab}	0.02^{a}	0.00^{a}	1.56^{a}	6.74^{bc}
L8_2		0.58^{a}	0.32^{a}	0.26^{abc}	0.12^{ab}	0.01^{a}	0.00^{a}	1.30^{a}	6.77^{bc}
L8_3		0.69^{a}	0.39^{a}	0.25^{abc}	0.10^{ab}	0.00^{a}	0.01^{a}	1.44^{a}	6.72^{bc}
L12_1		0.63^{a}	0.36^{a}	0.30^{abc}	0.11^{ab}	0.02^{a}	0.02^{ab}	1.45^{a}	6.76^{bc}
L12_2		0.62^{a}	0.38^{a}	0.27^{abc}	0.11^{ab}	0.01^{a}	0.01^{a}	1.40^{a}	6.72^{bc}
L12_3		0.73^{a}	0.42^{a}	0.31^{abc}	0.15^{ab}	0.03^{a}	0.06^{ab}	1.70^{a}	6.70^{b}
L94_1		0.50^{a}	0.34^{a}	0.19^{a}	0.08^{ab}	0.00^{a}	0.05^{ab}	1.16^{a}	6.75^{bc}
L94_2		0.54^{a}	0.30^{a}	0.24^{ab}	0.07^{a}	0.00^{a}	0.00^{a}	1.15^{a}	6.72^{b}
L94_3		0.79^{a}	0.41^{a}	0.27^{abc}	0.17^{ab}	0.05^{ab}	0.12^{abc}	1.81^{a}	6.74^{bc}
L103_1		0.65^{a}	0.40^{a}	0.31^{abc}	0.24^{bc}	0.19^{b}	0.04^{ab}	1.83^{a}	6.73^{bc}
L103_2		0.59^{a}	0.39^{a}	0.34^{abc}	0.24^{bc}	0.11^{ab}	0.07^{ab}	1.73^{a}	6.74^{bc}
L103_3		0.74^{a}	0.37^{a}	0.38^{bc}	0.23^{abc}	0.12^{ab}	0.02^{ab}	1.85^{a}	6.70^{b}
L117_1		0.69^{a}	0.39^{a}	0.35^{abc}	0.17^{ab}	0.04^{a}	0.07^{ab}	1.71^{a}	6.68^{b}
L117_2		0.88^{a}	0.53^{ab}	0.44^{c}	0.14^{ab}	0.08^{ab}	0.12^{abc}	2.18^{a}	6.64^{ab}
L117_3		0.62^{a}	0.40^{a}	0.31^{abc}	0.10^{ab}	0.03^{a}	0.07^{ab}	1.53^{a}	6.75^{bc}
L123_1		0.53^{a}	0.33^{a}	0.29^{abc}	0.14^{ab}	0.04^{a}	0.05^{ab}	1.37^{a}	6.73^{bc}
L123_2		0.68^{a}	0.40^{a}	0.32^{abc}	0.19^{abc}	0.04^{a}	0.10^{abc}	1.72^{a}	6.70^{b}
L123_3		0.84^{a}	0.50^{ab}	0.36^{abc}	0.16^{ab}	0.11^{ab}	0.15^{bc}	2.11^{a}	6.62^{ab}
Hay		1.45^{b}	0.72^{b}	0.71^{d}	0.33^{c}	0.18^{b}	0.23^{c}	3.62^{b}	6.50^{a}
s.e.d	no hay	0.111	0.075	0.050	0.044	0.038	0.031	0.294	0.040
	with hay	0.113	0.073	0.051	0.044	0.038	0.036	0.289	0.038
p value	no hay	0.082	0.341	0.009	0.008	<0.001	<0.001	0.045	0.068
	with hay	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Means in a column with different superscripts differ (p<0.05). Comparisons of the means were made between the treatments to both include the exclude the hay sample

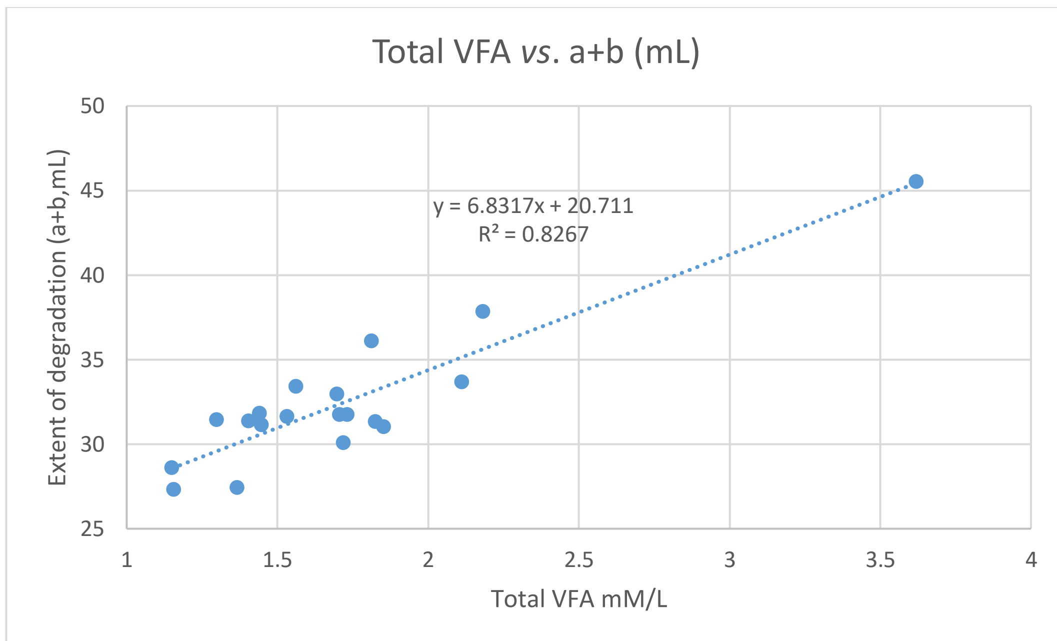


Figure 5.6: Correlation between extent of degradation (a+b, mL) and total VFA production mM/L measured at the end of the fermentation of rice straws and hay inoculated with rumen fluid (R^2=0.8267)

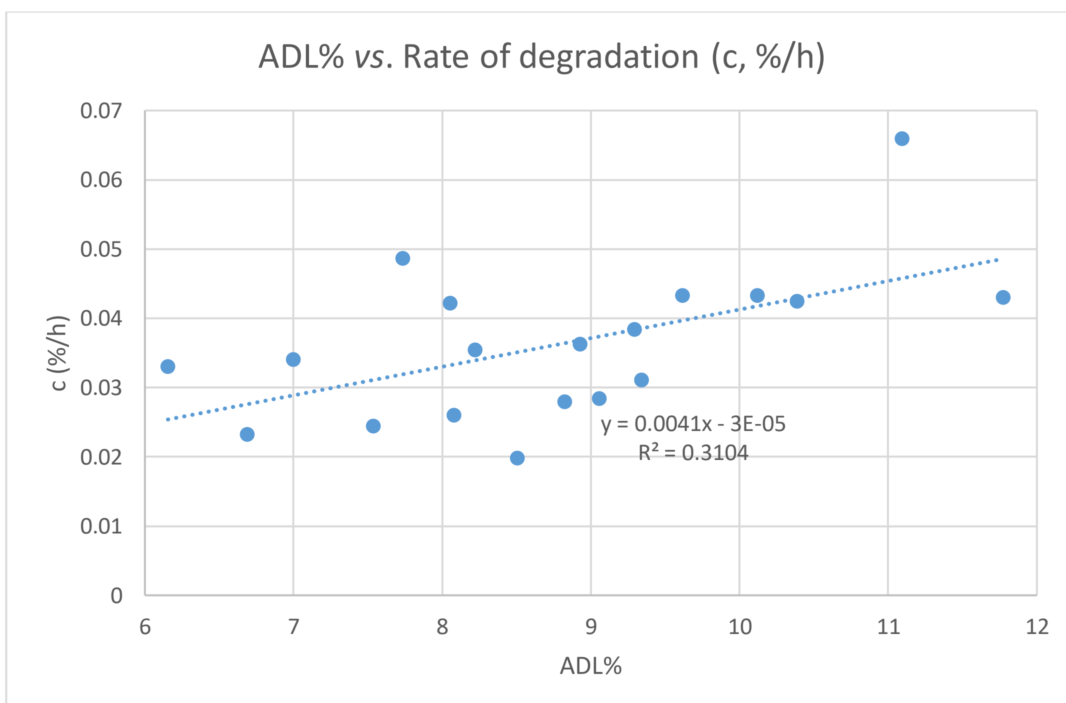


Figure 5.7: Correlation between acid detergent lignin (ADL%) and rate of degradation (c) (R^2=0.3104) in rice straws and hay inoculated with rumen fluid.

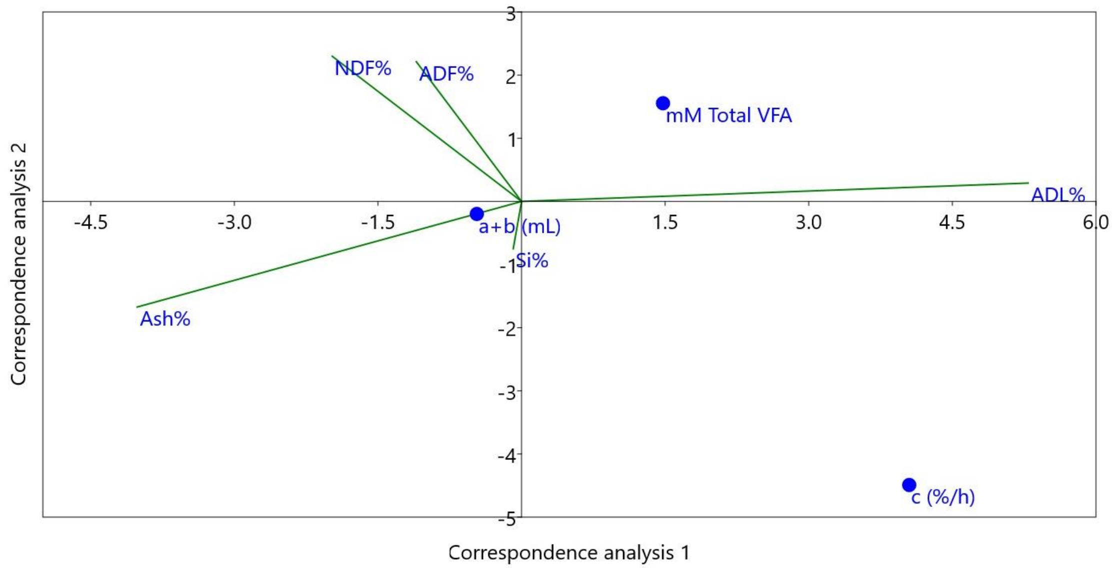


Figure 5.8: Canonical correspondence analysis illustrating the relationship between chemical composition of samples and rumen fermentation pattern *in vitro*. Plots show the direction of the gradient and those a longer line show a stronger correlation

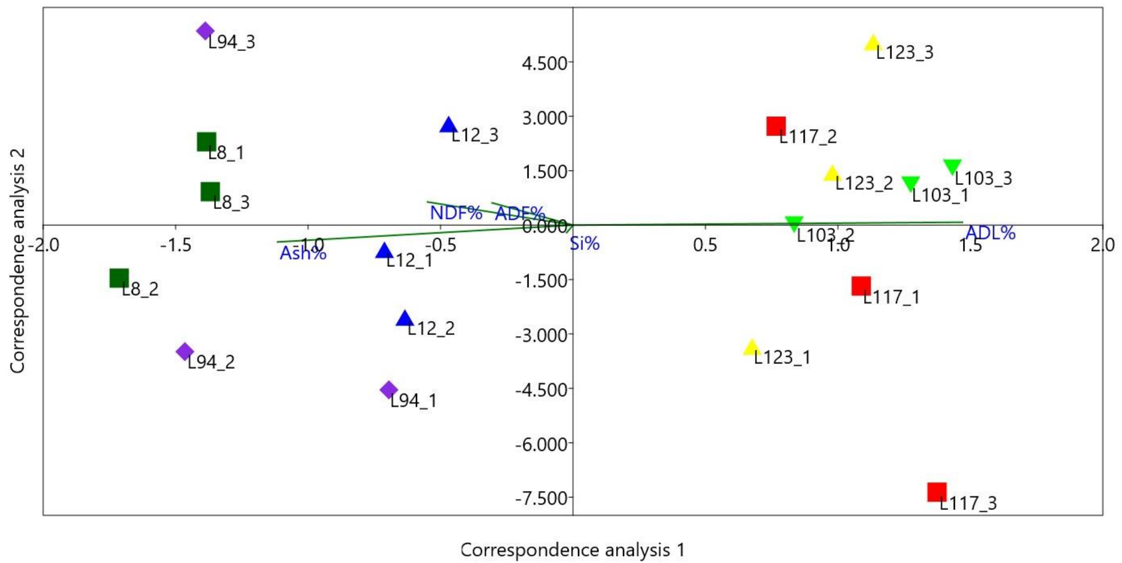


Figure 5.9: Canonical correspondence analysis illustrating the relationship between chemical composition of rice straw samples and rumen fermentation pattern *in vitro*. Plots show the direction of the gradient and those a longer line show a stronger correlation

ATTACHED BACTERIAL DENSITY

Total bacterial population in the rice straw samples were estimated with a real-time PCR analysis by measuring the total copy number of bacterial 16S rRNA genes. Incubation time had a significant effect on the copy number of bacterial 16S rRNA genes determined on rice (Figure 5.10, R^2= 0.7176, p = 0.01). The highest bacterial number in the samples of rumen-incubated rice straw was observed at 12h of incubation. The bacterial densities initially decreased after incubation but the numbers rapidly increased between 2h and 4h of incubation. Between 4h and 12h, the increase was much slower.

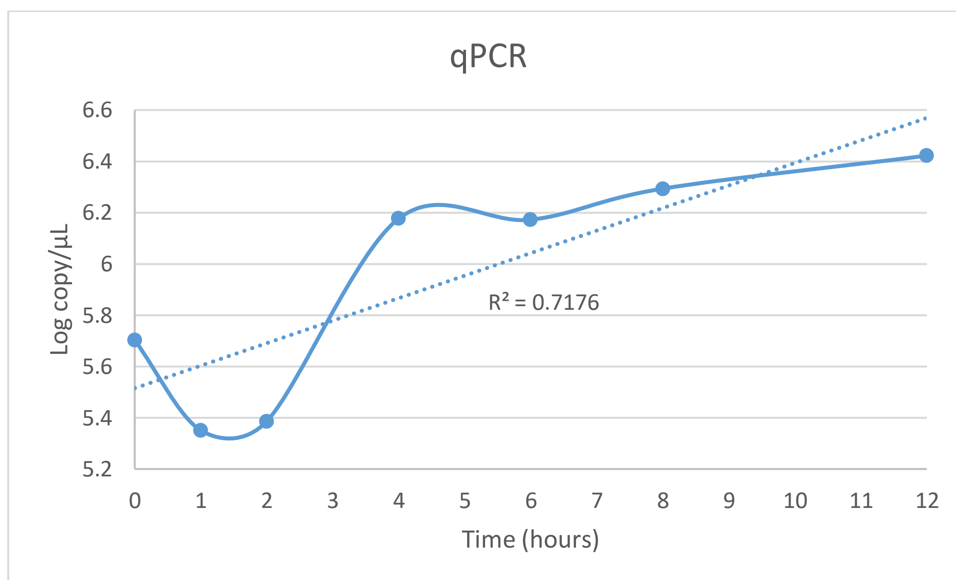


Figure 5.10 Total bacterial numbers (Log gene copies/μL) attached to rice straw following incubation for up to 12h. The points represent the log copy/μL and the dotted line represents linear estimate of the relationship (R^2 = 0.7176)

BACTERIAL COMMUNITY

Metagenomic analysis of bacterial communities attached to the rice straw showed statistically significant changes over the incubation time. There was also a clear difference between the communities occupying the straw before incubation and those that replaced the afterwards. The microbial population of the rice straw at the beginning of the incubation was dominated by the phylum *Proteobacteria* (62%), *Bacteroidetes* (16%), *Cyanobacteria*/Chloroplast (12%) and *Actinobacteria* (12%). At the 1h timepoint of incubation, the relative abundance of *Proteobacteria* had reduced to 22%, *Bacteroides*, *Cyanobacteria*/Chloroplast to 0.3% and *Actinobacteria* to 2.5%. There was a significant decrease in the relative abundance of bacteria in the phyla *Proteobacteria* which reduced from 61.5% to 22.5%, *Actinobacteria* (10% to 2.5%) and

Cyanobacteria/ Chloroplast (12% to 0.3%) in the first hour of incubation (Table 5.8). The levels continued to fall throughout the rest of the incubation period and ended at 2% for *Proteobacteria*, 0.02% for *Cyanobacteria*/Chloropast and 0.02% for *Actinobacteria*. The reduction in the abundance of *Proteobacteria* was driven mainly by the reduction of the genera *Novosphingobium*, *Sphingomonas*, *Methylobacterium*, *Pantoea*, *Pseudomonas*, *Sphingobium* and Rhizobium. The reduction in the levels of the genus accounted for the majority of the reduction of the phylum *Cyanobacteria*/Chloroplast. There was a reduction of bacteria in the genera *Sphingobacterium*, *Niastella* and *Mucilaginibacter* despite the general increase of bacteria in the phylum *Bacteroidetes*. The reduction in the relative abundance of bacteria in the phylum *Actinobacteria* was driven mainly by reductions in the genera *Curtobacterium* and *Microbacterium* (Figure 5.17, Table 5.9).

The rice straw incubated in rumen fluid showed a different composition of colonising bacterial phyla at significantly different levels depending on the hour of harvesting. The means of the relative abundance of bacteria phyla for the entire incubation period showed a dominance of *Bacteroidetes* (37%), *Firmicutes* (31%), *Fibrobacteres* (12%), *Proteobacteria* (9%) and *Tenericutes* (4%). The incubated straw also showed a higher diversity of phyla of the attached bacteria (Figure 5.11).

The composition of the bacterial community was not uniform throughout the incubation period; significant changes were observed in relation to the length of incubation. Within the first hour, there was a rapid increase in the relative abundance of the phyla *Bacteroides* and *Firmicutes* (Figure 5.12). Within the phylum *Bacteroides*, the genus *Prevotella* increase from zero to a relative abundance of 10% (Figure 5.13 and Figure 5.14). The increase in the abundance of the phylum *Firmicutes* was dominated by the genus *Butyrivibrio* which increased from zero to 3.8% (Figure 5.16). The levels of these two remains relatively constant until the 6th hour of incubation where their levels began to reduce. At this time point, an accompanying increase in the abundance of bacteria in the phylum *Fibrobacteres* was observed. At the end of the 12h incubation period, bacteria in this phylum dominated the population at a relative abundance of 40% (Figure 5.13). The genus *Fibrobacter* was responsible for this increase (Figure 5.14).

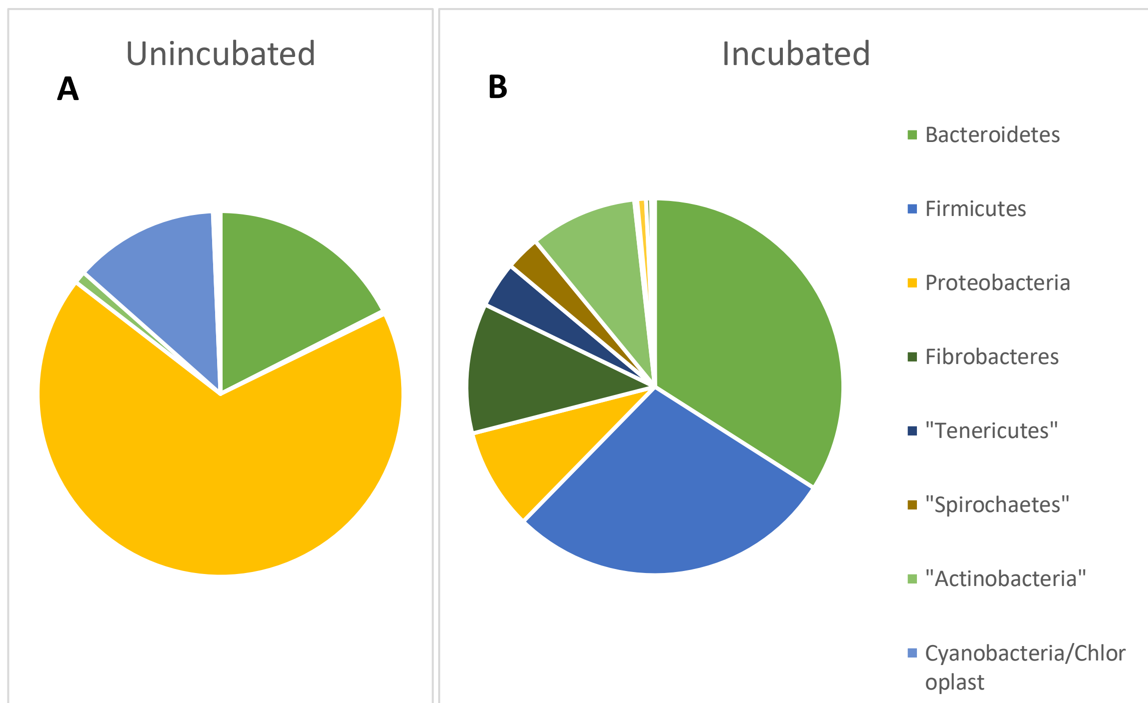


Figure 5.11: Taxonomic composition of microbiome at the phylum level attached to rice straw (**A**) before incubation in rumen fluid and (**B**) the mean of compositions of six time-points of a 12h incubation period. Pie chart showing the average distribution of the phyla (>0.05%) across all ruminal samples sequence using the Ion Torrent PGM. Samples standardised by total number of reads.

Bacteria of the phyla *Tenericutes* and *Spirochaetes* also showed a steady increase from the sixth hour of incubation but at much lower levels compared to *Fibrobacteres* (Figure 5.14 and 5.15). The genera *Anaeroplasma* and *Treponema* were the main drivers for the increase in the abundance of these phyla respectively (Figure 5.15).

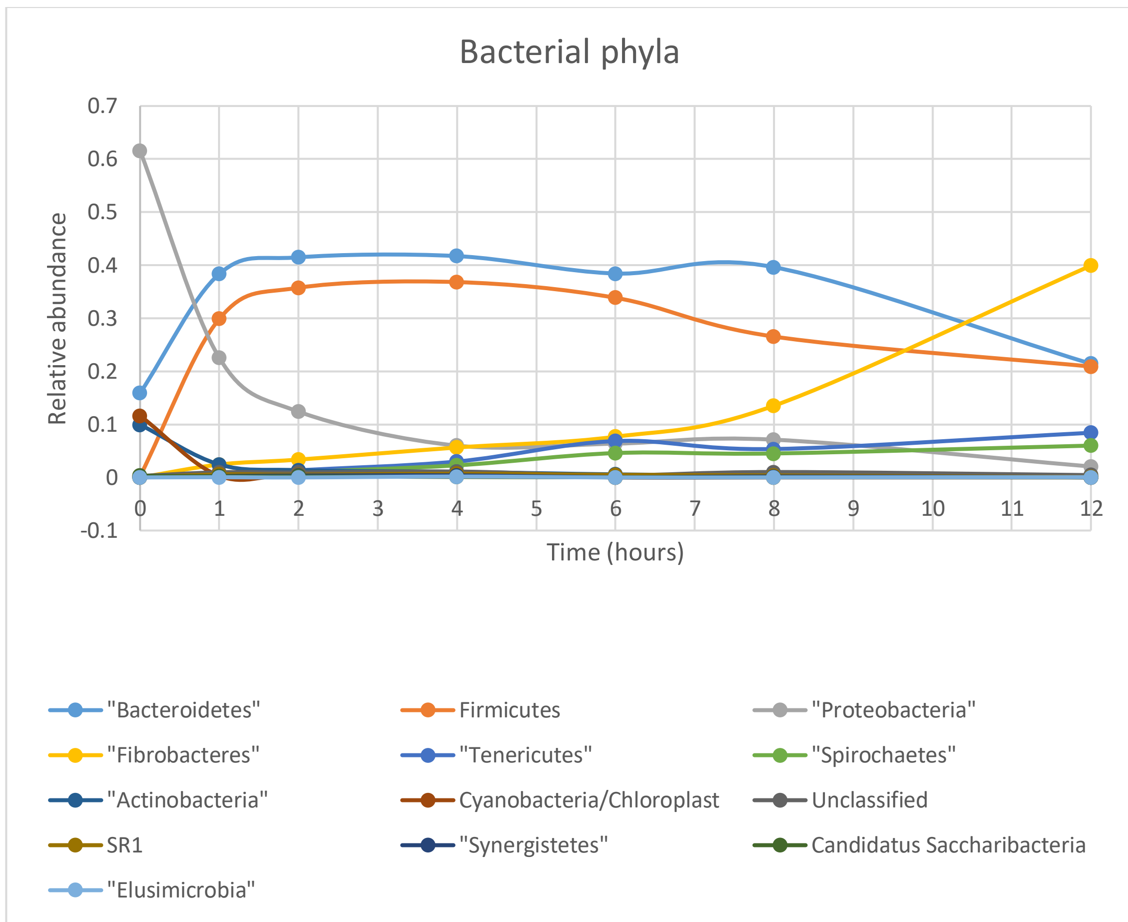


Figure 5.12: Scatter graph showing changes in the relative abundance of bacterial phyla over 12h incubation period. Phylum-level bacterial composition present at an average of more than 0.05% analysed using Ion Torrent PGM 16S rRNA gene sequencing. Reads were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II. Samples standardised by total and transformed by square root

Principal coordinate analysis (PCO) on operational taxonomic unit (OTU) level of bacteria showed three distinct clusters. The first separation (PCO1 = 37.65% of total variation) corresponded with differences between the un-incubated rumen fluid, un-incubated rice straw and the incubated samples. The secondary separation (PCO2 = 22.86% of total variation) corresponded to the time of incubation from zero to twelve hours (Figure 5.16). PERMANOVA showed a strong effect of incubation time (p=0.0001); however, pairwise comparisons did not show a significant effect between the straw types. Clustering based on Bray-Curtis similarly also showed clear separation between the incubation periods (

Figure 5.20). When the alpha diversity indices of rumen microbial community were compared, significant differences were observed over time. The Inverse Simpson index ranged from 11.2 to 212. The diversity started low (31.6) for un-incubated rice straw,

rose rapidly over 2h to 212, then fell to the lowest point at 12h with 11.2. A small increase at the 8h time-point was also observed. A similar pattern emerged for the Shannon index with diversity starting low (4.178), rising to maximum at the 2h time-point (5.645) and reducing up to the 6h time-point (4.961), then similarly rising to 5.272 at the 8h mark and subsequently falling to the lowest point at the 12h mark (3.997) (Figure 5.18). The patterns of the indices followed closely the successional changes observed for the taxonomic composition.

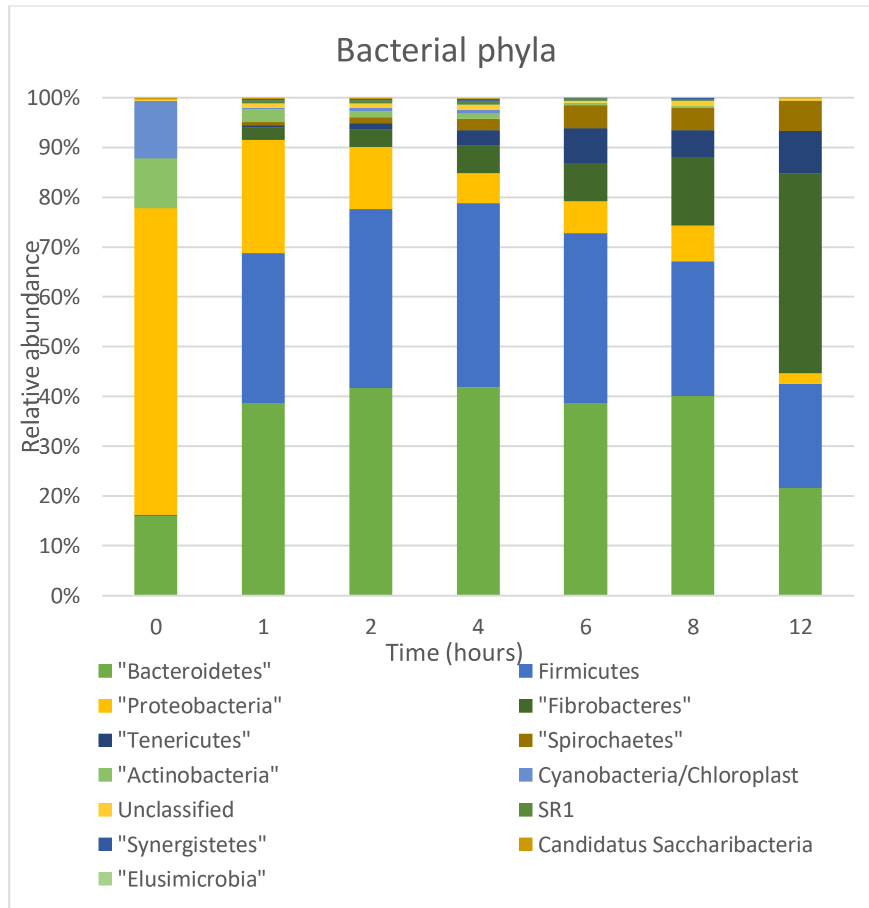


Figure 5.13 Stacked histograms illustrating phylum-level bacterial composition present at an average of more than 0.05% analysed using Ion Torrent PGM 16S rRNA gene sequencing. Reads were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II. Samples standardised by total number of reads and transformed by square root.

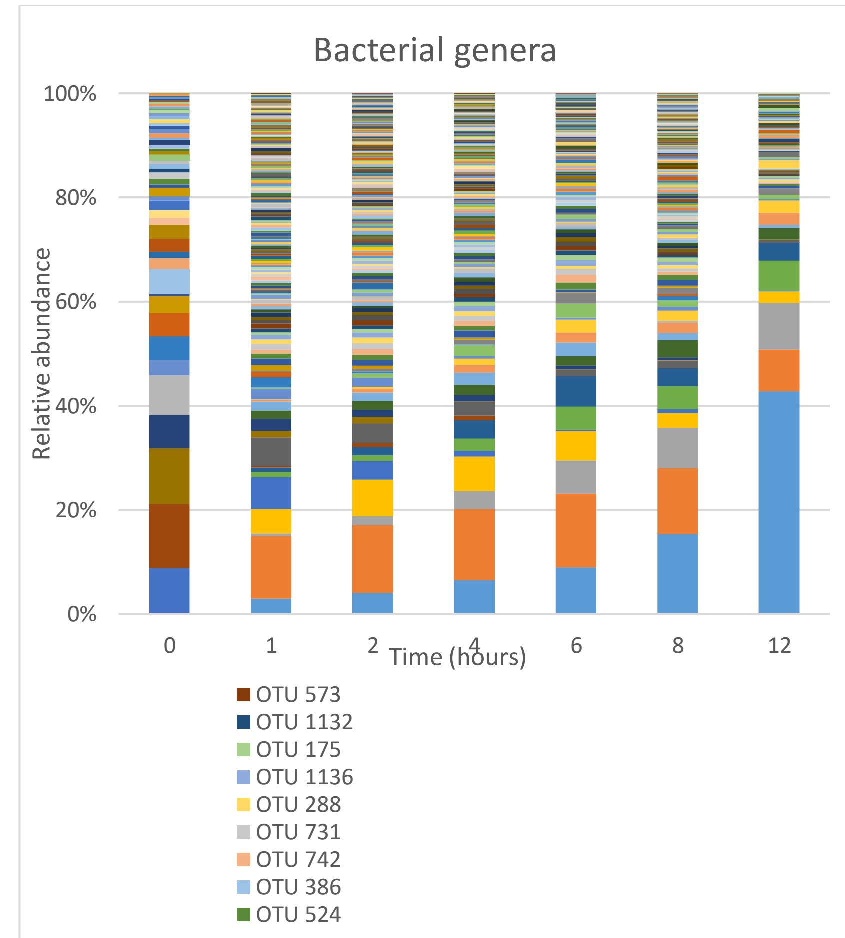


Figure 5.14 Stacked histograms illustrating genus-level bacterial composition present at an average of more than 0.05% analysed using Ion Torrent PGM 16S rRNA gene sequencing. Reads were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II. Samples standardised by total number of reads and transformed by square root.

Table 5.8: The relative abundance of bacteria phyla present at an average of more than 0.05% colonising rice straw leaves after 0, 1,2,4,6,8 and 12 hour of *in vitro* incubation is bovine rumen fluid

	Hour							s.e.d.	p- value
Bacterial phylum	0	1	2	4	6	8	12		
Acidobacteria	0.10^{d}	0.02^{c}	0.01^{bc}	0.01^{bc}	0.01^{ab}	0.00^{ab}	0.00^{a}	0.0207	<0.001
Bacteroidetes	0.16^{a}	0.38^{c}	0.41^{c}	0.42^{c}	0.38^{c}	0.40^{c}	0.21^{b}	0.0185	<0.001
Fibrobacteres	0.00^{a}	0.02^{b}	0.03^{bc}	0.06^{bc}	0.08^{cd}	0.14^{d}	0.40^{e}	0.0345	<0.001
Proteobacteria	0.62^{a}	0.23^{b}	0.12^{b}	0.06^{bc}	0.06^{cd}	0.07^{cd}	0.02^{d}	0.0361	<0.001
Spirochaetes	0.00^{a}	0.01^{b}	0.01^{b}	0.02^{bc}	0.05^{cd}	0.05^{cd}	0.06^{d}	0.0216	<0.001
Tenericutes	0.00^{a}	0.00^{b}	0.01^{bc}	0.03^{cd}	0.05^{de}	0.07^{e}	0.08^{e}	0.0204	<0.001
Cyanobacteria/Chloroplast	0.12^{c}	0.00^{b}	0.01^{b}	0.01^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.0117	<0.001
Firmicutes	0.00^{a}	0.30^{b}	0.36^{b}	0.37^{b}	0.34^{b}	0.27^{b}	0.21^{b}	0.0170	<0.001
SR1	0.00^{a}	0.01^{b}	0.01^{b}	0.01^{b}	0.01^{b}	0.00^{b}	0.00^{a}	0.0109	<0.001
Unclassified	0.00^{a}	0.01^{a}	0.01^{a}	0.01^{a}	0.00^{a}	0.01^{a}	0.00^{a}	0.0261	0.3

Means with different superscripts differ

Table 5.9: The relative abundance of bacteria genera present at an average of more than 0.05% colonising rice straw leaves after 0, 1,2,4,6,8 and 12 hour of *in vitro* incubation is bovine rumen fluid

Genus	Hour							p - value	s.e.d.
	0	1	2	4	6	8	12		
Fibrobacter	0.00^{a}	0.02^{b}	0.03^{bc}	0.06^{bc}	0.08^{cd}	0.14^{d}	0.40^{e}	<0.001	0.035
Anaeroplasma	0.00^{a}	0.00^{b}	0.01^{bc}	0.03^{cd}	0.05^{de}	0.07^{e}	0.08^{e}	<0.001	0.020
Prevotella	0.00^{a}	0.10^{bc}	0.11^{c}	0.12^{c}	0.12^{c}	0.11^{c}	0.08^{b}	<0.001	0.016
Treponema	0.00^{a}	0.01^{b}	0.01^{b}	0.02^{bc}	0.04^{cd}	0.04^{cd}	0.05^{d}	<0.001	0.021
Pseudobutyrivibrio	0.00^{a}	0.01^{b}	0.01^{b}	0.03^{c}	0.05^{c}	0.03^{c}	0.03^{c}	<0.001	0.017
OTU 20	0.00^{a}	0.00^{a}	0.00^{a}	0.01^{b}	0.02^{b}	0.02^{b}	0.02^{b}	<0.001	0.016
OTU 28	0.00^{a}	0.00^{b}	0.01^{bc}	0.01^{cd}	0.02^{d}	0.02^{d}	0.02^{d}	<0.001	0.013
Paraprevotella	0.00^{a}	0.01^{b}	0.01^{bc}	0.02^{bc}	0.02^{bc}	0.03^{d}	0.02^{cd}	<0.001	0.009
Butyrivibrio	0.00^{a}	0.04^{cd}	0.06^{e}	0.06^{e}	0.05^{de}	0.02^{bc}	0.02^{b}	<0.001	0.012
Ruminococcus	0.00^{a}	0.00^{ab}	0.00^{ab}	0.00^{ab}	0.00^{ab}	0.00^{bc}	0.02^{c}	0.002	0.020
Succinivibrio	0.00^{a}	0.00^{b}	0.00^{bc}	0.01^{cd}	0.02^{e}	0.01^{d}	0.01^{d}	<0.001	0.009
OTU 123	0.00^{a}	0.00^{ab}	0.00^{bc}	0.00^{bc}	0.00^{bc}	0.01^{bc}	0.01^{c}	0.002	0.015
OTU 131	0.00^{a}	0.00^{ab}	0.01^{bc}	0.02^{cd}	0.02^{d}	0.01^{bcd}	0.01^{cd}	<0.001	0.018
OTU 281	0.00^{a}	0.00^{a}	0.00^{ab}	0.00^{a}	0.00^{a}	0.00^{b}	0.01^{c}	<0.001	0.008
OTU 217	0.00^{a}	0.00^{ab}	0.00^{ab}	0.00^{ab}	0.00^{b}	0.00^{b}	0.01^{b}	0.01	0.017
OTU 64	0.00^{a}	0.01^{bc}	0.01^{bc}	0.02^{c}	0.02^{c}	0.01^{bc}	0.01^{b}	<0.001	0.020
OTU 76	0.00^{a}	0.00^{ab}	0.00^{ab}	0.00^{ab}	0.00^{ab}	0.01^{b}	0.01^{b}	0.009	0.019
Succiniclasticum	0.00^{a}	0.04^{e}	0.03^{de}	0.02^{cd}	0.01^{b}	0.01^{bc}	0.00^{b}	<0.001	0.014
OTU 32	0.00^{a}	0.00^{b}	0.00^{bcd}	0.00^{bc}	0.01^{d}	0.01^{cd}	0.00^{bc}	<0.001	0.007
OTU 83	0.00^{a}	0.01^{bc}	0.01^{bc}	0.01^{bc}	0.01^{c}	0.01^{bc}	0.00^{b}	<0.001	0.010
OTU 4	0.00^{a}	0.01^{c}	0.01^{c}	0.01^{c}	0.01^{bc}	0.01^{bc}	0.00^{b}	<0.001	0.007
OTU 375	0.00^{a}	0.01^{b}	0.01^{b}	0.01^{b}	0.01^{b}	0.00^{b}	0.00^{b}	<0.001	0.013
OTU 127	0.00^{a}	0.00^{bc}	0.00^{bc}	0.01^{bc}	0.01^{c}	0.01^{bc}	0.00^{b}	<0.001	0.008
OTU 144	0.00^{a}	0.00^{bc}	0.01^{bc}	0.01^{bc}	0.01^{c}	0.01^{bc}	0.00^{b}	<0.001	0.011
OTU 13	0.00^{a}	0.01^{c}	0.01^{c}	0.01^{bc}	0.01^{c}	0.01^{bc}	0.00^{b}	<0.001	0.012
OTU 148	0.00^{a}	0.01^{cd}	0.01^{cd}	0.01^{cd}	0.01^{d}	0.00^{bc}	0.00^{b}	<0.001	0.007

Sphingobium	0.03^{b}	0.02^{ab}	0.01^{ab}	0.00^{a}	0.00^{a}	0.01^{ab}	0.00^{a}	0.009	0.028
OTU 143	0.00^{a}	0.01^{c}	0.01^{c}	0.01^{c}	0.01^{c}	0.00^{bc}	0.00^{b}	<0.001	0.008
OTU 124	0.00^{a}	0.01^{d}	0.01^{d}	0.01^{cd}	0.01^{d}	0.00^{bc}	0.00^{b}	<0.001	0.007
OTU 399	0.00^{a}	0.00^{bc}	0.00^{bc}	0.01^{c}	0.00^{ab}	0.00^{bc}	0.00^{bc}	<0.001	0.012
OTU 67	0.00^{a}	0.01^{bcd}	0.01^{cd}	0.01^{cd}	0.01^{d}	0.01^{bc}	0.00^{b}	<0.001	0.011
OTU 421	0.00^{a}	0.01^{c}	0.01^{c}	0.01^{c}	0.00^{c}	0.00^{c}	0.00^{b}	<0.001	0.006
OTU 40	0.00^{a}	0.01^{c}	0.01^{c}	0.00b^{c}	0.00b^{c}	0.00^{bc}	0.00^{ab}	<0.001	0.012
OTU 45	0.00^{a}	0.01^{c}	0.01^{bc}	0.01^{bc}	0.01^{c}	0.00^{bc}	0.00^{ab}	<0.001	0.014
Sphingomonas	0.08^{c}	0.05^{bc}	0.03^{abc}	0.01^{ab}	0.00^{a}	0.01^{ab}	0.00^{a}	<0.001	0.044
SR1_*genera_incertae_sedis*	0.00^{a}	0.01^{b}	0.01^{b}	0.01^{b}	0.01^{b}	0.00^{b}	0.00^{a}	<0.001	0.011
Novosphingobium	0.10^{b}	0.01^{a}	0.01^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	0.028
OTU 89	0.01^{b}	0.00^{ab}	0.01^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	0.023
Aureimonas	0.01^{b}	0.01^{b}	0.00^{ab}	0.00^{a}	0.00^{ab}	0.00^{a}	0.00^{ab}	0.006	0.020
Streptophyta	0.12^{c}	0.00^{b}	0.01^{b}	0.01^{b}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	0.012
Microbacterium	0.03^{c}	0.01^{b}	0.01^{ab}	0.00^{ab}	0.00^{a}	0.00^{ab}	0.00^{a}	<0.001	0.021
Actinomycetospora	0.01^{b}	0.00^{ab}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.003	0.015
Bosea	0.01^{c}	0.01^{b}	0.00^{b}	0.00^{a}	0.00^{a}	0.00^{b}	0.00a	<0.001	0.009
Brevundimonas	0.01^{c}	0.00^{ab}	0.00^{b}	0.00^{ab}	0.00^{a}	0.00^{ab}	0.00a	<0.001	0.011
Caulobacter	0.02^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00a	<0.001	0.002
Chitinophaga	0.01^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00a	<0.001	0.001
Chryseobacterium	0.01^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00a	<0.001	0.001
Curtobacterium	0.02^{c}	0.00^{b}	0.00^{ab}	0.00^{ab}	0.00^{ab}	0.00^{ab}	0.00a	<0.001	0.016
Devosia	0.01^{b}	0.00^{ab}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00a	<0.001	0.019
Dyadobacter	0.02^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00a	<0.001	0.013
Pantoea	0.05^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00a	<0.001	0.006
Geodermatophilus	0.01^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00a	<0.001	0.010
Larkinella	0.01^{c}	0.00^{bc}	0.00^{ab}	0.00^{a}	0.00^{a}	0.00^{a}	0.00a	<0.001	0.014
Lysobacter	0.01^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00a	<0.001	0.016
Methylobacterium	0.06^{c}	0.02^{b}	0.01^{b}	0.01^{b}	0.01^{ab}	0.00^{ab}	0.00a	<0.001	0.025
Mucilaginibacter	0.01^{c}	0.00^{b}	0.00^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	0.012

Mycobacterium	0.01^{d}	0.00^{cd}	0.00^{bcd}	0.00^{abc}	0.00^{a}	0.00^{ab}	0.00^{a}	<0.001	0.011
Niastella	0.02^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	0.016
Nocardioides	0.01^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	0.009
OTU 156	0.01^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	0.003
Pseudacidovorax	0.01^{c}	0.00^{bc}	0.00^{bc}	0.00^{a}	0.00^{a}	0.00^{ab}	0.00^{a}	<0.001	0.013
Pseudomonas	0.04^{c}	0.01^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{ab}	0.00^{a}	<0.001	0.019
Quadrisphaera	0.01^{b}	0.00^{ab}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.003	0.018
Rhizobium	0.04^{c}	0.02^{bc}	0.00^{ab}	0.00^{ab}	0.00^{a}	0.01^{ab}	0.00^{a}	<0.001	0.028
Roseateles	0.01^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	0.014
Roseomonas	0.01^{b}	0.00^{b}	0.00^{ab}	0.00^{ab}	0.00^{ab}	0.00^{ab}	0.00^{a}	0.025	0.020
Sphingobacterium	0.07^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	0.017
Stenotrophomonas	0.02^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	<0.001	0.013
Xenophilus	0.01^{b}	0.01^{b}	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}	0.00a	<0.001	0.011

Means with different superscripts differ

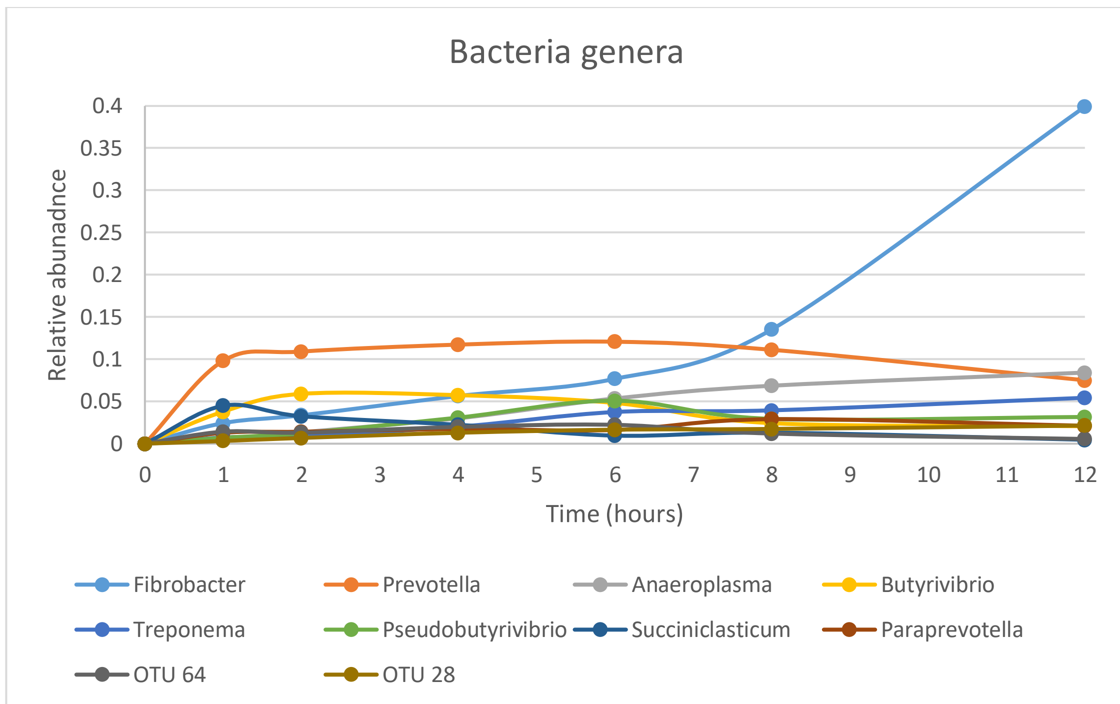


Figure 5.15 Scatter graph showing changes in the relative abundance of the main bacterial genera over 12h incubation period. The selected genera were absent at the beginning of the incubation (zero hour) and showed dynamic abundance over the incubation period. Analysed using Ion Torrent PGM 16S rRNA gene sequencing. Reads were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II. Samples standardised by total number of reads and transformed by square root.

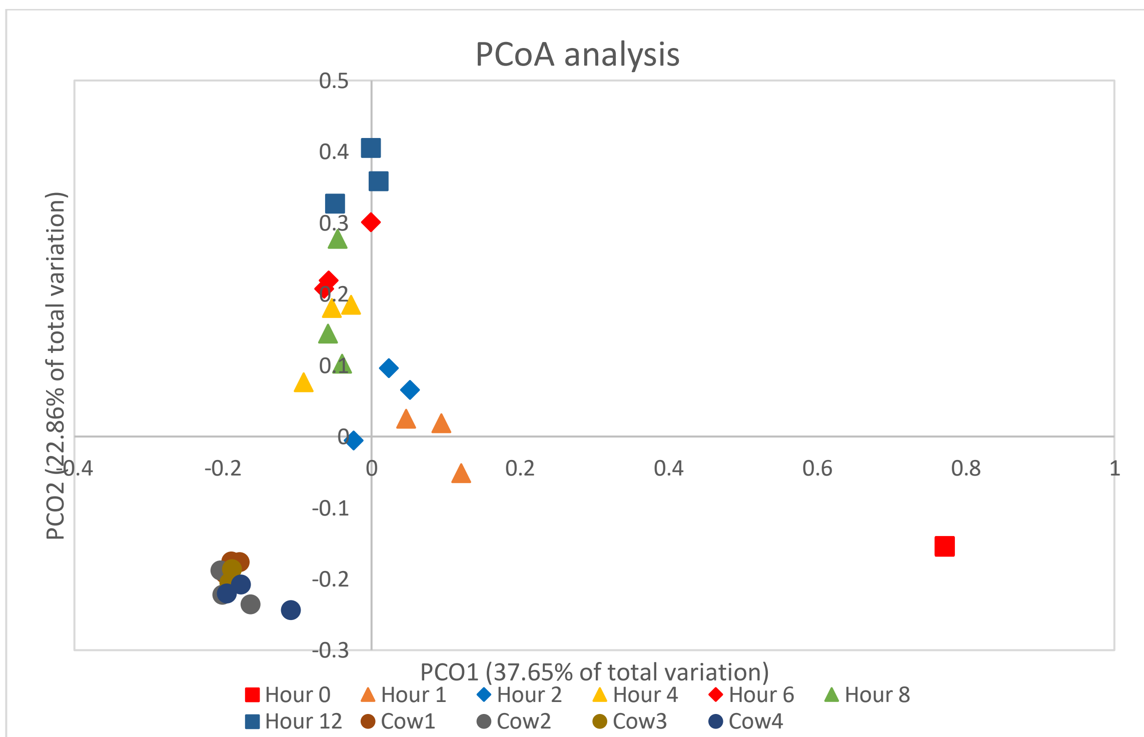


Figure 5.16: Visualisation of relative abundance matrices using principal coordinate analysis (PCO) ordination on a Bray-Curtis distance matrix to identify patterns in the data. Rumen bacteria analysed

using Ion Torrent 16S rRNA gene sequencing. Samples standardised by total number of reads and transformed by square root.

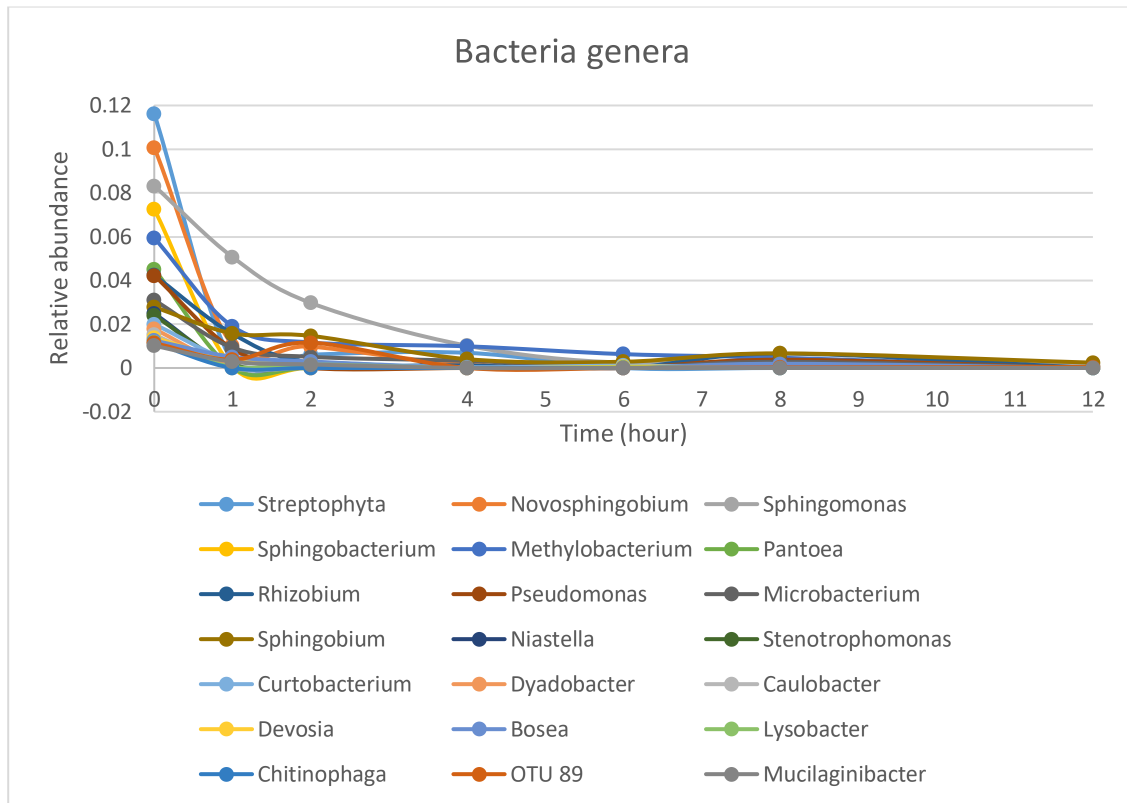


Figure 5.17: Scatter graph showing changes in the relative abundance of bacterial genera over 12h incubation period. The selected genera had a high relative abundance before the incubation (zero hour) which rapidly reduced at the incubations began. Rumen bacteria analysed using Ion Torrent PGM 16S rRNA gene sequencing. Reads were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II. Samples standardised by total number of reads and transformed by square root.

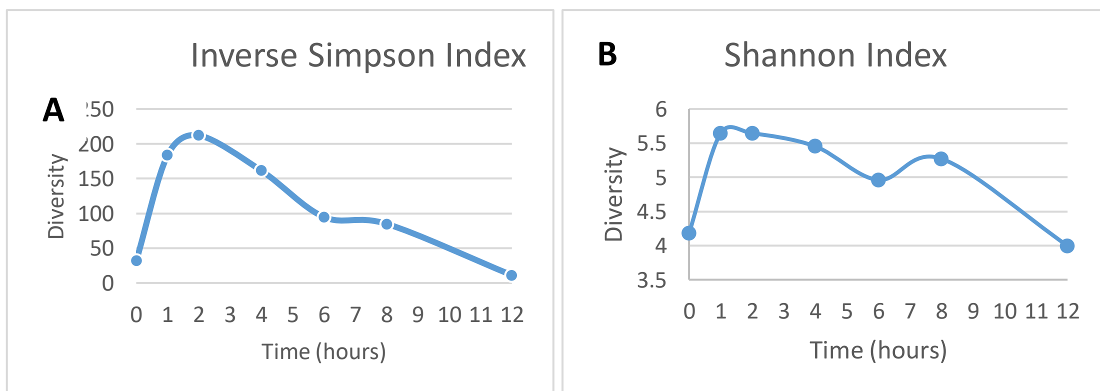


Figure 5.18 The Simpson (**A**) and the Shannon (**B**) indices representing changes in diversity of bacteria colonising rice straw incubated in rumen fluid over 12h

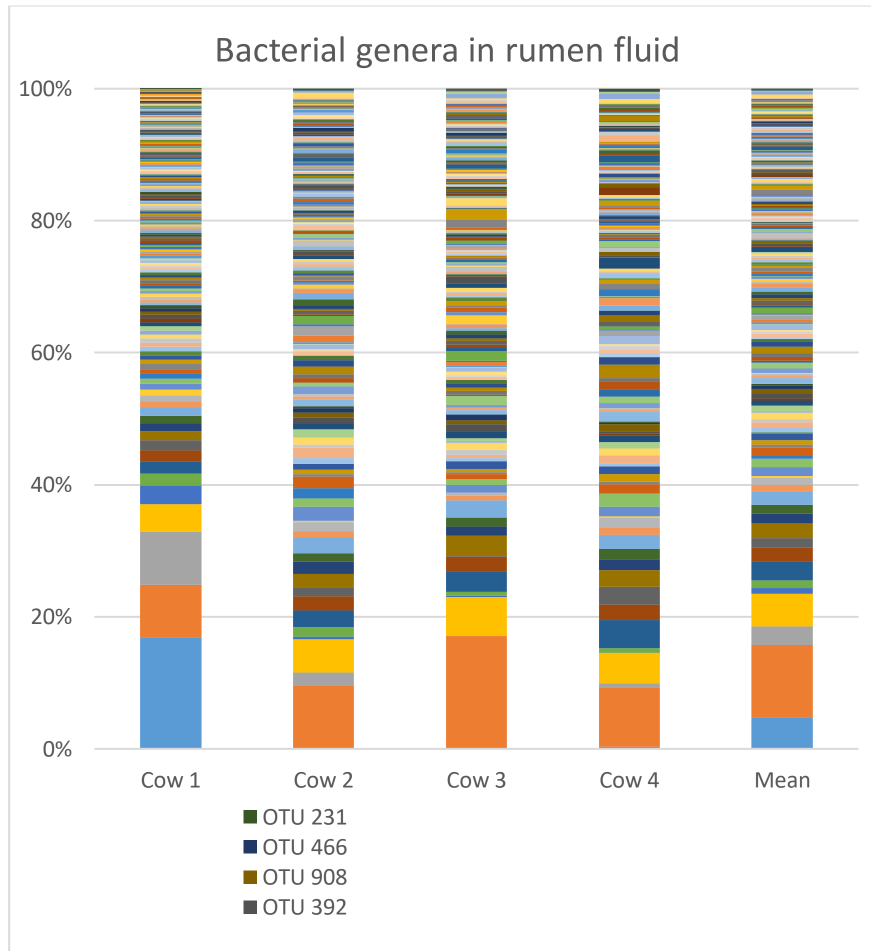


Figure 5.19: Stacked histograms illustrating genus-level bacterial composition present at an average of more than 0.05% showing the *in vitro* effect of on cow rumen bacteria analysed using Ion Torrent PGM 16S rRNA gene sequencing. Reads were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II. Samples standardised by total number of reads and transformed by square root

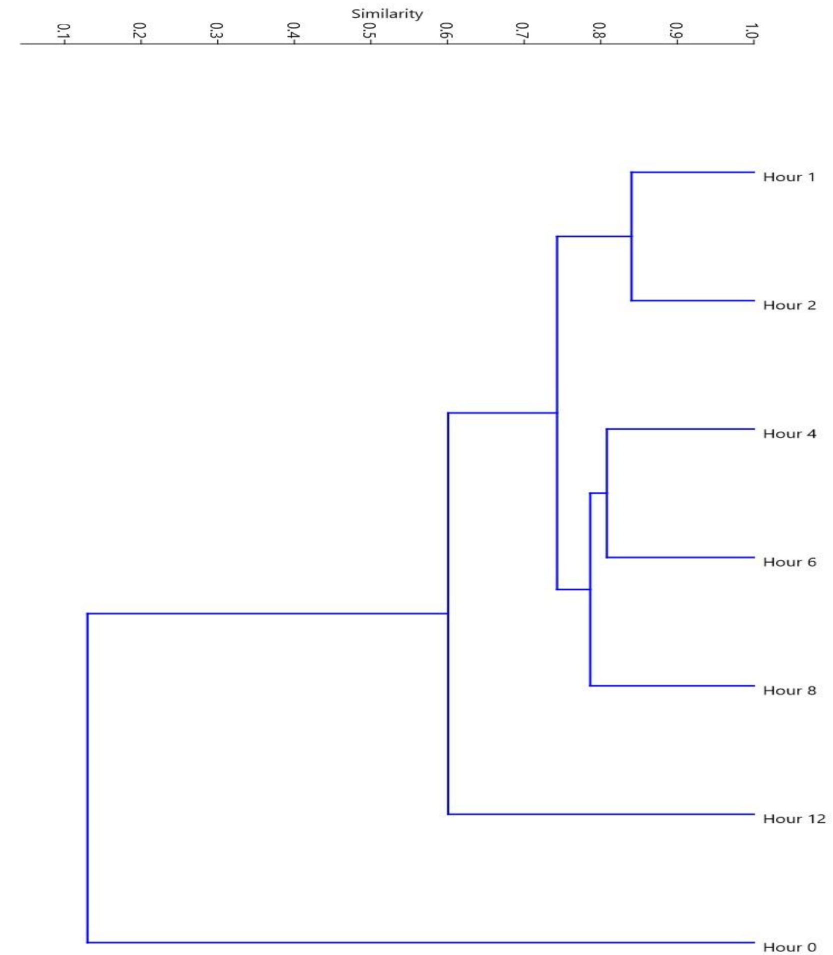


Figure 5.20: Cluster analysis of the *in vitro* colonisation of rice straw by cow rumen bacteria analysed using Ion Torrent PGM 16S rRNA gene sequencing. Dendrogram based on the UPGMA clustering of the Bray-Curtis distances. Samples standardised by total number of reads and transformed square root; minor genera discarded (<0.05%).

5.5 DISCUSSION

Rice straw consists predominantly of cell walls, comprised of cellulose, hemicellulose, and lignin (Van Soest, 2006). To break down these components cellulase, hemicellulase and ligninase are required (Schiere & Ibrahim, 1989). The animals themselves do not produce these enzymes but the rumen maintains microorganisms that do produce cellulase and hemicellulase. However, lignin cannot be broken down in the rumen due to the lack of ligninase. Lignin, however, has important effects on livestock production through effects on degradability and feed intake. Lignin plays a role in resisting compressing forces, providing protection against consumption by insects and mammals, and inhibiting the rate and degree of microbial degradation (Sarnklong et al., 2010). Another important component of the rice cell walls is silica and can be present in high concentrations ranging from 5% to 15%, depending on the rice variety and the availability of this mineral in the soil (Agbagla-Dohnani et al., 2003; Vadiveloo, 2000). Silica reduces palatability and the degradability of rice straw in the rumen due to its direct action in preventing colonisation by ruminal microorganisms (Agbagla-Dohnani et al., 2003).

In this experiment, chemical analysis confirmed the expected high NDF, ADF and lignin values in the range reported by Sarnklong et al. (2010), Agbagla-Dohnani et al. (2003) and Vadiveloo (2000). There were significant differences between the varieties; and between all rice straw varieties and hay (Table 5.5). Total silica levels in rice straw were within the ranges reported by Agbagla-Dohnani et al. (2003) and much higher compared to hay. Van Soest and Jones (1968) reported a negative correlation between silica and lignin content in 49 varieties of forages. The physiological explanation for this relationship is that silica may act like lignin components by increasing the structural rigidity of the plant (Raven, 1983). Consequently, lignin synthesis could be diminished in plants that accumulate silica (Van Soest & Jones, 1968). Such a relationship was not found in this experiment. Agbagla-Dohnani et al. (2001) were similarly unable to demonstrate such a relationship in rice straw. More studies are therefore needed to verify this hypothesis.

Silica levels in rice plants are usually higher than in other plants, which has led many to suggest that silica is responsible for the low digestibility of rice straw but, the evidence

for this view is weak (Santos et al., 2010). Wang et al., (2006) suggested that the negative impacts of silica on digestibility of rice plants may be due to its location in the fibre matrix rather than in its total level in the plant. Silica in rice straw is primarily found in the epidermis as a silicified waxy layer (Van Soest, 2006). Wang et al. (2007) demonstrated that dissolving of the cuticle wax layer of rice straw stem epidermis with NaOH resulted in a higher degradation by rumen microorganisms. Santos et al. (2010) reported that the association of silica with either ADF or NDF influenced gas production with varieties having more silica in the NDF rather than ADF showing a lower gas production. Further, long term storage of straw after baling reduced the *in vitro* fermentation despite having the same levels of silica before storage. They suggested that field drying of rice straw altered the chemical nature of the fibre matrix or silica, or an interaction of the two that resulted in lowered digestibility. These observations support suggestions of Wang et al. (2006) that the location of the silica in the plant may also alter its chemical form and reactivity with organic components and could affect degradation (Santos et al., 2010). They concluded that the digestibility of rice straw cannot be predicted through common chemical analyses.

The results of this experiment also suggest that the total levels of silica in rice straw may not be an accurate predictor of digestibility. While there were significant differences in *in vitro* gas production between the rice straw varieties (Figure 5.4), silica levels had minimal effects (Figure 5.5). Canonical correspondence analysis (CCA) of the relationship between the chemical composition of rice straw and *in vitro* fermentation parameters also confirmed that silica content had minimal effect compared to organic matter and fibre components (Figures 5.8 and 5.9). Further, the CCA triplot showed that the levels of ADL, Ash, NDF and ADF had the largest effects on *in vitro* fermentation in that order while silica content had the least. Results published by Agbagla-Dohnani et al. (2001) also suggested that the fibre components were more important in determining rice straw degradation than silica content. They reported that the degradation *in sacco* degradation of rice straw was positively related to hemicelluloses, whereas increasing cellulose and lignin tended to decrease degradation. Wang et al. (2006) reported that the increase in lignin levels during maturation resulted in lower gas production. Additionally, a gene mutant variety of rice with higher lignin levels in the straw had a significantly higher gas production rate

and a shorter lag time, but lower in potential gas production. The current study produced results consistent with those findings; there was a positive correlation between the ADL levels and the rate of gas production (Figure 5.7).

Ion Torrent sequencing of 16S rRNA gene revealed substantial temporal changes occurring within the first hour and after 6h of rumen incubation (Figure 5.20). The first major shift in the community composition during the first hour was phylum *Proteobacteria* being replaced by members belonging to the *Bacteroidetes* and *Firmicutes*. This shift was accompanied by a rapid reduction and a subsequent rapid increase in total bacterial DNA measure *via* real-time PCR (Figure 5.10). The second shift occurred at the 6h point where bacteria in the phylum *Fibrobacteres* largely replaced the bacteria from the primary colonisation.

Successional colonisation of forage by rumen bacteria has been previously reported. Huws et al. (2013, 2016) investigated the successional colonisation of fresh perennial ryegrass by the bacteria in the cow rumen and identified distinct primary (0–2h) and secondary (4h onward) bacterial communities. The authors demonstrated that a proportion of the primary colonising bacteria (*Succinivibrio* spp.) detached after 2h of incubation and were replaced with the population of secondary colonising bacteria, which included *Prevotella*, *Pseudobutyrivibrio*, *Roseburia*, and *Ruminococcus*. Cheng et al. (2017) investigated the colonisation of ground rice straw incubated *in sacco* for 72h. They reported that the proportions of *Anaerostipes*, *Coprococcus*, *Desulfovibrio*, *Prevotella*, *Pseudobutyribivrio*, *Ruminococcus*, and *Succiniclasticum* all significantly decreased in the secondary bacterial community when compared to the primary bacterial community. In addition, the *Prevotella* significantly decreased to ∼9% abundance in the secondary bacterial community from ∼29% in the primary bacterial community, whereas the proportions of *Clostridium*, *Dehalobacterium*, and *Oscillospira* increased in the secondary bacterial community. Successional occurrence of ruminal bacterial microbiota colonising rice straw and alfalfa was reported by Liu et al. (2016). In their experiment, the change to a secondary phase occurred after 6 hours was mainly associated with decreases in abundance of the taxa *Butyrivibrio*, *Prevotella*, *Ruminococcus*, and unclassified *Christensenellaceae* and increases in *Fibrobacter*, *Treponema*, unclassified *Bacteroidales*, and unclassified *Rikenellaceae* (Liu et al., 2016). Piao et al. (2014) also observed the same behaviour when air-dried

switchgrass was incubated in the rumen. In their case, pyrosequencing-based analyses identified discrete microbial profiles within the first 30min and after 4h of rumen incubation.

In this study, after the first hour of incubation, the bacterial population was dominated by the taxa *Prevotella* (9.8%), *Succiniclasticum* (4.5%) and *Butyrivibrio* (3.8%). A shift occurred at 6h where there was a significant reduction in these taxa and an increase in *Fibrobacter*, *Treponema* and *Anaeroplasma*. Liu et al. (2016) showed that the first phase of colonisation was accompanied by the greatest loss of biomass attributed to rapid utilization of soluble sugars and other easily fermentable nutrients. They suggested that bacteria in the second phase play a more focused role in plant fibre degradation reflected in the results showing that more fibre-degrading bacteria, such as *Fibrobacter* and *Treponema* observed in the samples of rice straw from 6h to 12h.

In general, the genera *Butyrivibrio*, *Prevotella*, *Anaeroplasma*, *Treponema*, and *Fibrobacter* were the major colonising bacteria. Similar dominance has been described by (Liu et al., 2016) and is partly consistent with the report of Huws et al. (2016). The latter reported that *Butyrivibrio*, *Fibrobacter*, *Olsenella*, and *Prevotella* spp. abundances were high in the community of rumen bacteria colonising fresh perennial ryegrass, indicating that these taxa play an important role in forage degradation in the rumen of dairy cattle (Huws et al., 2016).

The successional pattern of colonisation may be attributed to the different functions of bacteria during the process of fermentation. Liu et al. (2016) reported that *Butyrivibrio* abundance was positively associated with the crude protein (CP) content and was higher in alfalfa-associated microbiota than rice straw sample from 0.5 to 6h of incubation in rumen of cannulated cows. *Butyrivibrio* spp. are known to have proteolytic and polysaccharide-degrading activity (Kelly et al., 2010); the effect might therefore be partly explained by the higher content of CP in alfalfa hay compared with the rice straw. In this study, the levels of *Butyrivibrio* did not increase as robustly as compared to *Prevotella* and *Fibrobacter*. Maximum levels were attained at 2h and the numbers started falling after that time-point; the decline was more pronounced after the 6h time-point. The low CP content of rice straw may have accounted for this.

Prevotella was the most abundant genus in the un-incubated rumen fluid of three cows; in one of the cows, OTU 64 was the dominant bacteria (Figure 5.19). *Prevotella*

has been reported as the most abundant genus in the rumen of adult dairy cattle, and it is also associated with ruminal carbohydrate and protein fermentation (Chiquette, Allison, & Rasmussen, 2008). The present study showed that *Prevotella* spp. dominated in the first six hours of incubation and was largely replaced by the genus *Fibrobacter* from 6h onwards. *Fibrobacteria* is one of the main cellulose-degrading bacterial genera in the rumen, and it may have an important role in the degradation of low-quality forages such as rice straw (Tajima et al., 2001). Liu et al. (2016) reported that the abundance of *Fibrobacteria* was positively correlated with the content of NDF whereby a higher abundance of *Fibrobacteria* was observed in rice straw incubated for 16 and 48h in the rumen than in the corresponding alfalfa hay samples.

5.6 CONCLUSION

Results clearly demonstrate the substantial differences in the nutritional value of hay compared to rice straw, and it is obvious that hay should be first choice between the two if it is available and economically feasible. However, in a production system with limited feeding options, a better understanding of the degradation characteristics and colonisation patterns can potentially improve the nutritive value of rice straw. The role of silica in the degradation of rice straw by rumen bacteria is unclear. Results from this and other referenced studies show that the total amount of silica in rice straw may not be as important as its chemical form and interaction with the fibre matrix. As a result, chemical qualification of silica in rice straw may not be an accurate predictor of digestibility and more research is needed.

This study also documented the *in vitro* colonisation pattern of rice straw rumen bacteria. Colonisation occurred in a biphasic pattern. Within first hour of incubation, the epiphytic communities either decreased to low relative abundances or disappeared completely. The bacterial population was dominated by the taxa *Prevotella*, *Succiniclasticum* and *Butyrivibrio*. A second shift occurred at 6h where there was a significant reduction in these taxa and an increase in *Fibrobacter*, *Treponema* and *Anaeroplasma*. *Fibrobacteria* is one of the main cellulose-degrading bacterial genera in the rumen, and it may have an important role in the degradation of low-quality forages such as rice straw. Further investigation of the function of colonising bacteria, and especially the secondary bacterial community, is needed to improve the utilization of rice straw in ruminants.

CHAPTER 6: THE EFFECT OF CHEMICAL COMPOSITION ON *IN VITRO* COLONISATION OF RICE STRAW BY RUMEN MICROBIOTA

6.1 INTRODUCTION

The forage lignocellulosic complex is one of the greatest limitations to utilization of the nutrients and energy in fibre. Consequently, several technologies have been developed to increase forage fibre utilization by ruminants (Adesogan et al., 2019). In areas where rice straw is abundantly available from cultivating rice, farmers offer rice straw as the main roughage source to their animals (Sarnklong et al., 2010). Rice straw is sometimes chopped to reduce wastage and to facilitate feeding, but this does not alter the cell wall structure in such feeds (Devendra, Pearce, & Doyle, 1986). Buffaloes have been observed to consume more chopped than long rice straw (Castillo et al., 1982); however no differences were found between long and chopped straw in dry matter intake and digestibility by sheep (Devendra, 1983a). Investigation into the effect of chopping straws from other cereal crops have also failed to show a difference a difference in weight gain of animals (Mathison, 1976). This suggests that chopping is unlikely to improve the feeding value of rice straw (Devendra et al., 1986). Machine threshing incorporates chopping of straw; in some countries, notably Kenya, hand-operated chaff cutters are common (Kimenchu et al., 2014). However, farmers who do not own one will not cut straw before feeding.

In the rumen, degradation of cellulose requires direct association of microbial cellulases with the substrate and, hence, the rate of hydrolysis would be expected to be affected by the cellulosic surface area accessible to the enzyme (Devendra et al., 1986). Grinding would therefore have a large effect on degradation than chopping. The extent of any increase in cellulosic surface area is likely to be determined by the fineness of grinding. For instance, ball-milling results in extreme reduction in particle size to the point of the physical separation of cell wall components and this results in marked increases in *in vitro* digestibility. However, the high cost of the process means that it has no practical significance for animal production. (Moore, Effland, & Millett, 1972). Grinding methods commonly employed in the processing of animal feeds

results in only moderate increases in the exposed cellulosic surface area because of the length-width relationships of fibres (Walker, 1984). Many of these treatments are not for practical use on small-scale farms, as they require machines or industrial processing. This makes these treatments economically unprofitable for farmers as the benefits may be low. However, small machines to grind or chop rice straw may be feasible (Malik et al., 2015).

Several chemical and biological treatments have been applied in attempts to improve the feed quality of straw. These include treatment with sodium hydroxide (NaOH), ammonia, urea, pressure and heat, urine, enzymes, acids and fungi (Van Soest, 2006). The "Beckman method" involves soaking the straw in solutions of NaOH, draining and washing. The result is dramatic increases in digestibility but accompanied by considerable loss in dry matter including valuable soluble organic matter (Jackson, 1977). While NaOH cleaves the lignin bond thus increasing digestibility, it also increases the disintegration of the fibre reducing particle size and increasing passage of undigested fibre. As a result, the increased *in vitro* digestibility is difficult to replicate in animal digestion trials (Klopfenstein, 1978). Additionally, the intake of soluble sodium salts increases osmotic pressure and rumen washout (Jackson, 1977). Overall, ammonia treatment has produced the largest increases in rates of digestion and voluntary intake. Urea treatment is however more accepted by farmers in tropical countries. Farmer acceptance is related to their perceptions on costs, labour, equipment, health, safety, *i.e*. the exposure to ammonia vapour, economic and other social factors. Compared to NaOH, urea has the advantage being easy and safe to use, and provides a source of non-protein nitrogen (Van Soest, 2006). The disadvantage of ammonia and urea is that they are weaker bases than NaOH. Ammonia and urea a similar effect of disrupting the silicified cuticular barrier in leaves; the rise in digestibility likely results from this effect as well as cleavage of some lignin-carbohydrate bonds. Physical treatments such as steam pressure and explosion need further investigation. Crushing also needs examination in relation to breaking the silicified encrusting layer (Van Soest, 2006).

Any technique employed is designed to increase intake and increase the surface area for microbial colonisation to increase fibre utilization (Adesogan et al., 2019). The ability of the rumen microbiota to attach in a timely manner to ingested forages in the

rumen is central to ruminant nutrient use efficiency (Huws et al., 2014). Experiments with fresh forage have shown that rumen microorganisms rapidly attach to recently ingested feed particles. Huws et al. (2013) observed that of fresh whole perennial ryegrass was colonised by rumen bacteria within 15 minutes of ingestion. The authors also reported that the colonisation of fresh fodder was successional with a change in the diversity of attached bacteria from primary to secondary attached communities occurring within 2 - 4 h of colonisation (Huws et al., 2013).

Rumen microorganisms colonising the surfaces of forages are confronted with highly variable physiochemical conditions including nutrient type and availability due to the heterogeneity of the plant material. This heterogeneity may affect microbial attachment and subsequent biofilm formation (Huws et al., 2014). Degradation of fresh forage within the rumen is known to be affected by the amount of cuticle, waxes or lignin and the degree of cross-linkage to other cell wall polymers within the plant. Rumen bacteria do not bind to the waxy cuticle of forage particles due to the difficulty in degrading these structures in order to access the plant nutrients (McAllister et al., 1994). Huws et al. (2014) observed that different parts of fresh perennial rye grass were colonised by different taxa and numbers of bacteria when incubated *in vitro* in the presence of rumen bacteria. In addition, the biofilm coverage on the adaxial surface was greater than the abaxial surface of the leaves. They concluded that bacterial attachment to different parts of the plant material was different due to the differences in their surface chemistry (Huws et al., 2014).

Although rice straw has a different chemical composition to fresh forage, successional colonisation of rice straw by the rumen microbiota over time has also been observed in Chapter 5 (Section 5.5) where chopped straw leaves were incubated *in vitro* in the rumen fluid over a period of 12h. As with, fresh forage, temporal colonisation of the rice straw by the rumen bacteria was biphasic with a primary colonisation occurring between 0-2h and a secondary colonisation at 6h (Figure 5.12). It is likely that composition could similarly affect colonisation of rice straw; Wang et al. (2006) observed that that the increase in lignin levels during maturation resulted in lower *in vitro* gas production and long term storage of rice straw after baling reduced the *in vitro* fermentation (Santos et al., 2010).

6.2 AIMS AND OBJECTIVES

Increasing the efficiency of feed degradation requires rapid and effective colonisation of newly ingested feed by rumen bacteria to drive fibre degradation. Colonisation of forages by rumen microbiota is commonly investigated without consideration of the effects of different plant structures on attachment of the microbiota. These approaches often miss fundamental information on the interactions of key microbiota with various plant structures and the subsequent effect on degradation of various structures within the whole plant. Therefore, there is a need to understand colonisation by rumen microbiota in relation to plant chemistry. This would contributes towards developing novel strategies of improving ruminant nutrient use efficiency to ensure food security (Huws et al., 2014). It would also help in developing a cheap, efficient and environmental friendly pre-treatment technique for the industrialization of rice straw (Chen et al., 2011).

The dynamics of colonisation of rice straw by rumen bacteria holds the key understanding and possibly improving the breakdown of forage in the rumen. Previous studies observed that colonisation of rice straw is biphasic with distinct 0-1h phase and a 6h and onwards (Chapter 5). It has been proposed that the differences in the chemistry of plants influence microbial colonisation and observations by Huws et al. (2014) on fresh perennial rye grass supported that. The accumulation of silica on the surface of rice straw has been proposed as the primary barrier to rumen degradation (Van Soest, 2006). However, no studies have been carried out to characterise the bacterial colonisation of field-grown untreated rice straw.

This study therefore proposed to the further investigate the relationship between silica content and digestibility, *i.e.*, does the absolute content of silica in rice straw affect in vitro degradation? Does it have any effect when rice straw is milled?

This study also proposed that there was an effect of the levels of silica in rice straw leaves on the colonisation by rumen microbes as determined *via* 16S rRNA gene metabarcoding.

6.3 EXPERIMENTAL DESIGN

This chapter is a continuation of the investigations detailed in Chapter 5. Rice straw with a range of silica contents was obtained as described in Chapter 5 (Section 5.4). The separated leaves were milled and analysed for organic matter, silicon and fibre as detailed in Chapter 2 (Section 2.2). Based on the results observed in Chapter 5, six straws were selected for further experimentation based on the relationship between *in vitro* gas production and silica content to represent low, median and high gas production and silica content (Figure 6.1, Table 6.1).

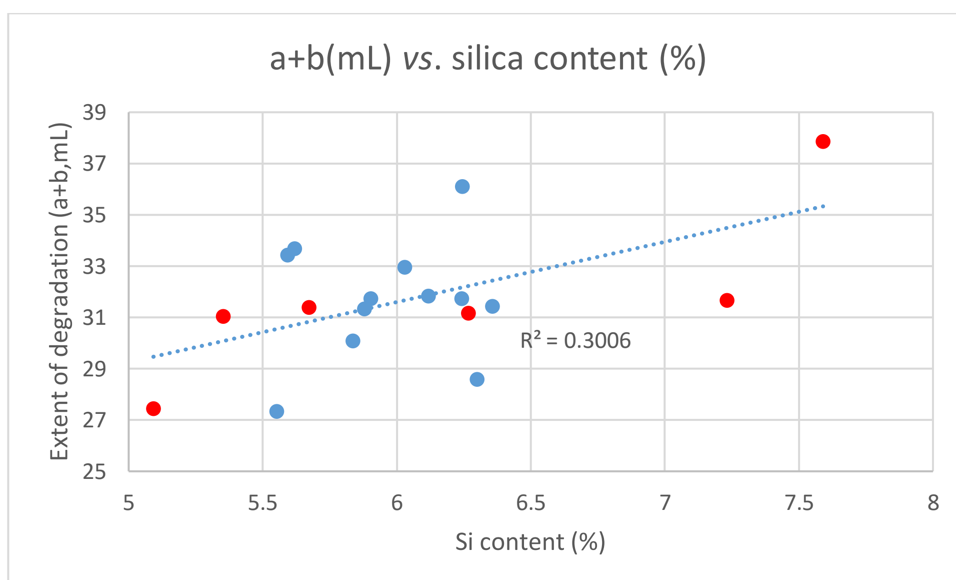


Figure 6.1: Correlation between extent of degradation (a+b, mL) and Silica content (%) (R^2=0.3006) in straw samples (from Chapter 5). The data points in red were the samples selected for experimentation in this chapter.

Table 6.1: Samples selected for *in vitro* degradation and colonisation. Selection was based on the Si content (%). Samples were chopped and milled to represent two levels of mechanical treatment and their effect on *in vitro* degradation.

Identity	**Treatments**		**Si content (%)**
L12-2	Chopped 1	Milled 1	5.67
L103-2	Chopped 2	Milled 2	6.24
L103-3	Chopped 3	Milled 3	5.35
L117-2	Chopped 4	Milled 4	7.59
L117-3	Chopped 5	Milled 5	7.23
L123-1	Chopped 6	Milled 6	5.09

6.3.1 *IN VITRO* DEGRADATION AND COLONISATION

Results published in Chapter 5 showed an unexpected positive correlation between silica content and *in vitro* gas production (Figure 6.1). In order to determine the degradability of the rice straw in the rumen, gas production over 96h was determined using the method of Theodorou et al. (1994) as detailed Chapter 2 (Section 2.2.5). However, the rumen fluid to buffer ratio was changed from 1:2 as used in Chapter 5 to 1:9. This was done to rule out that an overestimation of gas production due to the presence of feed particles suspended in the rumen fluid (Maccarana et al., 2016) was the cause of the results observed in Chapter 5.

The *in vitro* gas production experiment was carried out in a 6 x 2 factorial design with six rice straw varieties and two levels of mechanical treatment as the factors. Six chopped samples and six milled samples were incubated with pooled rumen fluid from four cannulated cows in triplicate (6 X 6) making total of 36 bottles plus 3 blanks (39). Cumulative gas production and VFA at the end of the incubation was measured all these bottles. Statistical analyses were done on Genstat 19th edition (VSN International Ltd., Hemel Hempstead, UK). Analysis of gas production volume and rate; and VFA production was done using two-way ANOVA, with these measurements as the dependent variables and rice straw variety as the fixed factor. Multiple comparisons of the means (Fisher's protected LSD, p=0.05) was done as the *post hoc* test.

The results in Chapter 5 also showed that the colonisation of rice straw was dynamic with two phases occurring between 0-1h and 6h onwards. The 12h hour point was therefore selected to as the primary time-point to determine the effect of different rice straw varieties on microbial colonisation. Six chopped samples were incubated as described for *in vitro* colonisation study (6 x 3) (Table 6.2). The replicates were harvested at 12 hours for colonisation analysis (18 bottles). The diversity of attached bacteria was assessed using Ion Torrent PGM of 16S rRNA (cDNA) as detailed in Chapter 2 (Section 2.2.7).

At the end of the gas production incubation (96h), the undigested straw was also harvested for analysis of bacterial colonisation to determine whether further changes in the attached bacteria occurred after 12h.

Table 6.2: Experimental design for *in vitro* degradation and colonisation

Treatment	Replicates						
	Full Gas Production (96h)				Colonisation (12h)		
	1	2	3		1	2	3
Chopped 1	1	2	3		A	B	C
Chopped 2	4	5	6		D	E	F
Chopped 3	7	8	9		G	H	I
Chopped 4	10	11	12		J	K	L
Chopped 5	13	14	15		M	N	O
Chopped 6	16	17	18		P	Q	R
Milled 1	19	20	21				
Milled 2	22	23	24				
Milled 3	25	26	27				
Milled 4	28	29	30				
Milled 5	31	32	33				
Milled 6	34	35	36				
Blank	37	38	39				

Following Ion Torrent sequencing of the 16S rRNA gene and bioinformatic processing, relative bacterial taxa abundance data were subjected to square root transformation. Statistical analysis of the differences in the relative abundance of attached bacteria at 12 and 96 hours was done using one-way ANOVA with Fisher's protected LSD as the *post hoc* test. The straw variety effects on transformed data were analysed based on their Bray-Curtis distance metric within the function unweighted pair group method with arithmetic mean (UPGMA). Canonical correspondence analysis (CCA) was also performed to investigate the relationships between the chemical composition of straw, the fermentation dynamics, and the relative abundances of the attached bacteria. CCA was carried out using default settings with unrestricted permutations. All tests were carried out using the PAST v3 statistical package (Hammer et al., 2001).

6.4 RESULTS

6.4.1 *IN VITRO* DEGRADATION

For further investigation into the relationship between silica content and digestibility of ground *versus* chopped feed, samples of six straws were selected from those studies in Chapter 5 to represent low, median and high gas production and silica content (Figure 6.1, Table 6.1). For all rice straw varieties, milled samples showed a significantly higher cumulative gas production compared to chopped samples. In addition, there were differences between cumulative gas production between the milled samples. Accession L12_2 had the highest cumulative gas production while L117_2 had the lowest in the milled samples. However, there was no difference between the between the chopped samples. There was no difference between the samples and the treatments in regard to the rate of degradation (Table 6.3). Similarly, there was a treatment effect on VFA production but no difference was detected within treatment groups (Table 6.4). The levels of ADL and silica were positively correlated with cumulative gas production in chopped samples. This is consistent with the results obtained in Chapter 5 (Figure 5.5). The opposite effect was seen in milled sample where there was a negative correlation between cumulative gas production and the levels of ADL and silica (Figure 6.3, Figure 6.4).

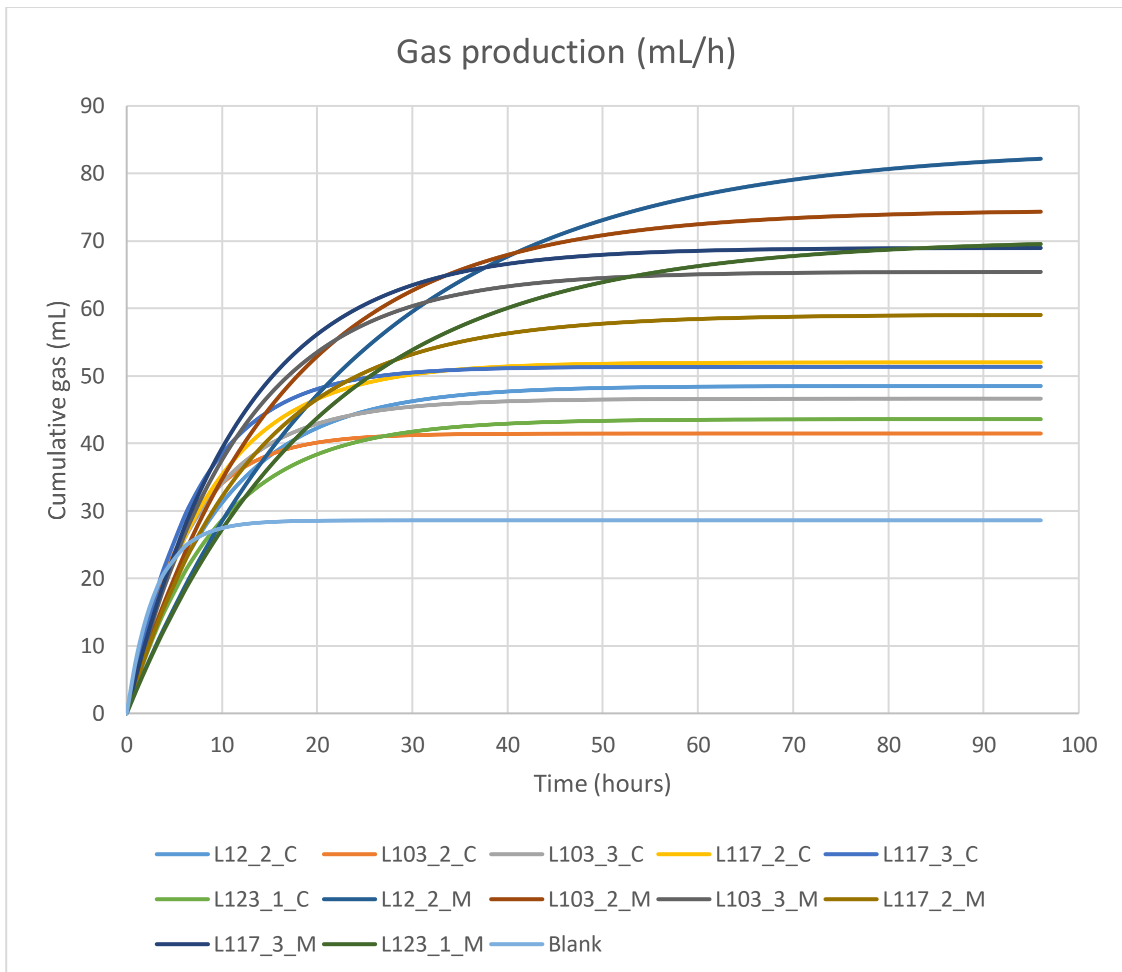


Figure 6.2: Cumulative gas production (mL/hour) from *in vitro* fermentation of in rumen fluid for 96 hours showing the effect of rice straw variety and physical treatment ("C" – chopped and "M" – milled)

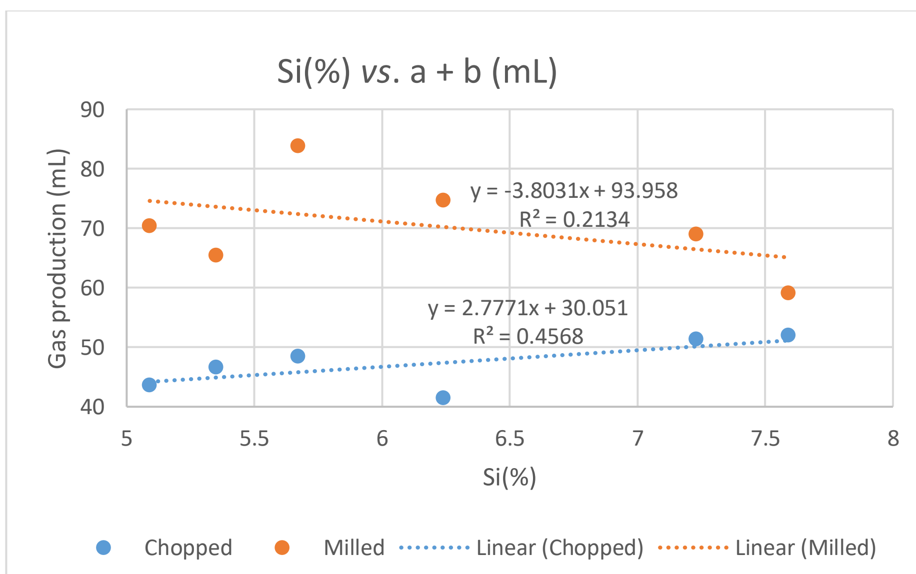


Figure 6.3: Correlation between extent of *in vitro* degradation (a+b, mL) and silica content (%) of chopped (R^2=0.4568) and milled (R^2=0.2134) of rice straw samples by rumen microbiota.

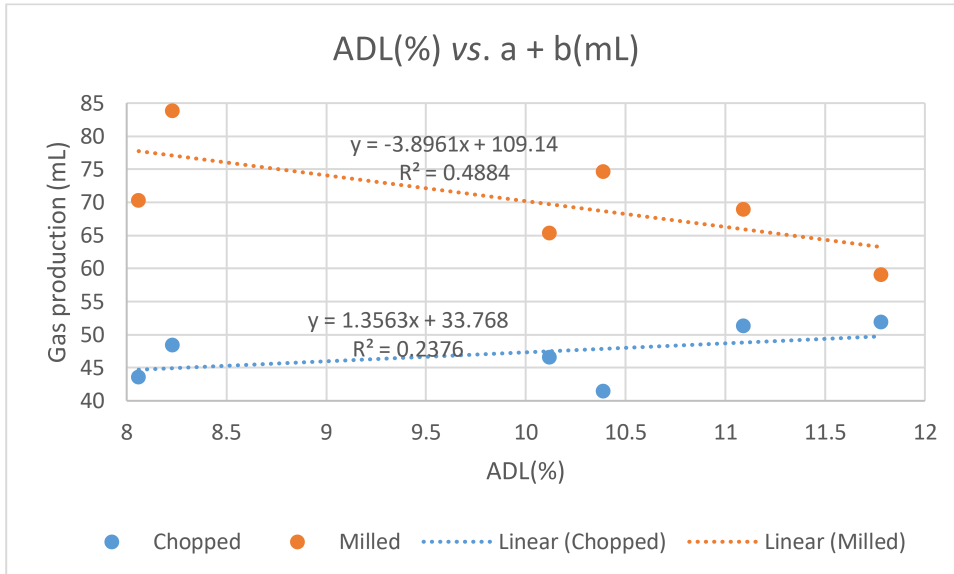


Figure 6.4: Correlation between extent of *in vitro* degradation (a+b, mL) and acid detergent lignin (ADL) content (%) of chopped (R^2=0.2376) and milled (R^2=0.4884) of rice straw samples by rumen microbiota.

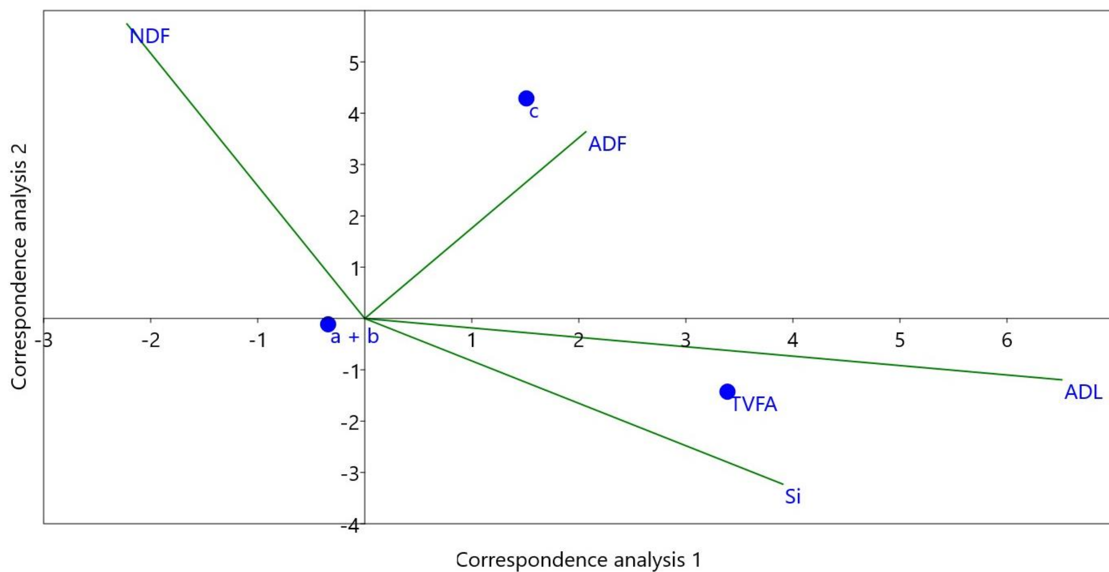


Figure 6.5: Canonical correspondence analysis illustrating the relationship between chemical composition of chopped samples and rumen fermentation pattern *in vitro*. Plots show the direction of the gradient and those longer show a stronger correlation

Canonical correspondence analysis of the relationship between the proximate analysis of the rice straw varieties and the fermentation outcome (*i.e.* cumulative gas production, rate of degradation and total VFA production) showed that the effect of the fibre components was the major driver of these outcomes. In the chopped straw treatment, ADL had the most effect on *in vitro* fermentation outcomes followed by

silica content, NDF and ADF levels in that order (Figure 6.5). The same order of influence was seen in the milled samples (Figure 6.6).

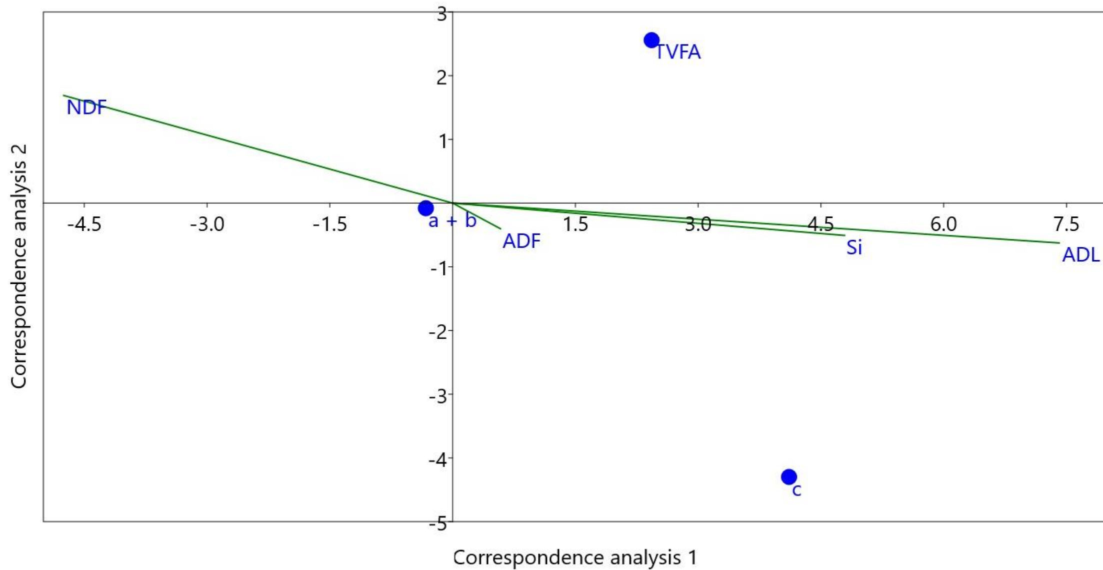


Figure 6.6: Canonical correspondence analysis illustrating the relationship between chemical composition of milled samples and rumen fermentation pattern *in vitro*. Plots show the direction of the gradient and those longer show a stronger correlation

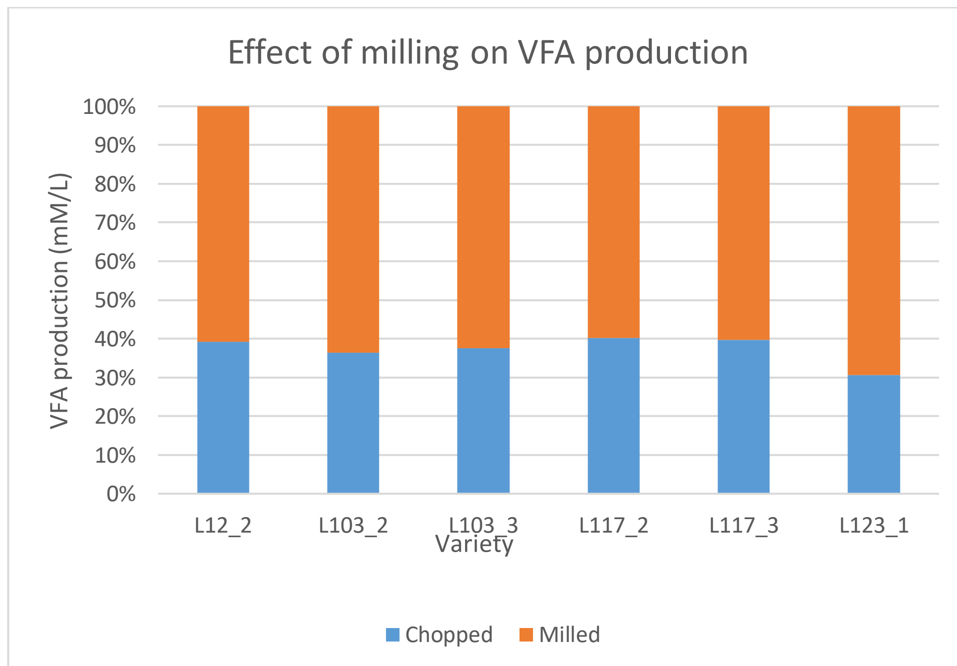


Figure 6.7: Stacked histogram illustrating the difference in total VFA (mM/L) production between chopped and milled treatment

Table 6.3: Volume of gas produced (a + b), and rate of degradation (c) of six rice straw varieties mechanically treated by chopping and milling

	a + b (mL)			c (%/h)		
	Chopped	Milled	Mean	Chopped	Milled	Mean
L12_2	48.51	83.87^{c}	66.19^{b}	0.103	0.042	0.072^{a}
L103_2	41.46	74.65^{bc}	58.05^{ab}	0.174	0.063	0.118^{b}
L103_3	46.63	65.43^{ab}	56.03^{a}	0.138	0.085	0.111^{b}
L117_2	52	59.08^{a}	55.54^{a}	0.115	0.080	0.097^{ab}
L117_3	51.35	68.98^{ab}	60.16^{ab}	0.138	0.084	0.111^{b}
L123_1	43.59	70.37^{abc}	56.98^{a}	0.108	0.049	0.079^{a}
s.e.d	Straw variety		2.918			0.010
	Treatment		1.685			0.006
	Variety x Treatment		4.127			0.014
p value	Straw variety		0.014			<0.001
	Treatment		<0.001			<0.001
	Variety x Treatment		<0.001			0.017

“a” and “b” parameters explain the potential of fermentation (“a + b” reflects the maximum potential of fermentation), and “c” parameter explained the speed/slope of the curve of fermentation. Means with different superscripts differ ($p<0.05$).

Table 6.4: Volatile fatty acids concentration (mM/L) and measured at the end of the incubation

Variety	Acetic			Propionic			Butyric			BCFA			TVFA		
	Chopped	Milled	Mean	Chopped	Milled	Mean	Chopped	Milled	Mean	Chopped	Milled	Mean	Chopped	Milled	Mean
L12_2	9.65	15.05	12.35	4.6	6.31	5.45	2.117	2.521	2.319	0.68	0.659	0.669	17.04	24.54	20.79
L103_2	6.77	15.26	11.01	3.03	6.93	4.98	1.273	3.5	2.387	0.516	1.103	0.809	11.58	26.79	19.19
L103_3	7.48	16.28	11.88	3.61	6.81	5.21	1.537	3.62	2.578	0.597	1.177	0.887	13.23	27.89	20.56
L117_2	9.47	13.89	11.68	4.91	6.44	5.67	3.03	3.77	3.4	0.753	1.01	0.882	18.17	25.11	21.64
L117_3	8.45	13.44	10.94	4.16	6.62	5.39	2.457	3.563	3.01	0.732	1.09	0.911	15.8	24.71	20.26
L123_1	6.18	17.22	11.7	3.2	7.37	5.29	1.294	3.323	2.309	0.471	1.056	0.763	11.15	28.98	20.06
s.e.d	Variety		1.685			0.63			0.3447			0.1425			2.746
	Treatment		0.973			0.364			0.199			0.0823			1.585
	Variety x treatment		2.383			0.891			0.4875			0.2016			3.883
p value	Variety		0.958			0.92			0.022			0.537			0.969
	Treatment		<0.001			<0.001			<0.001			<0.001			<0.001
	Variety x treatment		0.329			0.213			0.056			0.236			0.258

Table 6.5: Effect of rice straw varieties on relative abundance of bacteria genera present at 12h at an average of >0.05%.

Genera	Treatments						S.E.D.	Uncorrected p-value	Benjamini-Hochberg p-value
	L12_2	L103_2	L103_3	L117_2	L117_3	L123_1			
Anaerosporobacter	0.002^{ab}	0.003^{bc}	0.002^{a}	0.002^{a}	0.002^{a}	0.003^{c}	0.004	0.002	0.009
Anaerovorax	0.006^{ab}	0.005^{ab}	0.004^{ab}	0.008^{ab}	0.008^{b}	0.004^{a}	0.008	0.042	0.044
Christensenella	$0.006b^{c}$	0.003^{abc}	0.003^{abc}	0.006^{c}	0.003^{ab}	0.003^{a}	0.009	0.016	0.027
Coprococcus	0.003^{ab}	0.001^{a}	0.001^{a}	0.004^{b}	0.003^{ab}	0.001^{a}	0.007	0.005	0.015
Eisenbergiella	0.006^{b}	0.002^{a}	0.004^{ab}	0.007^{b}	0.006^{b}	0.004^{ab}	0.008	0.009	0.020
Flavonifractor	0.001^{bc}	0.000^{ab}	0.000^{a}	0.002^{d}	0.001^{cd}	0.001^{abc}	0.005	<0.001	0.007
Fretibacterium	0.003^{b}	0.001^{a}	0.001^{a}	0.003^{b}	0.002^{ab}	0.001^{a}	0.004	<0.001	0.007
Howardella	0.005^{ab}	0.003^{a}	0.004^{a}	0.007^{b}	0.003^{a}	0.003^{a}	0.006	0.004	0.014
Lachnobacterium	0.012^{ab}	0.018^{b}	0.009^{ab}	0.007^{a}	0.006^{a}	0.009^{ab}	0.015	0.037	0.042
Oscillibacter	0.007^{ab}	0.013^{ab}	0.005^{a}	0.018^{b}	0.016^{ab}	0.010^{ab}	0.017	0.028	0.037
OTU 103	0.004^{c}	0.001^{a}	0.001^{a}	0.003^{bc}	0.002^{ab}	0.002^{a}	0.005	0.001	0.007
OTU 11	0.033^{b}	0.019^{a}	0.032^{ab}	0.027^{ab}	0.029^{ab}	0.020^{ab}	0.013	0.025	0.037
OTU 110	0.002^{bc}	0.001^{ab}	0.001^{a}	0.002^{c}	0.002^{bc}	0.000^{a}	0.004	<0.001	0.007
OTU 115	0.002^{ab}	0.001^{a}	0.001^{a}	0.003^{b}	0.001^{ab}	0.001^{a}	0.006	0.009	0.020
OTU 12	0.013^{a}	0.010^{a}	0.010^{a}	0.026^{b}	0.013^{a}	0.010^{a}	0.012	0.002	0.009
OTU 121	0.002^{ab}	0.002^{a}	0.002^{a}	0.004^{b}	0.002^{ab}	0.001^{a}	0.005	0.009	0.020
OTU 127	0.003^{ab}	0.005^{c}	0.005^{bc}	0.003^{abc}	0.004^{abc}	0.002^{a}	0.006	0.011	0.022
OTU 128	0.005^{b}	0.005^{b}	0.004^{ab}	0.002^{ab}	0.004^{ab}	0.001^{a}	0.010	0.033	0.038
OTU 1486	0.001^{a}	0.004^{b}	0.003^{ab}	0.001^{ab}	0.003^{ab}	0.002^{ab}	0.008	0.031	0.037
OTU 159	0.003^{b}	0.000^{a}	0.001^{a}	0.001^{a}	0.000^{a}	0.000^{a}	0.006	0.002	0.009
OTU 165	0.003^{ab}	0.002^{a}	0.001^{a}	0.005b	0.003ab	0.002a	0.008	0.015	0.027
OTU 170	0.002^{ab}	0.004^{b}	0.004^{b}	0.001^{a}	0.002^{ab}	0.002^{ab}	0.006	0.028	0.037
OTU 172	0.001^{a}	0.003^{ab}	0.003^{ab}	0.003^{ab}	0.003^{a}	0.002^{ab}	0.006	0.04	0.044
OTU 205	0.000^{ab}	0.000^{a}	0.000^{a}	0.000^{ab}	0.004^{b}	0.000^{a}	0.014	0.028	0.037
OTU 22	0.007^{ab}	0.006^{ab}	0.007^{b}	0.004a	0.004^{ab}	0.004^{ab}	0.007	0.016	0.027
OTU 2210	0.002^{a}	0.003^{ab}	0.003^{a}	0.002^{a}	0.008^{c}	0.006^{bc}	0.007	<0.001	0.007
OTU 23	0.006^{ab}	0.003^{a}	0.004^{ab}	0.008^{b}	0.005^{ab}	0.003^{a}	0.009	0.017	0.028

OTU 24	0.004^{a}	0.006^{a}	0.005^{a}	0.004^{a}	0.017^{b}	0.012^{b}	0.008	<0.001	0.007
OTU 27	0.013^{b}	0.010^{ab}	0.007^{ab}	0.008^{ab}	0.007^{a}	0.006^{a}	0.009	0.031	0.037
OTU 31	0.008^{bc}	0.004^{a}	0.005^{ab}	0.010^{c}	0.007^{abc}	0.007^{abc}	0.007	0.009	0.020
OTU 37	0.003^{b}	0.001^{a}	0.001^{ab}	0.002^{b}	0.002^{ab}	0.003^{b}	0.007	0.015	0.027
OTU 38	0.005^{ab}	0.005^{ab}	0.004^{a}	0.008^{b}	0.006^{ab}	0.004^{b}	0.008	0.028	0.037
OTU 39	0.002^{ab}	0.001^{a}	0.002^{ab}	0.002^{ab}	0.002^{ab}	0.003^{b}	0.006	0.047	0.047
OTU 393	0.004^{ab}	0.003^{a}	0.004^{ab}	0.006^{b}	0.004^{ab}	0.004^{ab}	0.007	0.043	0.044
OTU 43	0.007^{ab}	0.008^{ab}	0.006^{ab}	0.007^{ab}	0.006^{a}	0.009^{b}	0.006	0.032	0.038
OTU 55	0.002^{ab}	0.003^{b}	0.003^{ab}	0.002^{ab}	0.003^{ab}	0.001^{a}	0.007	0.041	0.044
OTU 60	0.002^{a}	0.005^{b}	0.003^{ab}	0.003^{ab}	0.005^{b}	0.003^{ab}	0.006	0.029	0.037
OTU 668	0.000^{ab}	0.000^{a}	0.000^{a}	0.002^{b}	0.001^{ab}	0.000^{a}	0.008	0.005	0.015
OTU 70	0.005^{ab}	0.002^{a}	0.004^{a}	0.002^{a}	0.004^{ab}	0.008^{b}	0.009	0.006	0.016
OTU 71	0.001^{ab}	0.002^{b}	0.001^{b}	0.000^{a}	0.002^{b}	0.000^{a}	0.010	0.003	0.013
OTU 75	0.002^{abc}	0.001^{ab}	0.001^{ab}	0.003^{bc}	0.003^{c}	0.001^{a}	0.006	0.006	0.016
OTU 77	0.001^{a}	0.001^{ab}	0.002^{ab}	0.001^{ab}	0.000^{a}	0.003^{b}	0.010	0.025	0.037
OTU 82	0.002^{a}	0.005^{b}	0.004^{ab}	0.002^{ab}	0.003^{ab}	0.003^{ab}	0.009	0.043	0.044
OTU 9	0.015^{ab}	0.017^{ab}	0.035^{b}	0.008^{a}	0.019^{ab}	0.028^{b}	0.022	0.015	0.027
OTU 96	0.001^{a}	0.002^{b}	0.001^{ab}	0.000^{a}	0.002^{b}	0.001^{a}	0.005	0.004	0.014
Phocaeicola	0.005^{ab}	0.008^{b}	0.008^{b}	0.003^{a}	0.006^{ab}	0.006^{ab}	0.009	0.023	0.036
Prevotella	0.046^{bc}	0.028^{a}	0.044^{bc}	0.055^{c}	0.048^{c}	0.031^{ab}	0.012	0.002	0.009
Pseudobutyrivibrio	0.003^{ab}	0.005^{b}	0.002^{a}	0.002^{a}	0.005^{b}	0.003^{ab}	0.006	0.004	0.014
Saccharibacteria genera incertae sedis	0.004^{ab}	0.002^{a}	0.002^{a}	0.006^{bc}	0.009^{c}	0.002^{a}	0.007	<0.001	0.007
Sphingobacterium	0.000^{a}	0.000^{a}	0.000^{a}	0.000^{a}	0.002^{b}	0.000^{a}	0.013	0.031	0.037
Sphingobium	0.000^{a}	0.001^{b}	0.001^{ab}	0.000^{ab}	0.001^{b}	0.000^{a}	0.008	0.006	0.016
Treponema	0.045^{ab}	0.068^{b}	0.070^{b}	0.026^{a}	0.058^{ab}	0.071^{b}	0.025	0.01	0.021

Means in a column with different superscripts differ ($p<0.05$).

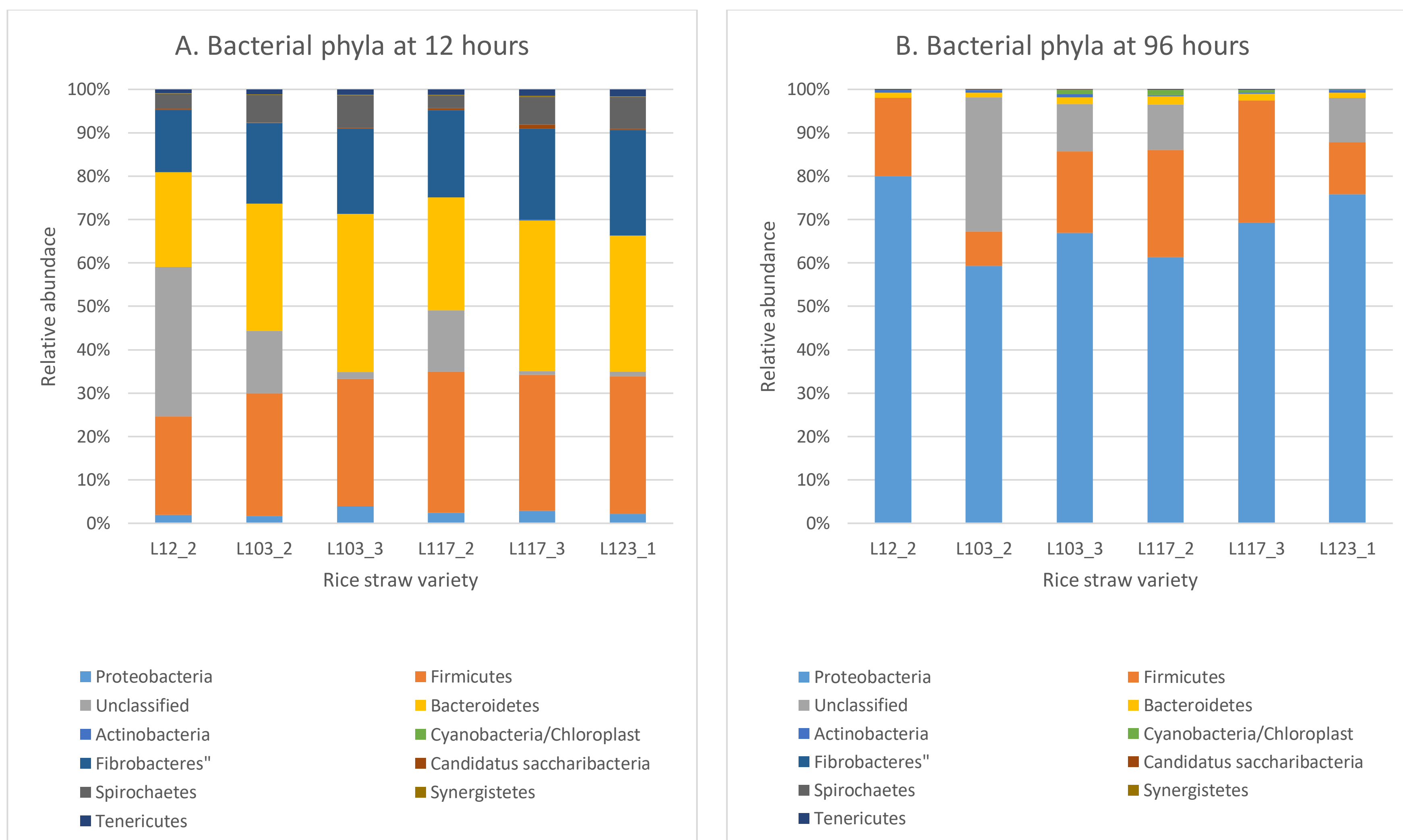


Figure 6.8: Stacked histograms illustrating phylum-level bacterial composition at **A**. 12h and **B**. 96h. Bacteria present at an average of more than 0.05% analysed using Ion Torrent PGM 16S rRNA gene sequencing. Reads were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II. Samples standardised by total and transformed by square root

6.4.2 BACTERIAL COMMUNITY

Metagenomic analysis of the bacterial communities attached to the rice straw showed statistically significant changes over the incubation time. There was a clear difference between the communities occupying the straw at 12h and 96h. The microbial population of the rice straw at 12h was dominated by the phyla Firmicutes (23 - 32%), Bacteroidetes (21 - 31%) and Fibrobacteres (14 - 24%) (Figure 6.8A). After 96h of incubation, the microbial population was composed primarily of the phyla Proteobacteria (59 – 80%) and Firmicutes (8 - 28%) (Figure 6.8B). The differences in the relative abundances of bacterial phyla between the straw varieties was not significant apart from the phyla Spirochaetes (p=0.012) and Cyanobacteria/ Chloroplast (p = 0.035).

At the genus level, the main colonising taxa was Fibrobacter (19 – 24%) in all samples. The dominance of Fibrobacter after 12h of incubation is consistent with the observations made in Chapter 5, although the relative abundance of the genus was much lower (Figure 6.10). There was a treatment effect of rice straw variety in numerous genera of bacteria colonising the straw after 12h (Table 6.5). Following 96h of incubation the bacteria colonising the rice straw were composed mainly of the genera *Comamonas* (26 – 44%), *Acinetobacter* (29 - 37%) and *Streptococcus* (9 – 26%) (Figure 6.11). There were no significant differences between genera after 96h of incubation apart from *Escherichia/Shigella* (p = 0.004), *Sphingobium* (p = 0.045) and OTU 57 (p = 0.009).

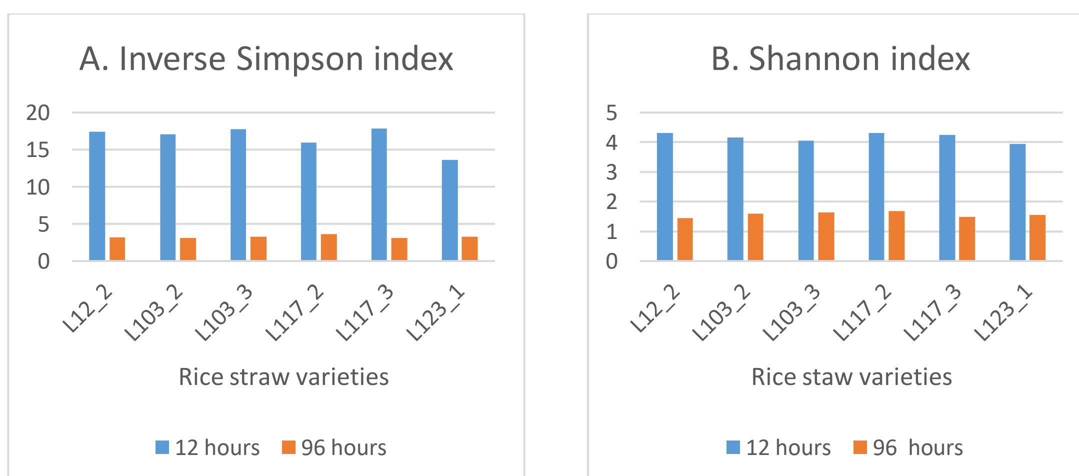
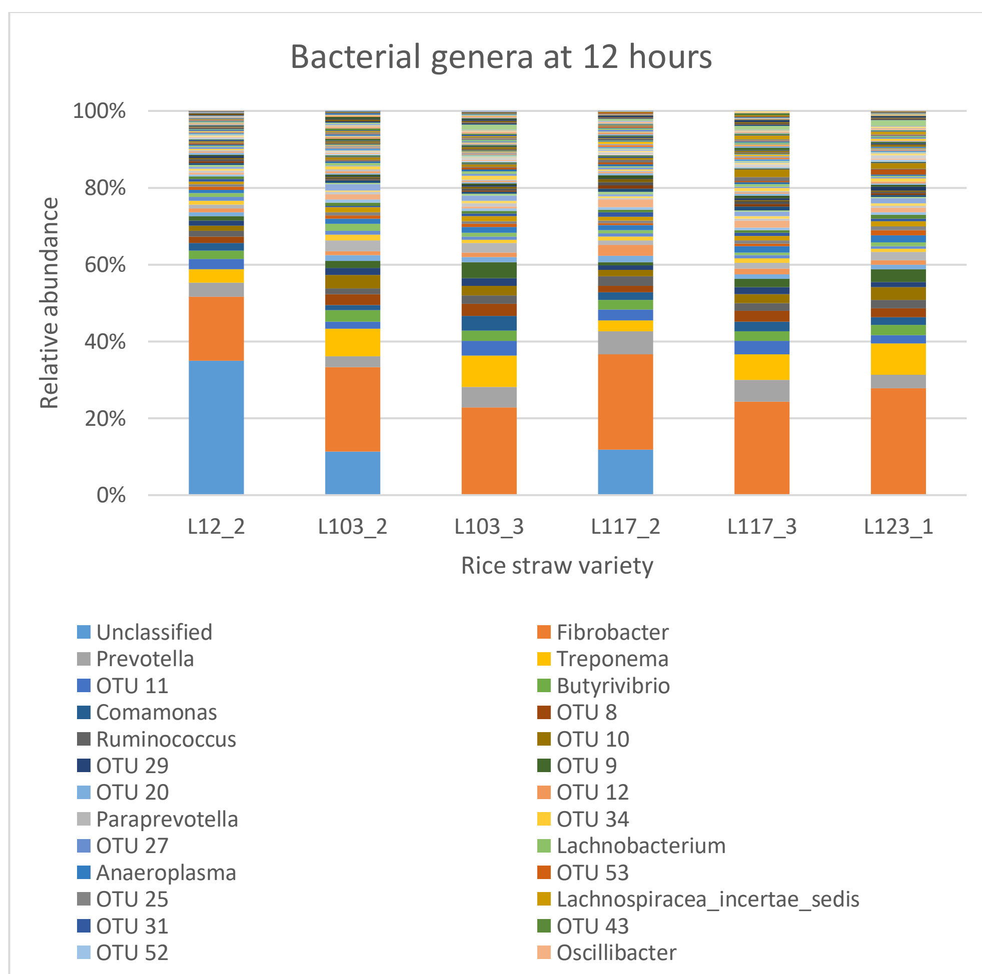


Figure 6.9: The Inverse Simpson (A) and the Shannon (B) indices representing changes in diversity of bacteria colonising rice straw incubated in rumen fluid at 12h and 96h

The changes in the bacterial composition attached to rice straw at 12h and 96h was accompanied by a reduction in the community diversity as illustrated by the inverse Simpson and the Shannon indices (Figure 6.9). There were no significant differences in the inverse Simpson index between the treatments at both time points. There was a significant effect of treatment on the Shannon index at 12h ($p = 0.02$) but no difference at 96h. The UPGMA dendrogram generated by the cluster analysis of bacterial genera at 12h placed the treatments into two major clusters (Figure 6.10); canonical correspondence analysis showed that the clustering was driven by levels of NDF, silica, ADF and ADL in descending order (Figure 6.12). On the other hand, the clustering demonstrated at 96h was influenced by the treatment levels of NDF, silica, ADL and ADF also in descending order (Figure 6.13).



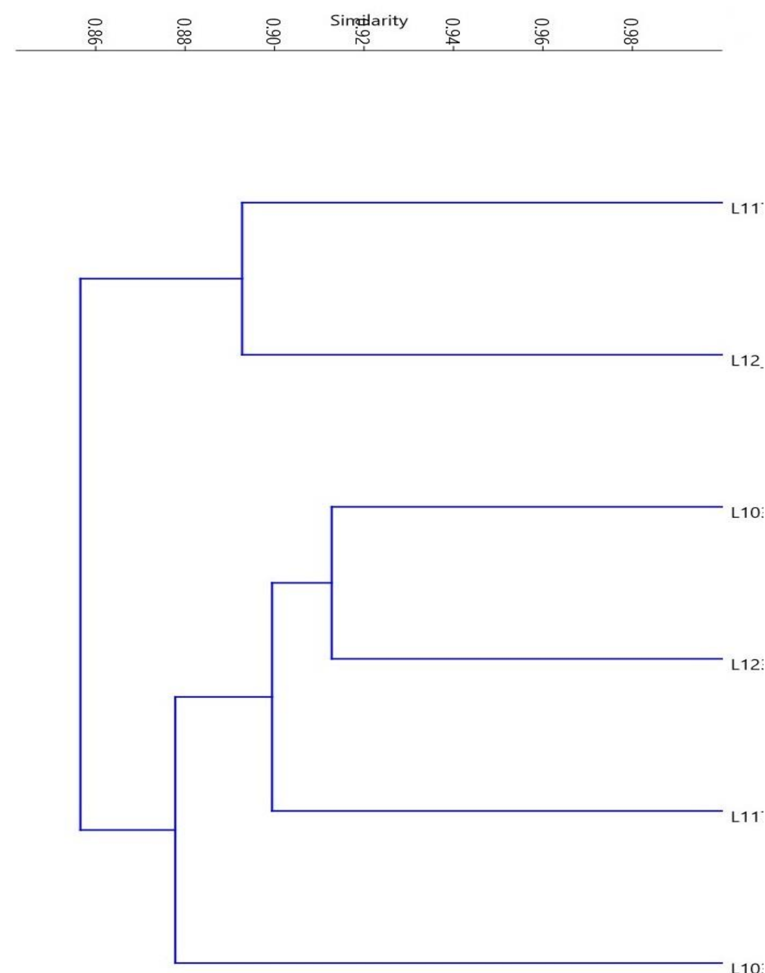
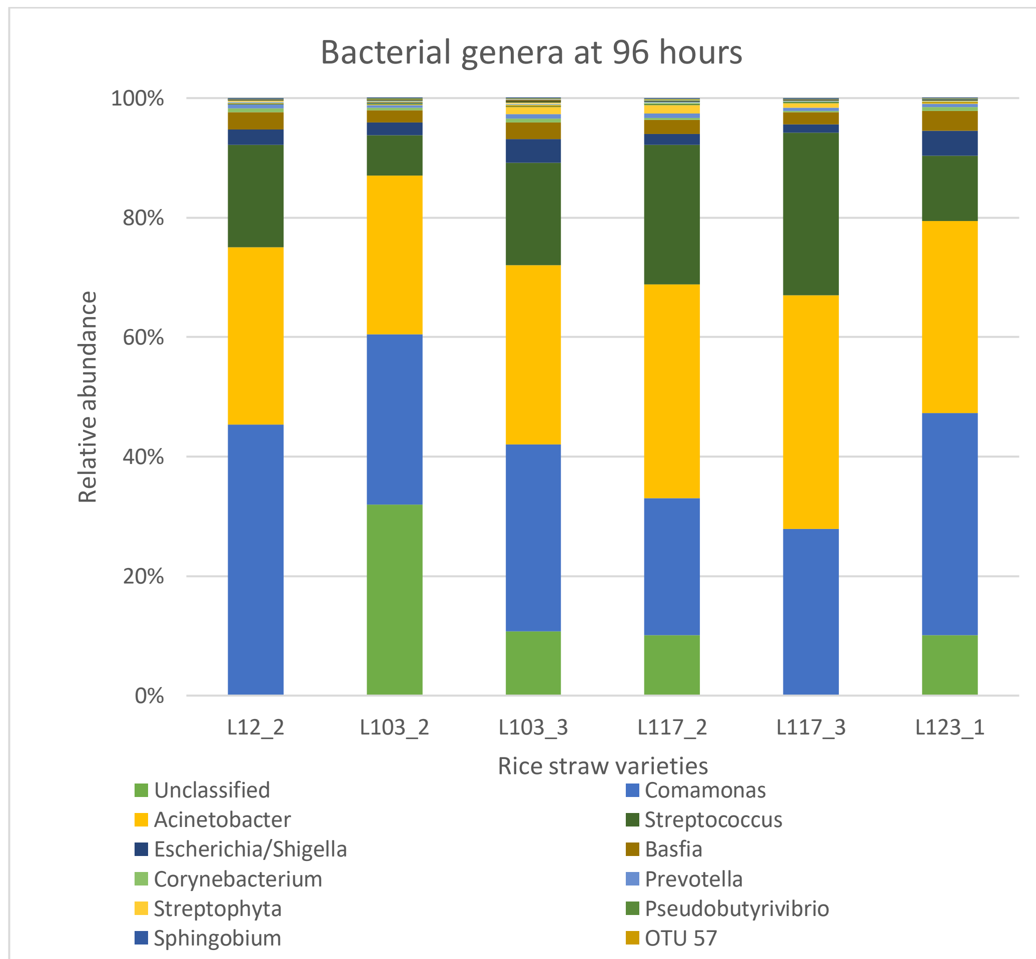


Figure 6.10: Stacked histogram and dendrogram illustrating genus-level bacterial composition and clustering. Bacteria present at an average of more than 0.05% analysed using Ion Torrent PGM 16S rRNA gene sequencing. Reads were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II. Samples standardised by total and transformed by square root. Dendrogram based on the UPGMA clustering of the Bray-Curtis distances



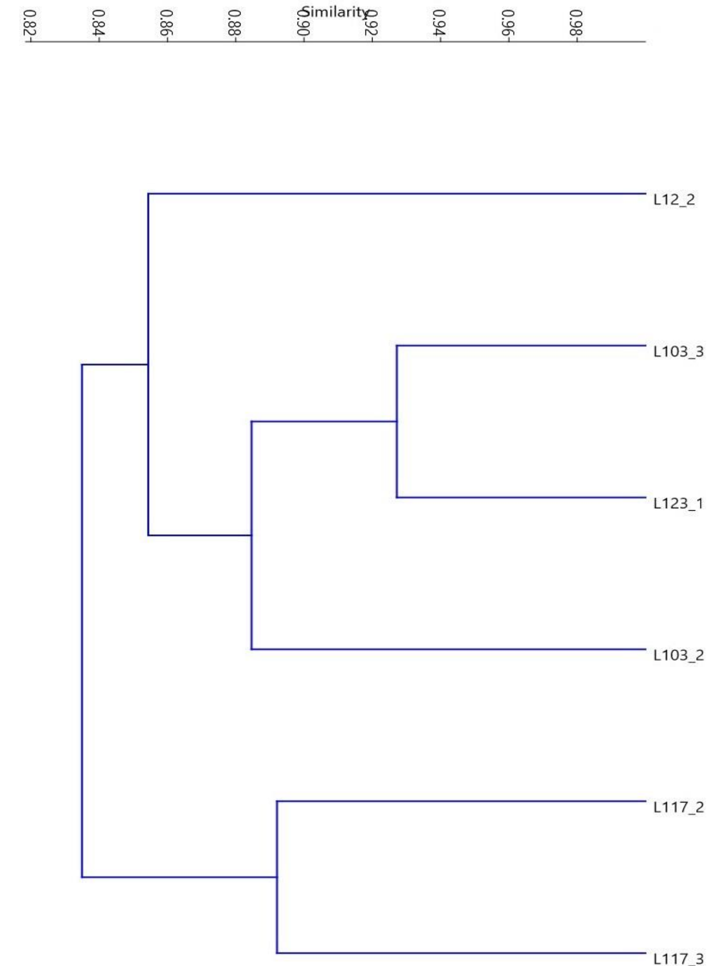


Figure 6.11: Stacked histogram and dendrogram illustrating genus-level bacterial composition and clustering. Bacteria present at an average of more than 0.05% analysed using Ion Torrent PGM 16S rRNA gene sequencing. Reads were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II. Samples standardised by total and transformed by square root. Dendrogram based on the UPGMA clustering of the Bray-Curtis distances

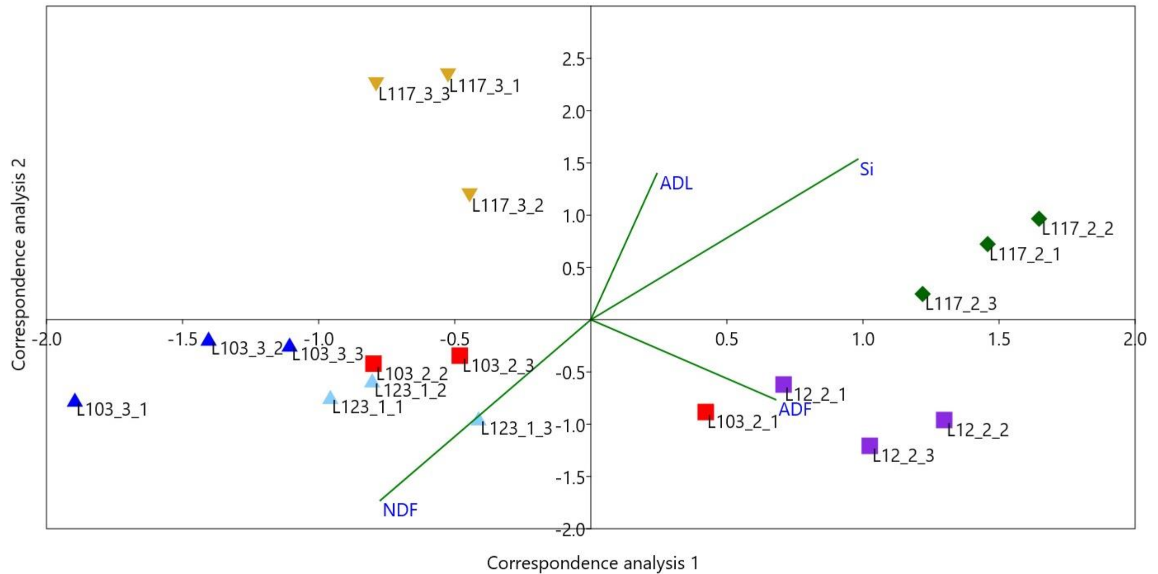


Figure 6.12: Canonical correspondence analysis illustrating the relationship between chemical composition of samples and *in vitro* bacterial colonisation pattern at 12h. Plots show the direction of the gradient and those longer show a stronger correlation

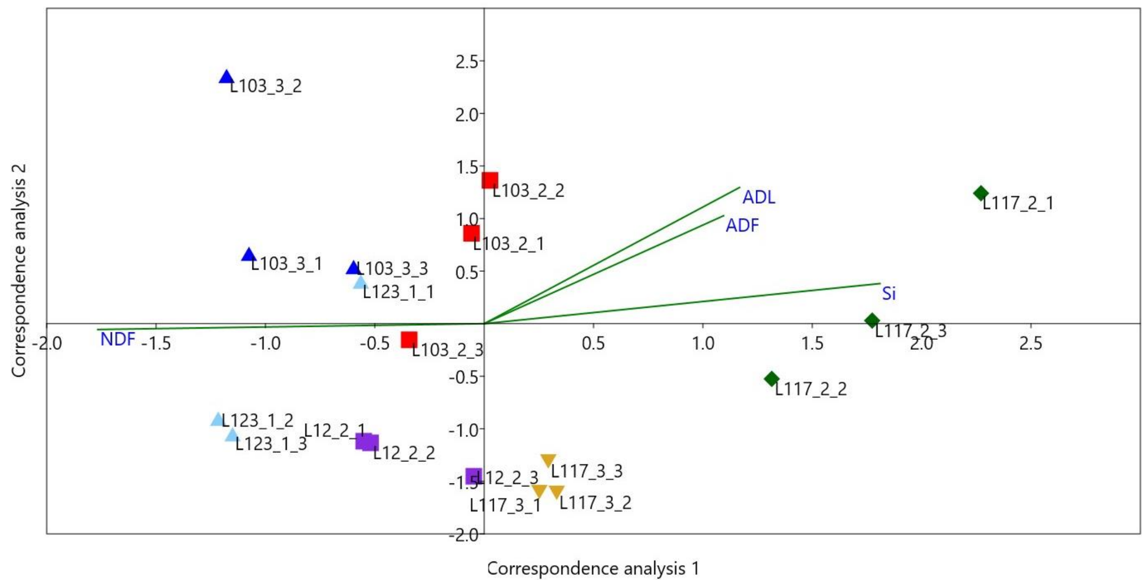


Figure 6.13: Canonical correspondence analysis illustrating the relationship between chemical composition of samples and *in vitro* bacterial colonisation pattern at 96h. Plots show the direction of the gradient and those longer show a stronger correlation

6.5 DISCUSSION

Digestion of feed depends in part on surface area presented for colonisation Here the effect of grinding as compared with chopped straw was investigated. The effect of the chemical composition of rice straw on *in vitro* gas production was not statistically significant, as was observed in Chapter 5. This is notable as the inocula, although collected from the same animals, were of different concentrations in the two experiments, *i.e*. 1:2 in Chapter 5 and 1:9 here. The change was made after the observation that the blank bottles (without the substrate) had a higher rate of gas production during the exponential phase of the curve compared to those with the treatment substrate. It was considered that this could have been an artefact due to microbial fermentation of rumen fluid constituents in preference for the relatively indigestible straw substrate, since the presence of feed particles suspended in the rumen fluid can lead to an overestimation of the actual gas production (Maccarana et al., 2016). Hence, the increase in the rumen fluid to buffer ratio to 1:9 was done to see if the statistical differences observed before was not due to this overestimation. However, despite a 5-fold dilution in inoculum, a similar pattern whereby gas production in control samples proceeded at a faster initial rate than in substrate containing samples was also seen in this experiment (Figure 6.2). The difference in the rate of gas production between the blanks and the treatments could have been due to the faster onset of microbial turnover in blanks compared to treatments, as reported by Cone (1998). On this basis, Williams (2000, p. 200) discouraged the adjustment of GP data using blank values prior to curve fitting particularly in the case of multiphasic curves as the phases present in the blank may not be relevant to what occurs in the presence of substrate. For instance previous results have demonstrated that increases in the proportion of rumen fluid was associated with a decrease in lag phase and a higher rate of gas production, while the effect on total gas production varied (Yáñez-Ruiz et al., 2016).

Consistent with the results from Chapter 5, even in the presence of an adjusted inoculum, a significant effect of rice straw variety on gas production was not detected; however, there was a significant effect of milling (Table 6.3). The higher levels of fermentation seen in milled treatments could be due to increased number of

attachment sites for fibre-degrading bacteria and thus bacterial colonisation (Broudiscou et al., 2003). Bacterial colonisation takes place on substrate surface, unlike fungal attack which would preferentially progress within the inner layers of roughage biomass (Broudiscou et al., 2001); and increase in surface area would therefore favour bacterial colonisation. The difference could also be due to a reduction in particle size. Particle size of roughage can have an impact on maintenance of rumen function, which has been associated with fibre digestibility and optimal pH for cellulolytic microorganisms (Gunun, Wanapat, & Anantasook, 2013). Zhao et al. (2009) reported that in goats fed rice straw with increasing particle size (1, 2, 4, and 8 cm) resulted in increased chewing activity and enhanced ruminal function. Yang et al. (2002) also reported that increased forage particle size in dairy cow diets improved ruminal pH and microbial synthesis in the rumen with no effect on feed intake. Decreasing roughage particle size increased VFA production particularly propionic acid and higher feed efficiency due to enhanced microbial protein synthesis (Kononoff & Heinrichs, 2003; Krause & Combs, 2003). Zhang et al. (2010) reported that chopped rice straw silage had better fermentative quality than long rice straw silage. In terms of applicability on farm, physical treatments are not often applicable on small-scale farms since they require machine or industrial processing (Yanti & Yayota, 2017). However, a small grinder or chopper may be practical for small-scale farmers (Sarnklong et al., 2010)

Silica levels in rice plants are usually higher than in other plants, which has led many to suggest that silica is responsible for the low digestibility of rice straw (Santos et al., 2010). However, the result of the experiment in Chapter 5 suggest that the total levels of silica in rice straw may not be an accurate predictor of digestibility. The positive correlation between the silica content and cumulative gas production observed in Chapter 5 (Figure 5.5) was also seen here in chopped samples (Figure 6.3). However, in milled samples there was a negative correlation between cumulative gas production and the levels of silica (Figure 6.3). This inverse relationship could be due to suppression of microbial cellulolytic enzymes by soluble silica proposed by Agbagla-Dohnani et al. (2001).

Results in Chapter 5 showed that the levels of levels of lignin had the largest effects on *in vitro* fermentation while silica content had the least (Figure 5.8). A similar result was

seen in this experiment in chopped samples (Figure 6.5) with the effect of lignin on gas production being increased by milling (Figure 6.4, Figure 6.6). Lignin plays a role in resisting compressing forces, providing protection against consumption by insects and mammals, and inhibiting the rate and degree of microbial degradation (Sarnklong et al., 2010). Huws et al. (2014) reported that stem material of perennial rye grass had more fibre and lignin content than leaves; additionally, they observed that the *in vitro* dry matter degradability (IVDMD) of stem material was lower than that of leaves at all harvesting times. They hypothesised that differential in recalcitrant cell wall structure between stem and leaves was the most likely explanation for the reduced IVDMD of stem material compared to leaves (Huws et al., 2014). This conclusion was supported by earlier results published by Chaves et al. (2006) who reported that the DM degradability for leaf was higher than for stem material for perennial ryegrass, tall fescue, Yorkshire fog, Phalaris and Paspalum grass species.

In Chapter 5, it was observed that colonisation of chopped straw proceeded in a successional manner. Here, it was further observed that the chemical composition of rice straw affected the colonisation by rumen bacteria. UPGMA dendrograms of the relative abundances of bacterial genera revealed that microbiota attached to the different rice straw varieties were markedly different from each other at 12h (Figure 6.10); the differences were much less pronounced at 96h (Figure 6.11). Canonical correspondence analyses of the relationship showed that the fibre components of rice straw had as much or even more influence on bacterial colonisation of rice straw than silica at both at 12h (Figure 6.12) and at 96h (Figure 6.13). Statistically significant differences were observed in several bacterial genera at 12h. These include *Anaerosporobacter* ($p=0.002$), *Anaerovorax* ($p=0.042$) *Christensenella* ($p=0.016$), *Coprococcus* ($p=0.005$), *Eisenbergiella* ($p=0.009$), *Flavonifractor* ($p<0.001$), *Fretibacterium* ($p<0.001$), *Howardella* ($p=0.004$), *Lachnobacterium* ($p=0.037$). Differences were also observed in *Oscillibacter* ($p=0.028$), *Phocaeicola* ($p=0.023$), *Prevotella* ($p=0.002$), *Pseudobutyrivibrio* ($p=0.004$), *Saccharibacteria genera incertae sedis* ($p<0.001$) *Sphingobacterium* ($p=0.031$), *Sphingobium* ($p=0.006$), *Treponema* ($p=0.01$) and numerous other OTUs (Table 6.5).

While many of these are not the dominant genera in the rumen, some of them have important functions. The genus *Coprococcus* is involved in important metabolic

pathways in the rumen. Shabat et al. (2016) found a greater proportion of *C. catus* in the rumen of dairy cows with a higher feed efficiency. In the more feed efficient cows, there were an increase in abundance of genes aligned to the acrylate pathway, which involves the conversion of lactate to propionate, and were annotated as *C. catus*. In general, the acrylate pathway rather than the succinate pathway for propionate production was more dominant in the efficient animals (Shabat et al., 2016). *Coprococcus* spp. are also involved, is the degradation of nitrotoxins in the rumen through reductive processes (Majak & Cheng, 1981). The genus is also a significant contributor to the metabolism of phenolic compounds in the rumen (Martinez-Fernandez et al., 2017). These findings suggest that *Coprococcus* genus is involved in key metabolic pathways in the rumen, and they should be considered when developing strategies to promote microorganisms which might improve the rumen efficiency and metabolize plant toxins in ruminants (Martinez-Fernandez et al., 2017).

Liu et al. (2017) reported that the abundance of *Flavonifractor* decreased gradually with increasing age in dairy cows. *Flavonifractor* has a weak capacity for sugar fermentation and utilization, with acetic and butyric acids as the major metabolic end products (Carlier et al., 2010). Methane production and volatile fatty acid concentrations were found to be age-related with Heifers (9–10 months) having lower methane production but higher methane production per dry matter intake. This was accompanied by age-related microbiota changes in the rumen reflected by a significant shift in bacterial taxa(Liu et al., 2017). Kim et al. (2014) observed that *Oscillibacter* abundance was positively correlated with starch content. Cattle fed high starch diets can have high bypass starch from rumen and *Oscillibacter* in the faeces of cattle may be associated with the high levels of bypass starch.

The genus *Prevotella* is the most highly represented genus in the rumen sequence databases making up >50% of rumen bacteria sequences (Jami & Mizrahi, 2012). The most important species are *P. ruminicola*, *P. brevis*, *P. bryantii* and *P. albensis* (Tajima et al., 1999).These bacteria utilise starch, non-cellulosic polysaccharides and simple sugars and are major converters of succinate to propionate and thus the maintenance glucose homeostasis in ruminants (Koike & Kobayashi, 2009). *Pseudobutyrivibrio* is an important producer of xylanase in the rumen (Krause et al., 2003); the enzymes degrade the linear polysaccharide xylan into xylose, thus breaking

down hemicellulose, one of the major components of plant cell walls. Shen et al. observed that the genus (2019) *Sphingobium* was an important member of the epimural microbiota involved in the host-microbe homeostasis *via* maintenance of the integrity of rumen epithelium in healthy animals and enhancing the activities of immune barrier in animal with lower rumen pH. In the rumen the genus *Treponema* are cellulolytic and break down of pectin (Liu et al., 2015).

The differences observed in the bacteria colonising the different varieties of straw could be due to differences in the available surfaces for colonisation. Stewart et al. (1997) suggested that distinct rumen bacterial species colonise plant surfaces in different ways, due to their distinct growth and survival requirements. Huws et al. (2014) observed more bacteria colonised the adaxial surface of leaves compared to the abaxial surface in fresh perennial ryegrass. They attributed this to differential chemo-attraction and physical barrier effects or differential electrostatic interactions that could be present on different sides of the leaf. Adaxial surfaces have been reported to have more waxes containing long-chain alkanes, alcohols, ketones, and fatty acids as compared to the abaxial surface (Gniwotta et al., 2005).

The observations that the nature of the substrate surfaces influences bacterial colonisation has necessitated detailed examinations of the surfaces and their interactions with microbes using electron microscopy (EM). Early EM studies by Lathan et al. (1978) investigated the attachment of mixed bacterial cultures on plant cells and observed differences in the attachment of rumen bacteria to plant cell types *e.g.* *Bacteroides* but not *Ruminococcus* adhered to intact mesophyll cell wall. Akin (1980) also found different morphological types of rumen bacteria on different plant surfaces. In regards to electron microscopy rice straw, Shen et al. (1999) observed that silica was deposited in epidermis polymerised with cuticle waxes. This cuticle wax layer was absent on the inner surface of stem (Shen et al., 1999). They observed that the cuticle wax silica polymerised layer acted as a barrier to rumen degradation of rice straw rather than being due to silica alone. As a result of the absence of cuticle wax silica layer and epidermis on the inner surface of stem, it had better degradability compared with sheath and blade. Further, urea treatment affected the cuticle wax layer to enhance the digestibility of parenchyma tissue through swelling of the wall of parenchyma cells and cracking of the wall of vascular tubes (Shen et al., 1999). Later,

Wang et al. (2007) also demonstrated that dissolving of the cuticle wax layer of rice straw stem epidermis with NaOH resulted in a higher degradation by rumen microorganisms. Agbagla-Dohnani et al. (2003) observed that rice straw stems incubated *in sacco* were less prone to colonisation in the variety where the epidermis was covered by a thick outer silica layer compared to a variety with no layer. As a result, damage to the epidermis was seen earlier in the latter than in the former under electron microscopy. They concluded that the silica layer impeded the access of microorganisms to the underlying sclerenchyma and lignified parenchyma. On both varieties of straws, parenchyma was degraded from the internal cavity and cut ends. The course of the degradation of parenchyma was similar in both varieties indicating that silica content and deposition had no effect on the degradation of these internal tissues (Agbagla-Dohnani et al., 2003). While this study appears to confirm the effect of silica on rice straw degradation was though incrustation (Van Soest, 2006), it has some limits on how far the results can be extrapolated. Firstly, the authors conceded that there was a lack of replication of the plot work due to the low amount of available plant material. Secondly, the two treatment were composed of two rice straws grown on culture media with different silica contents. As a result, one of the treatments had a silica content near the zero value. This is unusual for cultivated rice straws and is found only in experimental rice plants such as those grown on hydroponic solutions with no added silica (Balasta et al., 1989). Thus, the next logical step would be to carry out a similar electron-microscopy investigation on field-grown straw with varying chemical compositions to determine its colonisation by rumen microbiota.

6.6 CONCLUSION

In summary, the chemical composition of rice straw was found to have an effect on bacterial colonisation. While the presence is in rice silica has been hypothesised to be the main hindrance to microbial degradation, observations made in this experiment show that the levels of lignin and other fibre components have an equal or even bigger effect. These data illustrate the importance of investigating substrate colonisation to get a deeper fundamental understanding of the interaction between straw and rumen microbes. Furthering our understanding of this interaction is fundamental to the development of rice straw treatment strategies to improve its nutritional value.

CHAPTER 7: OVERCOMING THE CHALLENGES OF RUMINANT NUTRITION IN THE TROPICS

7.1 INTRODUCTION

In tropical zones across the world, ruminants depend on year-round grazing on natural pastures. Alternatively, the animals are fed with cut grass and crop residues. Most of these areas face seasonal dry periods in which the availability of pasture decreases. The quality is also lowered as a result of reduction in the content of digestible energy and nitrogen (Lukuyu et al., 2009). The practise of feeding rice straw in many parts of the tropics has resulted in management systems where animals contribute to efficient crop production without competing with crops for land or cause damage to the crops. Rice straw does not contain enough sugars, amino acids and minerals for efficient microbial growth. Feeding ruminants with only rice straw, without any supplementation of the other required nutrient sources, will result in poor performance of the animals (Devendra et al., 1986). The combination of low intake, low degradability, low nitrogen content and an unbalanced mineral composition means that rice straw alone may not even meet the animal's maintenance needs. Rice straw feeding systems vary in regards to the type and frequency of crop production, the availability of uncultivated land and the number of animals and can be classified into three groups: extensive systems, tethering systems, and stall-feeding systems (Devendra et al., 1986). The work presented here aimed to use a series of *in vitro* approaches to understand the molecular and chemical limitations to digestibility in the context of feeding ruminants in the tropics.

7.2 THE APPLICATION OF *IN VITRO* APPROACHES TO ESTIMATING DIGESTIBILITY OF TROPICAL FORAGES

The nutritional value of a feed depends on its chemical composition, the extent of rumen degradation and the digestibility of undegraded feed components, especially protein, escaping the reticulorumen to the abomasum and small intestine (Mohamed & Chaudhry, 2008). Therefore, the rumen degradation of a feed must be estimated to accurately assess its nutritional value *via* standardised methods that can be applied routinely with precision and accuracy. One way of estimating the degradation of feeds

in the rumen is to incubate them in rumen of fistulated live animals. This (*in vivo*) method measured the quantity of nutrients flowing to the duodenum or abomasum of fistulated animals. The method is laborious, expensive, requires large quantities of feed and is largely inappropriate for single feedstuffs, thereby making it unsuitable for routine feed evaluation (Mohamed & Chaudhry, 2008). Stern et al. (1997) observed that *in vivo* methods also subject to errors associated with the use of digesta flow-rate markers, microbial markers and inherent animal variations. An alternate to the *in vivo* method is the *in sacco* (*in situ*) method which measures the disappearance of dry matter or nitrogen from synthetic porous bags suspended in the rumen of fistulated animals and has been in use for many years (Ørskov & McDonald, 1979). The method however has the disadvantage of having poor reproducibility among laboratories due to variations in proteolytic activity of animals associated with variable diets and physiological status. Similar to *in vivo* methods, it also requires fistulated animals and thus raises animal welfare concerns and cost limits (Mohamed & Chaudhry, 2008). The alternative to *in vivo* and *in sacco* methods are *in vitro* methods which are cheaper and less-laborious approach to estimate the rumen degradation of feeds (Deaville et al., 1998). These alternatives include solubility in various solvents, the gas production technique and the degradation using enzymes (Mohamed & Chaudhry, 2008).

In vitro fermentation techniques (IVFT) involve incubations of substrates in rumen fluid. They have been used extensively to evaluate the nutritive value of ruminant feeds (Yáñez-Ruiz et al., 2016). *In vitro* techniques are considered less expensive than the *in vivo* and *in sacco* methods since they reduce the use of experimental animals. This is beneficial especially when in trials with a large number of treatments (Yáñez-Ruiz et al., 2016). Additionally, they offer the opportunity to recover both the metabolites and residue of microbial degradation for chemical analysis (Mohamed & Chaudhry, 2008). Compared to *in situ* methods, *in vitro* methods offer investigators the ability to control various factors (microbial, animal, environment) that would alter the feed degradation. As a result, the test feeds and treatments can be exposed to a uniform environment to detect significant effects. The techniques complement standard laboratory analysis of chemical composition and therefore offer a rapid and less expensive alternative to the determination of nutrient digestibility *in vivo* (Rymer et al., 2005).

In vitro gas production systems have been used extensively for rapid screening of the effect of various chemical substances, plant species, plant extracts and dietary ingredients on rumen fermentation (Yáñez-Ruiz et al., 2016). This was also the primary approach used in this dissertation in the experiments detailed in Chapters 2 – 6. Elsewhere, the gas method has been used to assess the actions of anti-nutritive factors on rumen fermentation of Mediterranean browses (Khazaal, Boza, & Ørskov, 1994) and African browses (Bonsi et al., 1995; Nsahlai, Siaw, & Osuji, 1994). Makkar et al. (1995) used the method to study interactions of tannins and saponins and to determine their effects on efficiency of microbial protein synthesis. They reported that the effects of simultaneous presence of tannins and saponins on rumen fermentation were additive and did not counteract effects of either tannins or saponins.

While *in vitro* studies can be valuable for screening of feeds, data should be interpreted carefully as results can be misleading when parameters of incubation are not carefully considered (Dijkstra et al., 2005). Further, the *in vitro* technique has been criticised for using the fermentation gas, a nutritional waste product, for estimation of ruminant feed degradation (Mohamed & Chaudhry, 2008). The gas is primarily from the fermentation of digestible carbohydrates by the activity of rumen microbes to (Pell & Schofield, 1993), and there's a strong correlation between gas production and feed degradation (Mohamed & Chaudhry, 2008). In addition, there's also a positive relationship between gas production and total VFA formation *in vitro* (Blümmel & Ørskov, 1993). However, gas production does not does not directly represent the extent of degradation and some feedstuffs with a low rate of gas production may have a higher *in vivo* digestibility than suggested from gas production (Menke et al., 1979). This has been attributed to further breakdown of feedstuff that occurs in other parts of the digestive system *in vivo* (Mohamed & Chaudhry, 2008). Further, *in vitro* gas production can also be affected by other experimental factors such as the nature of the buffer, and source and/or handling of the rumen fluid (El Shaer et al., 1987; Theodorou et al., 1994).

The use of rumen fluid for *in vitro* incubation of feeds is well established since its use by Tilley & Terry (1963) but does pose challenges such as accessibility (either from slaughtered stock or from fistulated animals) and ethical considerations. One approach without involving surgically prepared animals is to measure *in vitro* degradation using

various enzyme preparations involving cellulases, proteases, lipases and amylases individually or as mixtures (Chaudhry, 2005, 2007; Poos-Floyd, Klopfenstein, & Britton, 1985). However, the effect of enzymes *in vitro* may not be able to accurately predict the complex activity of microbes on feedstuff *in vivo* (Tamminga & Williams, 1998).

7.3 FEED RICE STRAW IN FARMING PRACTICE

In extensive systems, farmers collect and store straw after harvest. Ruminants are then released to graze on stubble and other available feeds such as weeds growing in the paddies, native pastures available on paddy bunds and roadsides, and browse from shrubs and trees (Figure 7.1). This practice is restricted to the period immediately after harvest to prevent animals from damaging cropped areas. It is common to see animals browsing on shrub and tree leaves as they are herded back to villages in the evening. Thus, there is a wide range of feed types available, but the amounts of each which are ingested, and their quality is generally unknown. This can lead to sub-optimal animal performance. Experiments detailed in Chapters 5 and 6 showed that rice straw had a lower degradation compared to hay as estimated *via in vitro* gas production. Further the chemical composition of rice straw, particularly the ADL% negative effect on degradation. Milling of the rice straw resulted in an increase in degradation. However, the effect ADL% remained even with this treatment. There was a positive correlation between the ADL% and the silica content. Santos et al. (2010) reported that the association of silica with either ADF or NDF influenced gas production with varieties having more silica in the NDF rather than ADF showing a lower gas production.

Figure 7.1: Putting cattle to grazing in a paddy field after harvesting (Mwea, Kenya, 2013) (Nagoya University SATREPS Rice Research Project. Retrieved from https://satreps.agr.nagoya-u.ac.jp/jpn/pic_slide-12.jpg)

During the growing season, ruminants are not allowed to go into the cropped areas. Farmers with a few ruminants may practise tethering. Large ruminants are tethered close to stacks of rice straw to enable self-feeding, particularly where the stacks are in the paddy fields, or when the straw has been transported to the farm house or yards (Figure 7.2). However, while it is convenient way of feeding straw, there is sometimes considerable wastage because of the manner in which the animals select straw from the stack. Reducing such wastage would improve the utilization of stored feed. Alternatively, large ruminants may be kept in stalls continuously or only during the night. The reasons for confining animals vary and depend upon the type of animals, the availability of feeds and climatic conditions. For example, small ruminants are often kept in total confinement in wet areas, dairy animals may be kept continuously in stalls when available grazing land or feed supplies are short, and draught animals may be kept completely confined during cropping seasons.

The long term storage of rice straw after baling has been shown to reduce the *in vitro* fermentation despite having the same levels of silica before storage (Santos et al., 2010). The authors suggested that field drying of rice straw altered the chemical nature of the fibre matrix or silica, or an interaction of the two that resulted in lowered digestibility (Santos et al., 2010). Voluntary intake of freshly harvested rice straw has been shown to be higher in sheep (Sharif, 1984) and in heifers (Santos et al., 2010) compared to dried straw. Therefore, feeding systems depending on long term storage may therefore have a disadvantage over systems where rice straw is fed in the paddies immediately after harvest. Further work is needed to better understand what

occurs in the rice plant during the field drying process to create this decrease in nutritional value. This would allow for formulation of strategies to maintain its nutritional value, rather than use treatments to improve the nutritional value of rice straw after field drying (Santos et al., 2010).

Figure 7.2: Rice straw is sold as a cattle feed (Mwea, Kenya, 2013) (Nagoya University SATREPS Rice Research Project. Retrieved from https://satreps.agr.nagoya-u.ac.jp/jpn/pic_slide-13.jpg)

7.3.1 FEEDING RICE STRAW WITH FORAGE SUPPLEMENTS

The rumen is the primary site of fibre digestion. Rumen microbes require certain nutritive elements for self-multiplication and for degradation of the cell walls of straw (Chenost & Kayouli, 1997). Rice straw is usually fed during periods where other more nutritious feeds are unavailable. However, it does not provide adequate nutrients for maintenance due to its deficiency in protein and other nutrients essential for normal digestion by rumen bacteria and the animal (Van Soest, 2006). Buffaloes, cattle and sheep fed rice straw alone have been shown to lose weight (Devendra et al., 1986). The straw is fed mostly in the long form, but it may also be chopped to limit selection and wastage. Supplementation of rice straw with protein, energy and/or minerals may optimize rumen function, also maximizing utilization of the rice straw and increasing intake (Malik et al., 2015).

The most common feeds provided with rice straw are roadside grasses (Trach & Thom, 2004). Other forages supplements include cassava (*Manihot esculenta*) (Sath, Borin, & Preston, 2008), gliricidia (*Gliricidia maculata*) (Devendra, 1990), leucaena (*Leucaena leucocephala*) (Devendra, 1983b; Dutta, Sharma, & Hasan, 1999) and Sesbania

(*Sesbania grandiflora*) (Van Thu & Preston, 1999). Forages from trees, crops residues and water weeds, including acacia (*Acacia arabica*), banana (Musa spp.), jackfruit (*Artocarpus heterophyllus*) (Hue & Ledin, 2008), pigeon pea (*Cajanus cajan*) (McMeniman, Elliott, & Ash, 1988), neem (*Azadirachta indica*), sweet potato vines (*Ipomoea batatas*) (Phesatcha & Wanapat, 2013) and water hyacinth (*Eichornia crassipes*) (Mako, 2013), are also commonly offered.

Supplementation with rice straw with green fodder is able compensate for the nutrient deficiency and also maintain or even boost the intake of basal diets (McMeniman et al., 1988). Further, only small quantities of green forage are required to improve the utilization of straw diets (Trach & Thom, 2004). The beneficial effects of supplementing rice straw with green forage even in small amounts are apparently due to the influence of cytoplasmic contents other than nitrogen or macrominerals on rumen microbial metabolism as demonstrated by Broudiscou et al. (2001). They observed that the inclusion of fresh *Medicago sativa* extract on a rumen microbial community maintained on a diet of rice straw and inorganic nitrogen lowered the outflow of fermented OM by 14% and enhanced the microbial OM outflow (+33%) and the efficiency of microbial protein synthesis in a dual outflow continuous culture (Broudiscou et al., 2001).

In developing countries, utilisation of fodder shrubs and trees is an important strategy for improving the nutrient intake of ruminants (Devendra et al., 1986). Fodder trees are deep rooted are therefore rarely affected by seasonal climatic changes; as a result the leaves of these plants remain green during dry periods when the availability of and quality of roadside grasses are low and hence they can be valuable supplements (Simbaya, 2002). In additional to their nutritional benefits, fodder trees also have the advantage of being easily accessible and relatively cheap, thus reducing requirements for concentrate supplements. Further, these trees have other beneficial effects in that they often provide fuel and in the case of leguminous types they may help to improve soil fertility by fixing atmospheric nitrogen (Viswanath et al., 2000).

The fodder trees species examined in this dissertation were *Acacia nilotica*, *Azadirachta indica*, *Guiera senegalensis*, *Lannea acida*, *Parkia biglobosa*, *Piliostigma reticulatum* and *Ziziphus mauritiana*. Fodder trees often contain antinutritive factors which may be detrimental to rice straw degradation and ruminant production (Myint

et al., 2010). As such, farmers should aim to optimise the level of browse supplementation to achieve a balance the benefits *versus* the negative effects (Kaitho et al., 1998). An inclusion rate of 16% of dry matter of dried *Leucaena* sp. was effective in reversing a weight loss to a significant weight gain in both sheep and cattle (Wahyuni et al., 1982). Higher levels of *Gliricidia* sp. (33% in DM) were needed to convert a substantial weight loss in cattle to maintenance (Devendra et al., 1986). Early recommendations, optimised the dietary level of 30 to 50% of the ration on DM basis or 0.9 to 1.5 kg/100kg body weight of fodder trees and shrubs (Devendra, 1988). Later studies narrowed the range to 30 to 45% of the ration DM or 0.75 to 1.125 kg/100 kg body weight (Kaitho et al., 1998). However, since the inclusion of green fodder in a rice straw diet at even lower levels can have beneficial effects (Broudiscou et al., 2001), the antinutritional effects of plant secondary metabolites can be avoided at lower inclusion rates. In general, any supplementation strategy should focus on locally available materials that can provide the necessary components for better utilization of rice straw (Malik et al., 2015). In the current study, the fodder tree samples had CP content of >10% demonstrating that leaves from the majority of tree species can be used as low-cost CP supplement for a rice straw-based diet. *A. nilotica* recorded the highest levels of total phenols ranging from 55mg/g to 94mg/g depending on the ecological zone. The phenols were composed primarily of condensed tannins. The other species recorded comparatively lower levels of total phenols, which were identified as different flavonoids (Section 3.4.2). High concentrations (>55g/kg DM) of condensed tannins generally have been shown to reduce voluntary feed intake and digestibility, and depress rates of body and wool growth in grazing ruminants (Min et al., 2003). Viswanath et al. (2000) reported that *A. nilotica* trees are commonly grown in rice fields in traditional agroforestry system in central India and that their dry pods are fed to cattle along with rice straw. However, there are no other studies showing the effect of this inclusion. Garba, Muhammad, and Maigandi (2015) observed that inclusion of *G. senegalesis* leaves in the rice straw diets of lactating does at the rate of 30% maintained milk yield compared to the animals supplemented with concentrate. Myint et al. (2010) reported that *Z. mauritiana* reduced both fibre and protein digestibility of a chopped rice straw and sesame meal diet in goats. They attributed this to the higher acid detergent insoluble

nitrogen (ADIN) content of the species. However, this experiment had a 50% inclusion rate, which is not practical in a production setting. For the rest of the tree species, no specific studies were found showing the effect of their inclusion in ruminant diets on rice straw degradation.

7.3.2 UNDERSTANDING THE DYNAMICS OF FODDER COLONISATION BY RUMEN BACTERIA

Rumen microbes are capable of obtaining energy from the breakdown of plant cell wall carbohydrates that are otherwise inedible by humans (Mizrahi, 2013). As a result, ruminant production can be undertaken in marginal land that cannot be used for food crop production (Kingston-Smith et al., 2010). Culturing techniques pioneered by Hungate (1966) provided the first glimpse of the complexity of the rumen ecosystem. However, many of the rumen microbes remain uncultured and uncharacterized. As discussed above, current nutritional concepts aim at high microbial efficiency; this cannot be achieved by measurement of *in vitro* gas production gas only (Makkar, 2004). Therefore, a better understanding of the roles played by the rumen microbes is key to the development methods to improve ruminant production (Yáñez-Ruiz, Abecia, & Newbold, 2015).

Advancements in sequencing techniques has enabled researchers study the function of the rumen bacteria and their interactions with other members of the rumen microbiome. The entry of high-throughput sequencing in particular has contributed to the better understanding of the rumen microbial ecosystem. The most prevalent approach is *via* sequencing of amplicons of specific microbial genes (Huws et al., 2018). The experiments in this dissertation applied amplicon sequencing of the 16S rRNA to generate the identity and the relative abundance of microbes.

Here feedstuffs typical of those used in tropics were evaluated. As previously described (here and others) the fermentation was affected negatively by presence of compounds such as tannin, silica and lignin. These are plant metabolites necessary for the health and resilience of plants but act as antinutritive factors by limiting ingestion and/or rumen degradation. In regard to fodder trees, results in Chapter 3 showed that high levels of phenols in *A. nilotica* had a significant effect on the relative abundance

of bacteria. This would translate as a limitation in ruminal microbial production. The fibre fractions had a stronger effect in the other species particularly in *G. senegalensis*, which had the highest levels of lignin and lowest *in vitro* degradation. The levels of lignin and silica were observed to have the largest effect on the *in vitro* degradation and colonisation of rice straw and detailed in Chapters 5 and 6. However, the negative relationship between silica and rice straw degradation alluded to in earlier publications was observed to be not as straightforward. Temporal colonisation of the rice straw by the rumen bacteria was observed to be biphasic with primary (0 – 1h) and secondary (6h onwards) events. Understanding rumen substrate-microbe interactions is necessary for development of novel strategies for improving ruminant nutrient use efficiency. Further investigation of the function of colonising bacteria, and especially the secondary bacterial community, is needed to improve the utilization of rice straw in ruminants. Results detailed in Chapter 5 showed that *Prevotella* spp. dominated in the first six hours of *in vitro* incubation of rice straw and was largely replaced by the genus *Fibrobacter* from 6h onwards. Chaucheyras-Durand et al. (2019) observed that supplementation of live yeast-based feed additive in early life of lambs promoted rumen microbial colonisation and fibrolytic potential. The supplemented lambs specifically showed a greater abundance in *Fibrobacter succinogenes* after weaning compared to control (Chaucheyras-Durand et al., 2019). *Fibrobacter* is one of the main cellulose-degrading bacterial genera in the rumen, and it may have an important role in the degradation of low-quality forages such as rice straw (Tajima et al., 2001). Further work is therefore needed to investigate whether it is feasible to increase the density of the genus in the second phase of colonisation.

7.4 FURTHER WORK

7.4.1 MANIPULATING RUMEN MICROBES

The rumen anaerobic bacteria, protozoa, anaerobic fungi, methanogenic archaea and phages interact with each other to form complex rumen ecosystem (Huws et al., 2018). Further, these microbes can engineer the rumen ecosystem in terms of colonisation and nutrient utilization thus offering a target for manipulation (Pereira & Berry, 2017; Shaani et al., 2018). The rumen microbiome composition is also influenced by the host factors including species, breed, individual specificity, host immunity and exposure to colonising microbes in early life. The rumen microbiome is highly resilient and will quickly revert to its original composition after manipulation as demonstrated by Weimer et al., (2010). This phenomenon has implications for likely success of attempts to modify the ruminal fermentation by targeted addition of microbes into the ruminant or into ruminant rations. Consequently, only a few examples exists where direct manipulation of the composition of the rumen microbial ecosystem has generated useful outcomes (Huws et al., 2018).

One of these successes relates to the leguminous tropical forage *Leucaena leucocephala*. While the forage is high in protein, it also produces the toxin mimosine which is converted in the rumen to 4-hydroxy-4(H)-pyridone (DHP). The toxic effects include salivation, live weight losses, poor animal performance and is goitrogenic (Wallace, 2008). However, Jones and Megarrity (1986) observed that Hawaii and Indonesia ruminant could graze on *Leucaena* without experiencing the toxic effect. Protection was also conferred to previously susceptible Australian ruminants infused with ruminal digesta from native Indonesian goats. Further investigations revealed that the goats possessed the bacterium, *Synergistes jonesii* capable of degrading DHP protecting animals from toxicity (Jones & Megarrity, 1986). *S. jonesii* is now used as an inoculum in many tropical countries as means of counteracting DHP toxicity (Wallace, 2008). This is a rare example where understanding the role of the rumen bacteria transformed livestock nutrition (Huws et al., 2018). The addition of live yeast into the cow's rumen has been shown to increase the abundance of fibre-associated *Fibrobacter succinogenes* on wheat bran and that of *Ruminococcus flavefaciens* on alfalfa hay *in sacco*. There was also an increase in attached fungi and NDF degradation

(Chaucheyras-Durand et al., 2016). These, and other results suggest an improvement in rumen fermentation efficiency by yeast supplementation (Desnoyers et al., 2009). The mechanism of action could involve nutrient supply or oxygen scavenging by the live yeast cells (Chaucheyras-Durand et al., 2016). Further work is needed to show the effect of live yeast in *in vitro* and *in vitro* colonisation and degradation of rice straw.

There is potential that with continued research and increasing understanding of the rumen microbial ecosystem, new discoveries that would enable scientists to improve the efficiency of rumen fermentation by effectively manipulating rumen microbes. The application of high-throughput sequencing platforms to study rumen microbes has allowed for a better understanding the complex microbial community found in the rumen. In this study, the Oxford Nanopore Technologies MinION, a novel sequencing platform, was shown to be capable of resolving treatment effect observed after Ion Torrent sequencing. The MinION has the advantage of being smaller, cheaper and easier to use compared to older platform; and offers the opportunity to rapidly expand the understanding of the rumen microbial ecosystem. Further work is required to develop standard protocols and bioinformatic pipeline in order to generate dependable result.

7.4.2 PLANT BREEDING

Aside from manipulating rumen microbes, the other approach of improving the efficiency of rumen metabolism is plant-breeding to reduce antinutritive factors such as plant secondary metabolites (PSM), silica and lignin. Concentrations of PSM vary significantly between plant species and also between accessions (Mueller-Harvey et al., 2019). The synthesis of PSM is under genetic control and that expression depends on the plant species and plant parts (Chezem & Clay, 2016).This means that plant breeding can target PSM composition and concentration (Mueller-Harvey et al., 2019). However, PSM have multiple and interrelated functions which provide plants with the plasticity to support their development and interaction with the environment (Mouradov & Spangenberg, 2014). Further, secondary compounds can help reduce rumen protein degradation (Kingston-Smith, Marshall, et al., 2013). Care is therefore

required to ensure that any breeding work with PSM-containing species results in appropriate results.

Silica and lignin are structural elements that strengthen and rigidify plants (Ghasemi et al., 2013). Silicon deficiency in rice results lowers grain yields (Yamamoto et al., 2012), increased susceptibility to fungal diseases (Ashtiani et al., 2012; Dallagnol et al., 2011) and increased susceptibility to lodging (Isa et al., 2010). Selective breeding to reduce the amount of silica or lignin in fodder may therefore have inadvertent negative effects on the plants yield, viability or resilience. Further work would need to improve the knowledge regarding the interactions between plants and the rumen microbes (Kingston-Smith, Marshall, et al., 2013). This would enable plant breeders to produce fodder that is not only easily broken down in the rumen but that also has sufficient mechanical strength to be self-supporting as it grows (Kingston-Smith & Thomas, 2003).

7.4.3 DIETARY INTERVENTIONS

The use of rice straw for ruminant production is increasing as developing countries strive to develop efficient livestock industries. As mentioned previously, supplementation with even small quantities of green forage can improve the utilization of straw diets (Trach & Thom, 2004). Further experiments would involve *in vitro* fermentation trials with the fodder trees as a supplement to a rice straw base diet rather than as the sole substrate. This would be a more practical evaluation of the usefulness of the fodder trees as they may be utilized in a tropical production system. Additionally, the effect of inclusion rate on fermentation and microbial population can be elucidated. *In vitro* experiments would be followed by *in vivo* trials to determine the palatability at different inclusion rates, the digestibility and the effect on rumen microbes and production.

7.4.4 RESEARCH TRANSFER

There has been a consistent effort to investigate the most efficient and practical ways to improve the efficiency of rumen fermentation. Much of the investigative effort has been made at the laboratory and experiment station level and only small changes in the traditional systems have occurred. The challenge that remains is to extend the accumulated findings in terms of efficient feeding systems that are realistic in small farm situations. To bridge the gap between research scientists and farmers, future research approaches should aim to produce quantitative information that can be easily adopted by ruminant production systems.

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