

High Throughput Approaches to Evaluate Between-Animal Variation in Digestion of Cattle

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ABSTRACT

Existing techniques to explore between-animal variation in digestion processes and their potential contribution to feed efficiency (FE) are laborious, expensive or invasive. The aim of this thesis was to refine and apply rapid techniques to assess the chemical (near infrared reflectance spectroscopy; NIRS) and physical (laser diffraction; LD) attributes of faeces, and characterise the rumen microbiome (16S next generation sequencing) associated with between-animal variation in FE. Preliminary work developed methods for LD, NIRS and 16S analysis. Differences in microbial communities were found between liquid and solid phases of rumen digesta. Nominal stem and leaf fractions of fresh grasses were incubated *in vitro* to assess the time course of rumen digestion using NIRS. Spectral regions associated with protein, starch, glucose and cellulose were highly degradable whilst those associated with lipids and lignin were less degradable. Principal component analysis (i.e. without the use of calibrations) of faecal NIR spectra from a compensatory growth study showed differences in digestion processes between restricted and *ad libitum* fed bulls. However, these differences were not apparent during a subsequent period of compensatory growth (when feed was offered *ad libitum*), confirming that differences in FE (feed conversion ratio; FCR) were not related to effects on digestion processes. A further study assessed relationships between all three techniques and FE; steers were offered one of 4 dietary treatments designed to alter methane emissions. A 56 day FE measurement provided residual feed intake (RFI) and FCR values for each steer. There were significant effects of the dietary treatments on measurements made using each of the techniques, but no consistent relationships with FCR or RFI within each dietary treatment group. Whilst the techniques used in these studies provided rapid and low-cost characterisation of between-animal variation in digestion processes, these differences made little contribution to between-animal variation in feed efficiency.

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ABBREVIATIONS

AA – Amino Acid(s)

AAx – Aberdeen Angus cross steer

ADF – Acid Detergent Fiber

ADFI – Average Daily Feed Intake

ADG – Average Daily Gain

ANOVA – ANalysis Of VAriance

BW – Body Weight

BW^{0.75} – Mid Point Metabolic Body Weight

C – Carbon

CP – Crude Protein

Cr₂O₃ – Chromic Oxide

DGGE – Denaturing Gradient Gel Electrophoresis

DI – DeIonised

DM – Dry Matter

DMD – Dry Matter Digestibility

DMI – Dry Matter Intake

DNA – DeoxyriboNucleic Acid

DOM – Digestible Organic Matter

DOMD – Digestible Organic Matter Content

DT – DeTrend

FA – Fatty Acid

FCE – Feed Conversion Efficiency

FCR – Feed Conversion Ratio

FD – Fattening Diet

FDR – False Discovery Rate

FFA – Free Fatty Acids

FISH – Fluorescent In Situ Hybridisation

FSG – Functional Specific Gravity

GH – Growth Hormone

GI – GastroIntestinal

GS – Grass Silage

H – Hydrogen
H – Shannon Index
He-Ne – Helium-Neon
IGF-1 – Insulin-Like Growth Factor 1
INVDMD – In Vitro Dry Matter Digestibility
Kc – Rate of Communion
Kd – Rate of Digestion
Kp – Rate of Passage
LC – Liquid-phase Clover
LD – Laser Diffraction
LE – Less Efficient
LG – Liquid-phase Grass
LIMx – Limousin cross steer
LW – Live Weight
ME – More Efficient
MPS – Median Particle Size
MSC – Multiplicative Scatter Correction
N – Nitrogen
N – Nitrogen
NDF – Neutral Detergent Fiber
NDFD – Neutral Detergent Fibre Digestibility
NIPALS – Non-linear Iterative Partial Least Squares
NIRS – Near Infrared Spectroscopy
NMDS – Non-metric Multidimensional Scaling
O – Oxygen
OM – Organic Matter
OMD – Organic Matter Digestibility
OTU – Operational Taxonomic Unit
PC – Principal Component
PCA – Principal Component Analysis
PCoA – Principal CoOrdinate Analysis
PCR – Polymerise Chain Reaction
PD – Phylogenetic Distance

PEG – PolyEthylene Glycol
PRG – Perennial RyeGrass
PUFA – Poly Unsaturated Fatty Acid
QC – Quality Control
QIIME – Quantitative Insights Into Microbial Ecology
qPCR – quantitative Polymerase Chain Reaction
RCC – Rumen Cluster C
RFI – Residual Feed Intake
rRNA – ribosomal RiboNucleic Acid
RT-PCR – Real Time Polymerase Chain Reaction
SC – Solid-phase Clover
SD – Standard Deviation
SDT – Standard normal variate and DeTrend
SG – Solid-phase Grass
SG – Specific Gravity
SNP – Single Nucleotide Polymorphism
SNV – Standard Normal Variate
STAMP – STatistical Analysis of functional and Microbial Profiles
SVD – Singular Value Decomposition
TMA – TriMethylAmine
TMR – Total Mixed Ration
T-RFLP – Terminal Restriction Length Polymorphism
VFA – Volatile Fatty Acid(s)
WC – White Clover
WSC – Water Soluble Carbohydrate

CHAPTER 1

GENERAL INTRODUCTION

1.1 ROLE OF THE RUMEN IN PHYSICAL AND CHEMICAL DEGRADATION OF FEEDS

The major difference between ruminant and monogastric digestion is the presence of additional compartments, the rumen, reticulum and omasum, in ruminants. The rumen and reticulum are essentially one structure normally referred to as the reticulorumen. The reticulorumen is a heterogeneous compartment that is heavily involved in both the physical reduction of particle size and the degradation of complex carbohydrates by the action of rumen micro-organisms. Feed enters the rumen directly from the oesophagus and tends to be found in the upper layer of long fibre (the 'rumen mat') soon after ingestion. Rumen contractions force rumen fluid through the rumen mat and thereby inoculate newly-ingested feed with microorganisms. As the fermentation proceeds, particles become denser and tend to be found in the dorsal rumen and eventually the reticulum.

Digesta within the rumen is mixed by primary and secondary contractions and relaxations of the reticulorumen wall and pillars. Primary contractions affect the whole of the rumen, and usually begin with a sharp contraction that is then followed by a secondary more sustained and powerful contraction that only affects part of the rumen. Ruminal mixing allows for circulation of undigested particles and ultimately results in small undigested particles moving to the dorsal reticulum where they can then be swept through the reticulo-omasal orifice.

Another aspect of rumen digestion is the physical breakdown of forage particles, which is partly the result of chemical breakdown, but this is greatly enhanced by the process of rumination. Rumination is the postprandial regurgitation of ingesta followed by mastication, reforming the bolus, and re-swallowing (van Soest, 1994). Rumination is closely related to reticuloruminal motility. It commences with a contraction of the rumen in which digesta is brought back up the oesophagus into the mouth. Ingesta is sucked into the oesophagus by aspiration of air causing negative pressure. Mastication reduces the particle size and liberates soluble contents, the bolus is then mixed with saliva and re-swallowed.

Microorganisms are an essential part of rumen function, with the diverse microbiota responsible for degradation of complex carbohydrates, production of volatile fatty acids (VFA) and synthesis of microbial protein that are key nutrients for host metabolism. The rumen confers the ability of ruminants to utilise feed components,

notably fibre, not digested by mammalian enzymes. Rumen fermentation of high roughage diets results in a high proportion of acetate, whilst high concentrate diets produce higher levels of propionate, and occasionally lactic acid. Fermentation of feed within the rumen also results in production of enteric methane, which is a form of energy loss to the animal and a major contributor to global greenhouse gas emissions. For every 100 g of ingested carbohydrate, approximately 4.5 g of methane is produced through methanogenesis (McDonald *et al.*, 2002). Protein enters the rumen as protein and non-protein nitrogen. Protein is degraded to amino acids (AA) through the action of microbes present in the rumen, and some is converted to ammonia which is absorbed across the rumen wall. MacRae and Ulyatt (1974) calculated that total nitrogen (N) absorbed was 10.0 g/d and 3.2 g/d within the rumen, and 18.8 g/d and 22.4 g/d in the large intestine for sheep fed on a diet of perennial ryegrass and short-rotation ryegrass respectively. Microbes are able to use non-protein N for synthesis of amino acids (AA) and protein, which contributes to the protein supply of the host animal. Undigested protein is passed out of the rumen and into the abomasum where microbial protein and undigested dietary proteins are digested by pepsin to peptides which are subsequently broken down further to amino acids which can be absorbed. Any undigested dietary protein is passed out in the faeces along with endogenous N losses.

Lipids ingested in the diet are converted to free fatty acids through microbial or plant-mediated lipolysis. Polyunsaturated fatty acids (PUFA) are then converted to saturated fatty acids through biohydrogenation via microbial action. α -linolenic acid is the main fatty acid in fresh herbage, due to the high levels present in glyco- and phospho- lipids of chloroplast membranes, however α -linoleic is the main substrate for biohydrogenation, so only low levels are available for host metabolism (Lourenco *et al.*, 2010).

A wide range of micro-organisms are present in the rumen at different concentrations: bacteria (10^{10-11} ml⁻¹), protozoa (10^{5-7} ml⁻¹), archaea (10^{5-7} ml⁻¹) and fungi ($<10^{4-5}$ ml⁻¹). Although cell numbers are fewer, protozoa make up a large proportion of the total microbial biomass in the rumen – 10 to 40 % of the rumen nitrogen (Van Soest, 1994). Archaea were recognised as a separate domain in 1977 (Woese and Fox, 1977) and rumen methanogens are an important class of Archaea that has received considerable recent interest.

Microorganisms that interact with feed particles are generally referred to as 'planktonic phase', 'loosely attached' or 'firmly attached', however they are also described as 'liquid-associated' or 'solid-associated'

Although it is difficult to measure, Craig *et al.*, (1987) estimated that solid associated bacteria make up 70-80% of the rumen population. Microbes rapidly colonise ingested forage, with estimates of initial colonisation within 5 minutes, and stabilisation of bacterial numbers within 15 minutes (Edwards *et al.*, 2007). However, Huws *et al.*, (2016) found considerable changes in microbial populations over time after feeding. Stomatal closure in fresh forages reduces accessibility and so decreases the rate of bacterial colonisation (Gudesblat *et al.*, 2009).

Material can leave the rumen either by passage through the reticulo-omasal orifice or absorption through the rumen wall. On average 65 % of apparent total tract digestion occurs in the rumen (ARC, 1980). Noble (1978) suggested that 70 % of the volatile fatty acids (VFA) produced in the rumen are absorbed across the rumen wall, whilst up to 70 % of the VFA that leaves the rumen are absorbed in the omasum. The presence of papillae on the luminal surface of the rumen increases the absorptive surface area. Diet has a significant effect on the number and form of rumen papillae, with more present when higher concentrate diets are fed. Leaves present in the omasum absorb water and nutrients.

1.2 IMPORTANCE OF DIGESTIVE EFFICIENCY

With an ever increasing human population and decreasing land mass, it is vital to increase efficiency of production of cattle. Feed represents up to 75 % of total variable costs in beef production systems (Ahola and Hill, 2012; Finneran *et al.*, 2010; Hersom, 2009), small increases in feed efficiency are therefore profitable to the farmer, as well as reducing environmental impacts. Reduction of variable costs must be both efficient and cost effective. The rumen is the main source of variation in digestive efficiency, with factors such as buffering, passage rates and microbial communities affecting both the rate and extent of digestion. Feed efficiency is typically measured over a period of 56 – 70 days (Archer and Bergh, 2000; Duthie *et al.*, 2015; Kearney *et al.*, 2004; Wang *et al.*, 2006), but studies have been reported over longer periods e.g. 120 days (Archer *et al.*, 1999). More recent work had found

that feed efficiency is moderately heritable, with values of 0.33 ± 0.01 and 0.23 ± 0.01 being reported for residual feed intake (RFI) and feed conversion efficiency (FCE) respectively (Berry and Crowley, 2013). These observations make it imperative to be able to measure digestive efficiency (or proxies) in large numbers of animals. Existing techniques for determination of feed efficiency and digestive efficiency are too laborious and costly to characterise digestion of large numbers of animals required for genetic evaluations. New methods need to measure, or provide proxies for, the physical and chemical breakdown of feeds between ingestion and faeces. Such methods should ideally be rapid and relatively cheap, and be able to be applied to samples that are relatively easy to obtain such as faeces or rumen fluid.

The objectives of the studies conducted as part of this thesis were to:

- I. Develop and refine a working method for analysis of (a) faecal samples using laser diffraction (LD) and near infrared spectroscopy (NIRS); and (b) rumen microbial communities using 16S based sequencing, with particular interest in populations of microbes from the liquid- and solid-phases of rumen digesta.
- II. Determine changes in chemical composition over the time course of rumen fermentation of fresh herbage *in vitro*, using NIRS.
- III. Assess the use of NIRS to investigate chemical differences in faeces resulting from feed restriction and realimentation of bulls (treatments that are known to affect feed efficiency).
- IV. To assess (a) the chemical (NIRS) and physical (LD) aspects of faeces, and differences in rumen populations (16S sequencing), associated with dietary differences; and (b) explore potential relationships with between-animal variation in digestion processes associated with feed efficiency (FCR or RFI).

CHAPTER 2

LITERATURE REVIEW

2.1 FEED EFFICIENCY

Feed makes up 50 to 75 % of variable costs in ruminant production systems (Ahola and Hill, 2012; Finneran *et al.*, 2010; Hersom, 2009), so it is important to maximise feed utilisation, whether at the level of digestion or metabolism. The rumen is the predominant source of variability in digestibility, especially for fibre, with possible sources of variation including (i) differences in rumen passage rates (ii) host effects on the microbial community, such as through the buffering of ruminal pH, and (iii) effects of chewing behaviour during both ingestion and rumination on particle size reduction. Whilst it is obvious that efficient cattle have increased levels of production relative to dry matter intake (DMI), methane production and manure production, there are a number of different metrics for expressing feed efficiency. Feed efficiency has been conventionally described using the ratio between feed intake and body weight gain known as feed conversion rate (FCR; Crews and Carstens, 2005). The reciprocal of FCR (i.e. the ratio between body weight gain and feed intake) is termed feed conversion efficiency (FCE). Since FCR is affected by body size, it has been suggested that selection for FCR would ultimately result in effects on cattle size of progeny of most efficient cattle (Arthur and Herd 2008). Selecting for lower FCR results in a correlated increase in growth rates, which in turn increases maintenance costs (Arthur and Herd, 2008). Residual feed intake (RFI) has been proposed as a measure of feed efficiency that is independent of growth rate and body weight (Koch *et al.*, 1963). RFI is the difference between actual and predicted intake, so a negative value indicates high efficiency (i.e. consuming less feed than predicted on the basis of measured body weight and weight gain). Basarab *et al.*, (2003) proposed additional elements in the RFI calculation, basing it on the regression of measured dry matter intake (DMI) on average daily gain (ADG), mid test metabolic body weight ($BW^{0.75}$) and ultrasonic fat depth at the 12th/13th rib at the end of the performance test period. In light of this, Arthur *et al.*, (2001) suggested that “*RFI should be the preferred trait for genetic improvement of post-weaning feed efficiency*” in Angus cattle.

Archer and Bergh (2000) reported genetic variation in RFI, both within populations and within individual breeds of cattle. RFI has been reported to be positively correlated to DMI (Kelly *et al.*, 2010; Lawrence *et al.*, 2012). It is moderately heritable, with heritability estimates ranging from 0.28 (Koch *et al.*, 1963) to 0.58

(Crews *et al.*, 2003). In addition to these genetic evaluations, researchers have explored genomic associations with feed efficiency. Barendse *et al.*, (2007) carried out a genome wide association study using the MegAllele Genotyping Bovine 10K single nucleotide polymorphism (SNP) chip. Over 160 SNP were associated ($P < 0.01$) with RFI when each SNP was tested individually.

Variation in feed efficiency is mainly related to differences in either digestion or metabolism and digestion, though Richardson *et al.*, (1999) suggested that a further very small part of variation in RFI could be explained by variation in body composition. Factors such as protein breakdown (McDonaugh *et al.*, 2001), animal activity (Richardson *et al.*, 2000) and heat increment have been investigated as possible reasons for the variation in feed efficiency between-animals (Herd *et al.*, 2004). Methane production represents a significant energetic loss to the animal, with a significant negative relationship between RFI and enteric methane emissions observed in beef cattle (Hegarty *et al.*, 2007). Between-animal variation in feed efficiency has also been investigated at the hormone level. Hormones, in particular insulin like growth factor 1 (IGF-1), are important for growth and development (Duan *et al.*, 2010). IGF-1 is associated with differences in regulation of muscle metabolism (Schiaffino and Mammucari, 2011). Hill and Herd, (2003) suggested that variation in metabolism at the endocrine, autocrine and paracrine levels contribute to variation in feed efficiency.

Herd *et al.*, (2004) carried out a review on factors contributing to variation in feed efficiency and proposed that variation in feed efficiency (expressed as RFI) resulted from: digestion (14 %); heat increment of feeding (9 %), body composition (5 %), activity (5 %) and other processes such as protein turnover and ion pumping (67 %). In a more recent review by Herd and Arthur, (2009), variation in RFI was explained by tissue metabolism and stress (37 %), digestibility (10 %), heat increment and fermentation (9 %), physical activity (9 %), body composition (5 %) and feeding patterns (2 %).

2.2 RUMEN FUNCTION AND DIGESTIVE EFFICIENCY

2.2.1 Digestion kinetics

Digestion is the time-dependent degradation, or hydrolysis, of feed into compounds that can be absorbed by the animal (Mertens, 2005b). When feed enters the rumen it can leave by one of two routes: absorption across the rumen wall or passage through the reticulo-omasal orifice. The extent of digestion in the rumen is controlled by the relationship between the rate of passage (k_p) and the rate of digestion (k_d). This can be described using the following equation, where k_d and k_p are expressed per h (Hvelplund *et al.*, 2009):

$$\text{Digestibility} = \frac{k_d}{k_d + k_p}$$

This equation can be applied to digestion of total DM or components of the diet, such as fibre, protein or starch. Hvelplund (2009) also showed that this approach could be used to estimate the k_d of starch for use in feed tables, with whole-tract starch digestibility estimated as $1.139 - 3.580 \cdot \text{RD} + 3.078 \cdot \text{RD}^2$, where RD is the rumen digestibility of starch. This equation is valid within the range of $0.58 < \text{RD} < 0.90$, with an R^2 of 0.45. Mertens *et al.*, (1977) showed non linearity for the first 6 h of fermentation observed in digestion curves and proposed models to take account of this lag.

Waldo *et al.*, (1972) produced a model (below) to describe cellulose digestion kinetics within the rumen. The model suggested that (1) the amount of potentially digestible cellulose is dependent on its digestion rate and passage rate and (2) the amount of digestion of potentially indigestible cellulose is dependent on its passage rate. The potential digestibility of cell walls is reduced by lignin. Lignin also affects the rate of digestion of the cellulose that is potentially digestible.

$$(1) \quad \frac{dA}{dt} = -k_1 A - k_2 A$$

$$(2) \quad \frac{dB}{dt} = -k_2 B$$

Where A is the amount of potentially digestible cellulose, B is the amount of indigestible cellulose, k_1 is the digestion rate, k_2 is the passage rate and t is the time in h.

Many animal factors affect the rate of passage of feed from the rumen, for example, intake levels, osmolarity of rumen fluid, milk production status and pregnancy status. Hanks demonstrated that non pregnant beef cows have a longer retention time than pregnant beef cows, although rumen fluid kinetics were not affected by pregnancy status (Hanks *et al.*, 1993).

2.2.2 Factors affecting digestibility

2.2.2.1 Chewing behaviour

Cattle spend between 5 to 12 h a day eating, and 3 to 11 h a day ruminating, depending on the physical properties of the ration and the level of intake (Dulphy *et al.*, 1980). Sheep ruminate for longer periods of time, with the duration proportional to levels of neutral detergent fibre (NDF) ingested (Welch and Smith, 1970). Passage of particles through the reticulo-omasal orifice is directly affected by the comminution of feed particles, as particle size is the rate limiting step for passage from the rumen (Luginbuhl *et al.*, 1990). Different feeds have different comminution rates (k_c), for example, Arroyo and Gonzalez, (2013) reported different rates for sunflower meal, $k_c = 0.577$ per h, and Italian ryegrass $k_c = 0.089$ per h. The mechanical breakup of particles has more of an effect on particle size than chemical digestion, thus faecal particle size is strongly linked to chewing efficiency of the animal (Hummel *et al.*, 2008).

2.2.2.2 Maturity and lignification

Lignin reduces the availability of cellulose and hemicellulose for digestion (Morrison, 1975), by both cross-linking polysaccharides and preventing microbial digestion (Moore and Jung, 2001). Lignin content of forages increases with maturity and this has a strong negative effect on digestion of cellulose (Kamstra *et al.*, 1958) and nutritional value for cattle. Plant cell walls also contribute to ruminal fill and so

the effect of lignification on digestion also affects voluntary intake (Jung and Allen, 1995).

2.2.3 Effects of diet on rumen pH and microbial populations

Diet has a marked effect on rumen pH, which in turn has an effect on microbial production and utilisation and ultimately affects the absorption of organic acids (Nagaraja and Titgemeyer, 2007). The occurrence of sub-acute ruminal acidosis was investigated on 10 commercial dairy herds in Italy (Morgante *et al.*, 2007). The average ruminal pH between herds varied from 5.59 (± 0.11) to 6.30 (± 0.6), and variation was also found between-animals. In addition ruminal pH naturally fluctuates throughout the day, with shifts of 0.5 to 1.0 pH units common in a 24 h period (Krause and Oetzel, 2006). This fluctuation is triggered by fermentable carbohydrates present in each meal. Increasing feeding frequency from two to six times a day can reduce the variation in pH, but the effect may be diminished if it also resulted in increased feed intake. However Robles *et al.*, (2007) reported that increasing feeding frequency had no effect on DMI. Organic acids produced from fermentation are buffered, with a large proportion of these buffers produced in the saliva; half of the bicarbonate entering the rumen comes from the saliva (Owens *et al.*, 1998).

Ruminal acidosis lowers the rate of microbial protein synthesis, which further increases the risk of acidosis. Low pH has also been associated with reduced feed intake due to increased osmolarity (Beauchemin, 2007), in addition this work found that NDF digestion within the rumen reduced from 52 to 44 % in cows experiencing repeated episodes of acidosis. Terminal-restriction fragment length polymorphism (T-RFLP) was used in an *in vitro* study to examine changes in microbial communities when pH ranged between 5.5 and 7.0 as a result of carbohydrate sources from differing sources (Poulsen *et al.*, 2012). A decrease in pH to 5.5 was found to favour the production of butyric acid. It has been suggested that the optimum pH range for methanogenic activity is between 6.0 and 6.5 as it has been reported that methane production was reduced at pH 5.5 and pH 7.0 (Poulsen *et al.*, 2012).

2.3 ESTIMATING DIGESTIBILITY

2.3.1 Total tract digestibility

The traditional requirement to estimate feed digestibility is based on the need to compare feeds and design diets for animals at different levels of production. The 'gold standard' method for estimating digestibility is a full digestion experiment in which animals are adapted to diets for a period of up to 3 weeks, followed by recording of total intake and faecal output for 5-10 days (and urine collection if determining total nitrogen (N) excretion), as described by Thornton and Minson (1973). Animals are normally kept in metabolic cages during the measurement period to facilitate collection of faeces and urine. Feed intake and refusals are also recorded. Faecal samples are taken over a number of days, which can overcome any issues associated with day-to-day variation in faecal output.

2.3.2 Nylon bag technique

Nylon (dacron) bags are also used to determine rumen digestibility of feeds (O'Donovan, 1966; Ørskov *et al.*, 1980). In this technique, feed samples are placed in a dacron bag, sealed and placed into the rumen with weights to ensure the bags remain submerged in rumen liquor. Samples are removed at specific time points, washed and dried to determine weight of the residue. The pore size plays an important role in the release of particles, and therefore digestion of feed, pore sizes are typically ranging from 10 to 53 μm (Lindberg and Knutsson, 1981; Ørskov *et al.*, 1980). The disappearance of dry matter in cereals (Nording and Campling, 1976) and roughage (Neathery, 1969), and the disappearance of the cell wall fraction (Uden *et al.*, 1974) have been assessed using this technique. A major drawback of this technique is that animals require a rumen cannula, which is impractical for characterising large numbers of diets or animals.

2.3.3 In vitro rumen digestion techniques

Tilley and Terry (1963) first proposed using a 2-step *in vitro* technique to assess digestibility of feeds. In this technique dried feed samples are digested in strained fluid from donor sheep. After 48 h, a second digestion stage is carried out in which

samples are digested in pepsin for a further 48 h. Samples are shaken occasionally to simulate rumen contractions. The pepsin-cellulase technique, as described by Jones and Hayward (1973), uses enzymes as an alternative to rumen fluid to assess digestibility of feed stuffs. This technique has proved useful to assess dry matter digestibility (DMD) and organic matter digestibility (OMD) in forages (Nousiainen *et al.*, 2003) and silages (Barchiesi-Ferrari *et al.*, 2011). Another technique used to assess *in vitro* digestibility is the Daisy (II) technique, which uses feed samples placed into bags and incubated. This technique has been used to predict NDF and DMD for feeds (Cattani *et al.*, 2009). The nylon (dacron) bag and *in vitro* techniques are sufficient for determining digestibility of feed, but not practical for assessing digestibility of feed between individual animals as only a handful of donor animals are used.

2.3.4 *In vitro* gas production technique

The *in vitro* gas production technique (Menke and Steingass, 1988) has been used to assess digestibility of feed from the appearance of fermentation gas (Getachew *et al.*, 2005). Recent work has assessed the use of additives on fermentation (Carro *et al.*, 2005).

2.3.5 Marker techniques for estimation of digestibility and passage rates

Indigestible markers, both internal and external, are commonly used to assess total-tract digestibility, as well as passage rates. Generally, markers are applied at a constant dose are used for digestibility studies, whilst pulse dosed particles are used for studies of passage rates and digesta flow. There is an inverse relationship between the faecal concentration of a marker and digestibility since the marker is contained within a smaller quantity of faecal DM when digestibility is higher (van Soest, 1994). Examples of markers that have been used include chromic oxide (Cr_2O_3), acid insoluble ash and *n*-alkanes which will be discussed further in Section 2.4.1.

Inert particles, such as plastics, and occasionally feed particles recovered from the digestive tract, have been used for passage rate studies in cattle (Ehle *et al.*, 1982; Welch, 1986). Plastic particles are not recommended for rate of passage studies as

feed particles naturally change in density over the course of fermentation (Kaske *et al.*, 1992). Particle density slowly increases whilst feed is in the rumen due to hydration, release of gases and physical breakdown of particles.

2.3.6 Prospects of digestibility techniques for comparison of animals

The *in vivo* techniques are generally costly and laborious, with each having their own shortcomings, especially if determining digestibility of a large number of animals. The laborious nature of total faecal collections for determining digestibility is discussed above, however this technique still remains the most reliable (Khan *et al.*, 2003). A number of laboratory methods, including chemical analysis, *in vitro* digestion procedures with enzymes or rumen fluid, and near-infra-red spectroscopy (NIRS), have been developed to predict feed values. However, these laboratory methods involve either no animals, or just a few donor animals, and so are not suitable to assess variation in digestibility due to animal factors. In this situation, we need to use animal-based measurements, but it would be preferable to avoid total faecal collections. The aim of the work undertaken for this thesis was to investigate rapid techniques that can be used to describe differences in digestibility between animals without the need to record feed intake or make total collections of faeces. The next three sections describe the methods that will be investigated for this purpose – particle size analysis to look at the physical aspects of digestion (Section 2.4), 16S based sequencing techniques to assess rumen microbial populations (Section 2.5) and near-infra red reflectance spectroscopy to look at the chemical aspects of digestion (Section 2.6).

2.4 PARTICLE SIZE ANALYSIS

2.4.1 Retention time in the rumen

Retention time in the rumen is an important factor for the control of intake and digestibility (Welch, 1986). Passage of particles is limited by the outflow rate of small particles from the reticulorumen. Passage is also dependent on specific gravity of particles, as particle size and specific gravity are often interrelated (Hristov *et al.*,

2003). Rumen contents are usually stratified into layers, with a rumen mat that is able to float on top of a more fluid phase. The rumen raft consists of newly ingested particles, which become entangled and act as a filter bed to trap larger particles. Trapped gasses within the mat cause buoyancy. Particles only become small and dense enough to pass through the mat after fermentation.

2.4.1.1 Critical size theory

The rate of escape of particles is inversely related to rumen retention time (Shaver *et al.*, 1988). Particles above a certain size tend to be retained in the rumen (Okine and Mathison, 1991). Poppi *et al.*, (1980) suggested a critical size of 1.18 mm for sheep, however they found that 1 – 3 % of particles in the abomasum were larger than this. The critical size theory implies that all particles > 0.5 mm and < 1.18 mm have equal mean relative resistance (Poppi *et al.*, 1985) and are equally likely to leave the rumen of sheep (Poppi *et al.*, 1980). However, in reality, this is an over-simplification as several factors affect the rate at which digesta leaves the reticulorumen. Other studies have shown that digesta above the 1.18 mm threshold can leave the rumen, ranging up to 3.6 mm (Cardoza, 1985) or 5 mm (Welch, 1986) in cattle. Material present in the reticulorumen of both cattle and sheep is mostly smaller than the size threshold of 1.18 mm (Poppi *et al.*, 1981). Little further reduction in particle size occurs once digesta has left the rumen of cattle (Okine and Mathison, 1991).

2.4.1.2 Specific gravity of particles

Specific gravity, or relative density, can be defined as the ratio of the density of a substance to that of a standard substrate. Functional specific gravity (FSG) is considered a more accurate physiological measurement as it takes into account the effect of gas filled spaces and water holding capacities (Siciliano-Jones and Murphy, 1991). Particle size and specific gravity (SG) are the primary factors that affect the retention of particles within the rumen (Hristov *et al.*, 2003). Denser particles tend to escape from the rumen more rapidly, however complex relationships between SG and diet have been identified in previous studies. In a study using plastic particles of

different sizes and density, Clauss *et al.*, (2011) showed that retention time decreased as SG of particles increased from 0.92 to 1.44 g/ml, but then increased once a density of 1.50 g/ml was reached. Particles with a SG of 1.2-1.4 g/ml had the highest probability of leaving the reticulorumen. It has also been shown that feeding silage, rather hay, reduces gas production and reduces water holding capacities, thereby increasing SG of particles (Nelson and Satter, 1992; Wattiaux *et al.*, 1992). In another study, diet had no effect on mean retention time of light (FSG < 1.02 g/ml) or heavy (FSG > 1.02 g/ml) particles within the rumen. Particles with a FSG > 1.02 passed through the rumen more rapidly than those with a FSG < 1.02 (Hristov *et al.*, 2003).

2.4.2 Effect of intake level on particle size distribution

Several studies have assessed faecal output and faecal particle size at varying intake levels (Kovacs *et al.*, 1997; Luginbuhl *et al.*, 1990; Luginbuhl *et al.*, 1994; Maulfair *et al.*, 2011). In one study (Luginbuhl *et al.*, 1990) steers were fed coastal Bermudagrass hay at four levels of intake and faecal and ruminal upper strata samples taken 2, 6, and 12 h after feeding. With increasing feeding level, linear increases in mean faecal particle size and sizes 6 and 12 h after feeding were found. In a similar study, Kovacs *et al.*, (1997) fed steers with a mixed diet containing ryegrass and maize silage and concentrate at three levels of intake and samples taken 3 and 7.5h after feeding. The results demonstrate that level of intake had no effect on the distribution of small medium and large particles in the rumen but there was a significant effect of time after feeding demonstrating particle breakdown in the reticulorumen. Maulfair *et al.*, (2011) noted that DMD of grass fed to cows tended to decrease when particle size of feed was increased, this was achieved by chopping feed to different lengths (short, medium, long and extra-long).

2.4.3 Faecal particle size analysis

Digesta, and faeces, are comprised of particles of varying sizes, or conglomerates (Fritz *et al.*, 2011). Differences in the distribution of particles can show differences in retention time within the digestive tract and may be related to differences in digestion.

2.4.3.1 Wet sieving techniques

Faecal and digesta particle distribution is commonly assessed using sieve analysis. Wet sieving is traditionally carried out using a series of stacked sieves, which gradually decrease in mesh size. Faecal samples are placed on the top mesh with a constant stream of water and shaken to separate particles. Faecal samples are commonly soaked in water to ensure good dispersion prior to analysis (Clauss and Lechner-Doll, 2002). Particles retained on each of the stacked sieves are dried and proportions of each of the fractions, or particle sizes, calculated (Fritz *et al.*, 2011). Particles are separated by length or width depending on sieving technique used (Schadt *et al.*, 2012), this means that particles with at least one dimension greater than the sieve mesh size can still pass through, giving inaccurate readings. Mertens (2005a) suggested multiplying the mean particle size by 4.8 to estimate the mean particle length of corn silages, whilst Igathinathane *et al.*, (2009) observed particle length deviations of 17 - 22 times during length based separations of particulate material.

2.4.3.2 Laser diffraction

A lack of standardisation of the sieve sizes used in wet sieving, coupled with the laborious nature of the technique limits its application to large numbers of samples and this has led to other techniques being explored to determine particle size distribution. Laser diffraction is one such example (Olaisen *et al.*, 2001). Laser diffraction is a rapid technique used to assess particle size distributions of both wet and dry samples (with the aid of a dispersal agent) ranging from 0.01 μm to 2000 μm . Laser diffraction is based on the ability of particles to scatter light, the intensity of light scattered by a particle is directly proportional to the particle size. The angle of the laser beam and particle size have an inversely proportional relationship where the angle increases as particle size decreases and vice-versa i.e. large particles scatter light at smaller angles and small particles scatter light at larger angles, relative to the laser beam. Samples are pre-diluted to a target obscuration (similar to a target concentration, with 100 % representing no light passing through the sample). A helium-neon (He-Ne) laser beam is passed through the sample in a flow cell, with scatter of light being recorded on a series of detectors (Figure 2.1; Malvern

Instruments, 2012). The intensity data is analysed to calculate the size of the particles responsible for creating the scattering pattern, using the Mie Theory of light scattering. Particle size is reported as a volume equivalent sphere diameter. Particles are then assigned to a range of pre-determined size categories using the volume equivalent sphere diameter. The equivalent sphere approach is used as it only requires one parameter to record size compared to reporting a particle in 3D using length/width and depth.

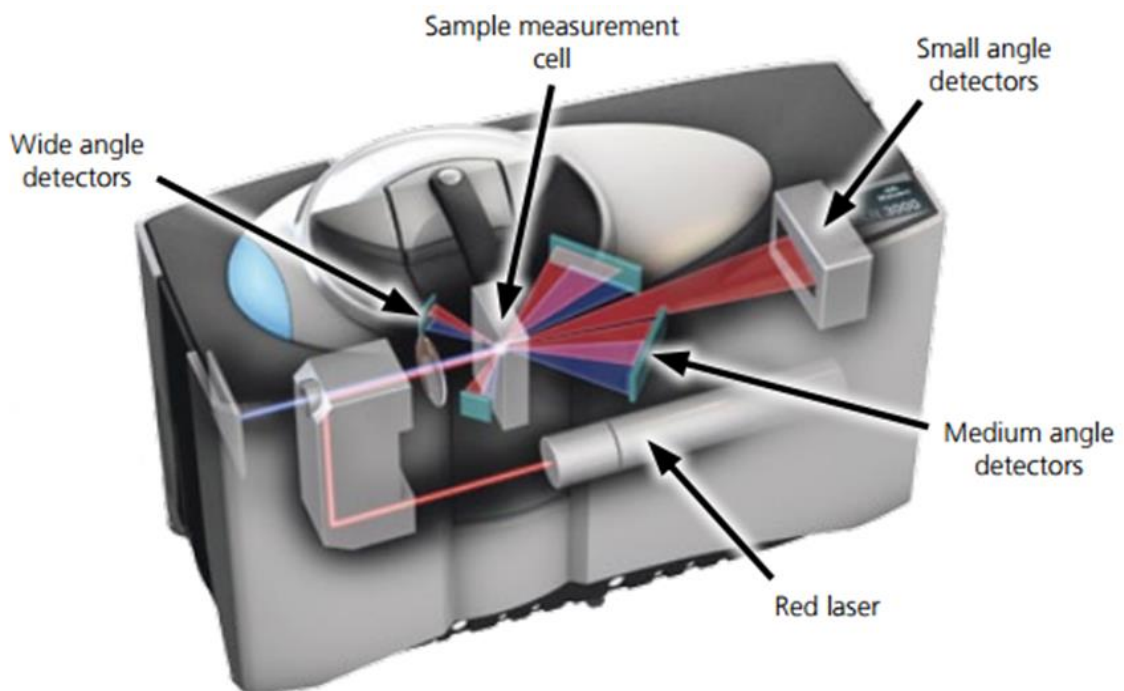


Figure 2.1 - Laser diffraction instrument, showing layout of flow cell, laser beam and detectors (Source - Malvern Instruments, 2012)

Common applications of LD to assess particle sizes have included studies of soil (Fenton *et al.*, 2015), cement (Malvern (a), 2015), inks (Duffy, 2015) and in pharmaceutical product development (Malvern (b), 2015). The LD method has been previously reported to be influenced by shape of each particle being measured (Eshel *et al.*, 2004), this can lead to particles being reported in particle size categories larger than they actually are. The shape of sand particles have also been found to effect particle size distribution whilst using the LD technique (Polakowski *et al.*, 2014). Surface roughness of particles has been shown to have a negligible effect on particle size distribution (Muhlenweg and Hirleman, 1998). Faeces consist of undigested

matter, the shape of which is typically long and rod like in shape, which deviates from the sphere measured by the LD technique. Therefore differences in faecal particle size likely influence the assignment of particles to each particle size category. However, as described above, measurements of long and thin particles are also an issue when using traditional wet sieving techniques. One further constraint is the LD instrument used in these studies is limited to a maximum particle size of 2 mm. Whilst Poppi *et al.*, (1980) suggested that particles no larger than 1.8 mm escape the rumen, Cardoza (1985) and Welch (1986) noted particles as large as 3.6 mm and 5 mm respectively have been reported in the faeces of cattle, leading to the potential of missing essential information. Although inherent issues with the LD technique exist, the technique still has potential to estimate faecal particle size as a rapid replacement for wet sieving techniques.

2.5 RUMEN METAGENOMICS

2.5.1 Assessing microbial communities within the rumen

The rumen contains a diverse population of microbes, protozoa and fungi that are responsible for breakdown of ingested feed, and are therefore essential to productivity and health of the host.

Most rumen microbes are obligate anaerobes, with only a few being facultative anaerobes (Stewart and Bryant, 1988), making them extremely difficult to culture under laboratory conditions. Recent advances in molecular techniques have allowed a shift from culture based and microscopic methods to culture independent methods to describe microbial communities.

2.5.2 Culture based methods

Traditionally, identification and investigation of the metabolism of ruminal microbes has been carried out using culture based methods. Hungate (1950) developed a roll-tube technique for isolation and cultivation of individual colonies of strict anaerobes. The technique allowed for growth in tubes sealed with rubber stoppers under oxygen free conditions. The Hungate technique allowed for identification of *Fibrobacter*

succinogenes, *Ruminococcus albus* and *Ruminococcus flavefaciens* (Stewart *et al.*, 1997). The development of anaerobic cabinets allowed isolation of ruminal bacteria on petri plates (Leedle and Hespell, 1980). Anaerobic techniques have resulted in identification of *Streptococcus bovis*, *Ruminobacter amylophilus*, *Succinimonas amylolytica* and *Selenomas ruminantium* (Dehority, 2003). All of the culture based techniques tend to underestimate the numbers of, and diversity of microbes present (Safaei *et al.*, 2006).

2.5.3 Culture independent methods

Molecular methods have been able to overcome some of the issues associated with culture-based techniques, whilst increasing taxonomic resolution. Woese (1983) suggested 16S rRNA gene as a microbial marker as it is phylogenetically conserved, consisting of hypervariable and conserved regions that allow differentiation of taxonomic groups as well as amplification (Gentry *et al.*, 2006; Yu and Morrison, 2004a). The 16S rRNA gene (*rrs*; ribosomal ribonucleic acid) is about 1500 base pairs long and ubiquitous in archaea and bacteria.

Real time polymerase chain reaction (RT-PCR) has been used to quantitatively estimate populations of archaea, fungi and protozoa. In one such study, Tajima *et al.*, (2001) used it to assess populations of 12 bacterial species during the transition from a hay- to grain-based diet of cows. A substantial increase in *Prevotella bryantii* was noted with an initial peak of 263-fold, however this fell to 10-fold 28 days after the diet switch, whilst *Fibrobacter succinogenes*, a fibrolytic species, showed a 57-fold reduction by day 28.

Another technique, Fluorescent in situ hybridization (FISH), has been used to visualise microbes using fluorescently labelled probes that hybridize to rRNA within an undamaged cell. However the limited number of probes targeting rumen bacteria means that this approach has limited potential (Kong *et al.*, 2010), though it has been used for some studies. Kong *et al.*, (2010) assessed the rumen microbial communities of cows fed barley silage or grass hay diets, with or without added flaxseed. *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were present in both the liquid and solid fractions of the rumen, with an increased abundance on the silage-based diets (75.2 to 87.3 %) compared to cows on the hay-based diet (31.8 to 49.5 %).

Proteobacteria, *Ruminococcaceae*, Deltaproteobacteria and Gammaproteobacteria were detected, although in lower numbers. Increases in these groups were also reported after the addition of flaxseed to the silage-based diet (45.2 to 49.5 %) and the hay-based diets (31.8 to 49.5 %). In another FISH study, Yanagita *et al.*, (2000) showed that *Methanomicrobium mobile* accounted for 54 % of total rumen methanogens.

Denaturing gradient gel electrophoresis (DGGE) separates amplicons of variable regions of *rrs* based on the electrophoretic mobility of the DNA (deoxyribonucleic acid). Differences in sequences affect melting points, so that amplicons migrate to different points on the gel. Kocherginskaya *et al.*, (2001) reported greater diversity of rumen microbes from steers offered a corn-based diet compared with a hay-based diet. Genus specific primers have been used to analyse *Prevotella* and *Treponema* in the rumen (Bekele *et al.*, 2011). Yu *et al.*, (2008) reported that the V3 region of the rRNA gene is the best target for archaea. This was achieved by separating and assessing *rrs* PCR (polymerase chain reaction) fragments from ruminal bacteria. Whilst DGGE is able to identify novel bacteria, it is unable to identify the full diversity as only abundant species are detected (Klieve *et al.*, 2007).

2.5.4 Next generation sequencing

Recent advances in sequencing technologies and bioinformatics have allowed the use of high-throughput sequencing techniques to assess microbial populations at lower costs. There has been a tremendous increase in sequence depth per run from approximately 1×10^3 using Sanger sequencing, 5×10^5 using Roche 454 sequencing, to 1×10^8 using Illumina GAIIx (Caporaso *et al.*, 2011). Illumina technology uses sequencing of the 16S rRNA gene, but is much better at identifying less abundant taxonomic group. The hypervariable regions (V1 through to V9) of the 16S rRNA gene allow bacteria and archaea to be identified at the species level due to its diversity. Next generation sequencing, using barcoded samples, allows for multiple samples to be assessed in a single run, with samples being “demultiplexed” bioinformatically after sequencing.

Caporaso *et al.*, (2011) demonstrated that the Illumina GAIIx with barcoded samples could be used to assess microbial populations of 25 environmental samples and 3

mock communities to a depth of 3.1 million reads per sample. The primers were developed against the V4 regions of the 16S rRNA gene, which meant they were able to detect both archaea and bacteria present in samples. An Illumina adaptor and linker sequence present within both forward (F515) and reverse primers (R806), with an additional 12-base error correcting Golay barcode added to the reverse primer. These same primers were later used on Illumina MiSeq and Illumina HiSeq platforms to assess differences in microbial communities from host and free living environments (Caporaso *et al.*, 2012) and results were consistent across sequencing platforms. Data analysis for both of these studies was carried out using the QIIME software (Quantitative Insights Into Microbial Ecology; Caporaso *et al.*, 2010b).

McCann *et al.*, (2014 b) reviewed early use of 16S rRNA based sequencing to explore diet effects on the rumen microbiome. Regardless of diet used in studies, Firmicutes and Bacteroidetes were the most abundant bacteria present in the rumen at the phylum level (Jewell *et al.*, 2015; Myer *et al.*, 2015). The genus Prevotella, from the phylum Bacteroidetes, was most abundant within the rumen (Morgavi *et al.*, 2015).

2.5.5 Sampling for rumen microbiome analysis

There have been major advances in methods for analysis of the rumen microbiome, however there are still issues relating to sampling rumen digesta for the analysis. Several studies have shown that there are noticeable differences in rumen microbiome from liquid and adherent (solid) fractions of the rumen (Brulc *et al.*, 2009; Jewell *et al.*, 2015; Veneman *et al.*, 2015). Jewell *et al.*, (2015) also noted greater diversity in samples taken from the liquid fraction of the rumen. There are also significant effects of sampling time (relative to feeding). Huws *et al.*, (2016) described primary (1 – 2 h) and secondary (4 – 8 h) microbial colonisation of ingested herbage in the rumen of 3 cows. This was simulated by incubating fresh grass *in sacco* within the rumen. Decreases in Clostridiales were noted during the secondary colonisation stage relative to the abundances of primary colonisers.

2.5.6 Relationships between the rumen microbiome and feed efficiency

It is well-established that the rumen microbiome differs greatly between-animals (Henderson *et al.*, 2015; Ross *et al.*, 2012). Several recent studies have assessed the link between the rumen microbiome and feed efficiency measurements. The Firmicutes to Bacteroidetes ratio has been associated with feed efficiency in cattle (Jami *et al.*, 2014), but the majority of studies taking place in humans and mice (Ramirez-Ramirez *et al.*, 2012; Turnbaugh, 2006). Jami *et al.*, (2014) reported correlations ($R^2 = 0.72$) between milk-fat yield and Firmicutes to Bacteroidetes ratio in dairy cows. Prevotella and the family Bacteroidales were found to be more abundant in the rumen microbiome of efficient (low RFI) Brahman bulls in the 454 Pyrosequencing study reported by McCann *et al.*, (2014a). Whilst Myer *et al.*, (2015) found no significant changes in overall rumen microbiome diversity, there were differences in relative abundances of Firmicutes, Lentisphaerae, Dialister and Lactobacillus related to ADG and ADFI (average daily feed intake). The same trend was seen when assessing the jejunal microbiome from the same cohort of animals: no differences in diversity, but significant differences in relative abundances of Proteobacteria, *Lachnospiraceae*, *Coriobacteriaceae* and *Butyrivibrio* between efficient and inefficient steers (Myers *et al.*, 2016). McCabe *et al.*, (2015) noted a large reduction in the relative abundance of an uncharacterised *Succinivibrionaceae* in cattle on restricted feeding. A decreased acetate:propionate ratio was also noted, which has previously been reported in efficient cattle (Christopherson *et al.*, 2008). Jewell *et al.*, (2015) compared microbial communities in efficient and inefficient lactating Holstein cows using samples taken from both the solid and liquid phases of the rumen. They found that *Anaerovibrio*, *Butyrivibrio*, Clostridiales, Prevotella, and *Ruminococcaceae* were more abundant in inefficient cows (high RFI). However it must be noted that although primers were designed to detect bacteria and archaea, archaea were removed from all further analysis. Even though archaea are only present at relatively low levels within the rumen (0.3 to 3.3 %; Janssen and Kirs, 2008), they are methanogens and as noted earlier in this chapter methane represents a significant loss of energy to the animal. More recently Poulsen *et al.*, (2013) identified the methyl-coenzyme M reductase gene, which is indicative for methanogenesis, in the previously lesser studied Thermoplasmata. Thermoplasmata, recently reclassified as Methanomassiliicoccales (Seedorf *et al.*, 2014), were found to

have enhanced growth in the presence of methylamine and this was coupled with increased methane production. The relative abundance of Thermoplasmata (Methanomassiliicoccales) was found to explain more of the variation in methane emissions than Methanobacteriales. Methane production was reduced with the addition of rapeseed oil supplementation, however it was discovered that only Thermoplasmata were significantly reduced in numbers and that levels of Methanobacteriales were unaffected.

In addition to analysis of effects related to individual taxonomic groups, other studies have explored relationships with overall microbial communities using principal coordinate analysis (PCoA). However, Rius *et al.*, (2012) was not able to find differences linked to RFI in cows and there was no PCoA grouping for feed efficiency (RFI) based on the rumen (Myer *et al.*, 2015) or jejunal microbiome (Myer *et al.*, 2016). Shabat *et al.*, (2016) proposed that a more diverse microbial community would produce a larger number of metabolites and that this would result in reduced feed efficiency.

2.6 NEAR INFRARED SPECTROSCOPY

Near infrared spectroscopy (NIRS) is a rapid, non-destructive technique and in recent years has represented a significant development for feed and forage analysis. The technique was introduced to measure moisture and protein in grains (Hymowitz *et al.*, 1974; Williams, 1975) and forages (Norris *et al.*, 1976). Since then, the number of analytical applications of NIRS has expanded in the agricultural field as well as many other industries, including the polymer industry, petroleum and fuel industries and biomedical and clinical applications (Pasquini, 2003). However, NIRS remains a secondary method being reliant on primary laboratory techniques. The major advantages of NIRS include that it is a rapid and low cost analytical method and requires no chemical reagents. Once calibrations are in place, it is a rapid and less expensive technique compared to conventional analytical methods. The technique has also been used to characterise the chemical composition of faeces and predict other attributes of the diet which will be discussed further below.

2.6.1 Principles of NIRS

Near infrared is the region of the electromagnetic spectrum between the fundamental mid infrared (mid-IR) and visible regions. The NIR region extends from 12,800 to 4,000 cm^{-1} , or 780 to 2,500 nm (Miller, 2001) (Figure 2.2). NIR spectra are a direct result of organic molecules absorbing light and are characterised by absorption bands caused by stretching vibrations of hydrogen (H) bonds with carbon (C), oxygen (O) or nitrogen (N) atoms. Spectra consist of harmonic overtones of mid-IR fundamental bands and combination bands (Barton, 1989). These are lower in intensity than fundamental bands (Burns and Ciurczak, 1992). This allows thicker or larger samples, using transmission or reflection NIRS respectively, to be analysed by this method compared to mid-IR. When radiation interacts with a sample, it is either absorbed or diffusely reflected. Therefore the NIR diffuse reflectance contains information about the composition of samples (Norris, 1989).

NIR in the Electromagnetic Spectrum

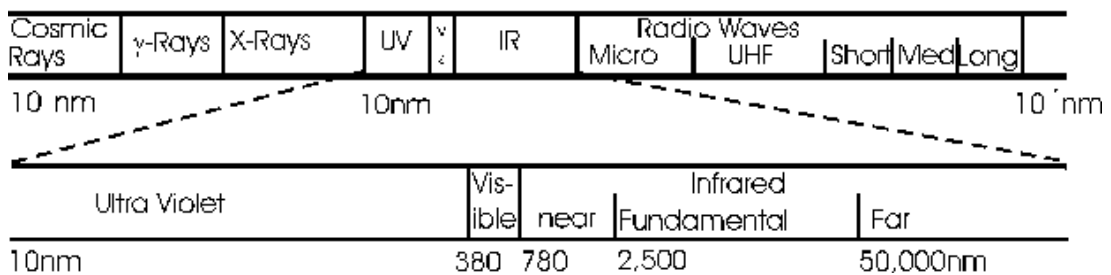


Figure 2.2 – Regions of the near infrared spectrum (Source – IMPublications).

NIR spectrometers are generally composed of a light source, monochromator, sample holder or sample presentation interface and a detector (figure 2.3) and can be used for transmittance or reflectance measurements. Reflectance measurements are widely used with agricultural products and these spectra are generally plots of $\text{Log } 1/R$ (R =Reflectance) against wavelength. A typical near infrared scanning monochromator will yield 700 readings for a sample between 1100 to 2500 nm. The spectra appear as smooth, rolling lines with few well defined features. They consist of many overlapping bands as reflectance spectrum is the summation of the spectra of its major chemical components (Norris, 1989).

Sample preparation and presentation to the NIR instruments varies widely. Dried and finely ground samples are often employed but whole grains or fresh, unground

samples can also be scanned. Instruments can be stationary in a laboratory or mobile, for example NIR mounted on Haldrup harvester for use in plant breeding (Welle *et al.*, 2007), or handheld NIRS for assessment of meat quality (Zamora-Rojas *et al.*, 2012).

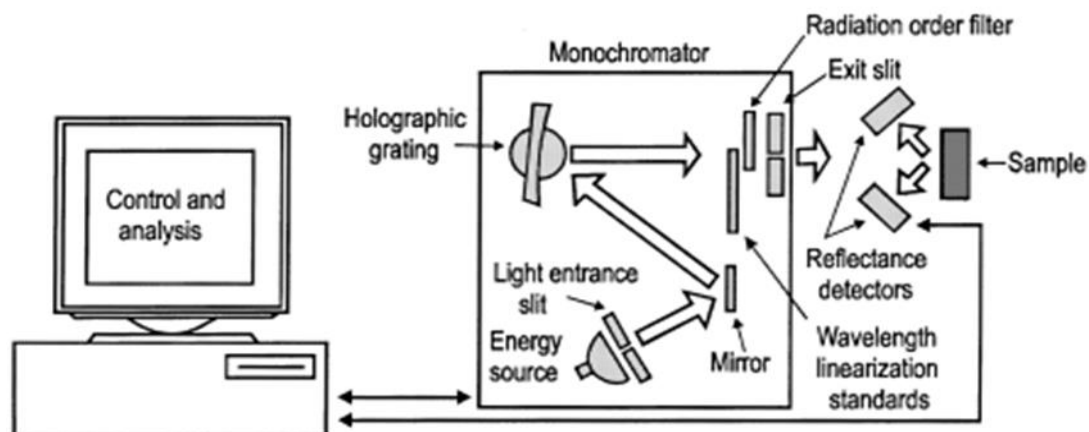


Figure 2.3 – composition of NIR spectrometer. (Source - Foley *et al.*, 1998).

2.6.2 Pre-treatment of spectra

NIR spectra contain information relating to the chemical and physical properties of the sample, variations observed are due to chemical composition and physical properties, such as scatter and path length effects due to particle size. NIR spectra are complex and many pre-treatments and transformations have been proposed and used to improved resolution, reduce effects of interfering factors and aid spectral interpretation. The most common pre-treatments are derivatives, standard normal variate (SNV), detrend and multiplicative scatter correction (MSC).

The use of the first or second derivative (Norris and Williams, 1984) are useful as they can resolve overlapping peaks into component absorptions and to a large extent remove baseline variations. The first derivative is able to separate overlapping peaks and it linearises the background to a constant level. The second derivative zeros the background and transforms peak maxima to peak minima. Band intensities and location of peaks occur in the same regions as the original Log 1/R spectra (Burns and Ciurczak, 1992). An advantage of this technique is that weak peaks not observed in original spectra are revealed. However derivatives are dependent on the operators choice of segment and gap size (Barnes *et al.*, 1989).

MSC is a set dependent transformation used to compensate for amplification (additive) or offset (multiplicative) effects on the spectra data (Gayo and Hale,

2007). Each spectrum is fitted to the average spectrum using least squares. As MSC is set dependent, changes to the raw data must result in recalculation.

Spectral pre-treatments which can be applied to individual spectra have also been proposed as these allow comparisons of samples using difference spectra (Deville and Givens, 1998). Two such transformations are SNV and Detrend (Barnes *et al.*, 1989). The SNV transformation subtracts the mean spectrum from each individual spectrum and is then scaled with the spectrum standard deviation. SNV reduces interferences due to particle size and scatter. Detrend removes linear tilting of the spectra and is able to account for variation in baseline shift. The variation is caused by densely packed and powdered samples. A second order polynomial is fitted to the spectral data and the resulting curve is subtracted from the spectrum (Barnes *et al.*, 1989).

2.6.3 Chemometrics

NIR spectra are highly collinear (Barnes *et al.*, 1989), with adjacent wavelengths having high correlation coefficients. With a large number of data points it is not practical to use univariate analysis. The challenge to the chemist is to extract analytically useful information related to the chemical composition from the reflectance data.

2.6.3.1 Quantitative analysis

Different techniques have been used to develop calibration equations to enable chemical composition to be predicted.

Multiple linear regression relationships between absorptions at a number of wavelengths are assessed against analytical chemistry data. However this technique uses only a small number of wavelengths and suffers from problems with wavelengths selection. Principal component regression overcomes collinearity issues that are common with least squares regression (Naes and Mevik, 2001). A principal component model is created with observed variation in prediction variables, however response variables are not taken into consideration. Partial least squares regression models both predictor and response variables simultaneously and can be used as a prediction tool. Prediction is straightforward once a calibration model is built.

The calibration model is dependent on the dataset used to create the model. Validation must be carried out to ensure the model is representative of the samples, using cross validation and/or an independent validation set. Cross validation removes a set of samples before performing calibrations. The omitted samples are subsequently checked against the model and then put back into the dataset. This process is repeated until all of the samples have been cross validated with the calibration test set. Test set validation uses data from a dataset that does not form part of the validation dataset. Test set validation is favoured due to the fact it uses independent dataset to calculate the validation.

2.6.3.2 Qualitative analysis

The major differences between quantitative and qualitative analysis is that calibration of variables is not necessary for qualitative analysis. Principal component analysis (PCA) is a multivariate chemometric technique, capable of handling large numbers of data points and collinearity. Each sample is represented as a point in a multi-dimensional space. A principal component can be described as a linear regression in multidimensional space (Camo Software[®], 2013). The first principal component (PC) accounts for as much of the variation in the data as possible. PCs are calculated using eigenvectors and eigenvalues, the eigenvector with the greatest eigenvalue is PC1. The second PC is calculated orthogonal to PC1. Next a plane can be built onto the projection when 2 PCs are present (Wold *et al.*, 1987). The distance in space from the plane determines the position of each sample on the score plot. The distance of a sample along the x axis of the score plot is directly related to the distance of the PC on the x axis. Loadings plots are useful for interpreting which interpreting relationships between variables. These are able to show how much each data point (or peaks in the case of NIRS spectra) contribute to each PC (Camo Software[®], 2013). Peaks with highest loadings have the highest contribution on that specific PC. Other examples of qualitative analysis used for analysis of NIR spectrum is cluster analysis, discriminant analysis and canonical variate analysis.

2.6.4 Use of NIR to predict diet quality

NIRS has been used extensively to predict the chemical composition of feeds and forages and have been used to predict animal performance for the production of meat, milk or wool.

2.6.4.1 Prediction of feed chemical composition

Several early studies used NIR to evaluate the moisture content in grains and oilseeds (Hart *et al.*, 1962). This technique was later applied to forages, Norris *et al.*, (1976) used NIR to analyse several components of temperate and tropical forages. Correlation coefficients ranged from 0.80 for dry matter intake (DMI) to 0.99 for crude protein (CP). Park *et al.*, (1983) later used NIR to predict soluble nitrogen and fibrous fractions in crested wheat grass. Aufrere *et al.*, (1996) found predictions of neutral detergent fibre (NDF) and acid detergent fibre (ADF) for feeds for swine and ruminants had R^2 ranged between 0.88 – 0.92 and 0.90 – 0.95 respectively. NIR studies into forage quality allowed an increase in the ease of which forage quality can be determined.

2.6.4.2 Prediction of digestibility

Several studies have looked at the potential of using NIRS to predict measurements made using animals that had been fistulated in the oesophagus or rumen. Holechek *et al.*, (1982) obtained prediction coefficients that ranged from 0.92 to 0.97 for the determination of *in vitro* dry matter digestibility (INVDMD) and CP respectively, whilst Volesky and Coleman (1996) found prediction coefficients between 0.61 and 0.79 for Big Bluestem (*Andropogon gerardii*) and total grass/forbs respectively.

Prediction of digestibility using NIR has also been coupled with the use of markers. Using a faecal carbon isotope ratio ($\delta^{13}\text{C}$) it was found that the legume content of diets could not be reliably predicted from extrusa samples collected from oesophageal fistulated steers, R^2 of 0.13 (Coates *et al.*, 1987). Polyethylene glycol (PEG) has been used to assess digestibility of feed by determination of PEG within the faeces using NIRS using both dried forage (Hassoun *et al.*, 2013) and grazing sheep (Hassoun *et al.*, 2016).

2.6.5 Spectral regions of interest

Regions of the spectra are associated with different compounds. A full summarised list of compounds associated with each wavelength and spectral region can be found in Workman and Weyer (2012). Spectral regions 2308, 2346 and 2308 nm are typically associated with aliphatic C-H absorptions, associated with lipids (Bertrand, 2002; Decruyenaere *et al.*, 2009; Murray, 1987; Wetzels, 1983), these are also repeated in the first overtone region at 1760 and 1725 nm. Two regions of the spectrum are associated with moisture, these can be found at 1940 and 1450 nm (Shenk *et al.*, 1992). Moisture has the ability to interact with neighbouring molecules including starch and protein (Coleman and Murray, 1993). It has been suggested that the spectral region 1900 – 2000 nm can be highly influenced by moisture in silages (Baker *et al.*, 1994). Nitrogen containing compounds, including protein, can be found at 2040, 2080, 2180 and 2220 nm (Bertrand, 2002; Murray, 1987). These regions are also repeated in the first overtone region at 1500 and 1530 nm. Spectral region 2070 and 2110 nm are typically associated with carbohydrates (Bertrand *et al.*, 2002). Spectral regions 2078 – 2100 and 2268 nm have previously been associated with fibre (Decruyenaere *et al.*, 2009) whilst 2260 – 2280 nm has been found to be associated with lignin and cellulose (Coleman and Murray, 1993).

2.6.6 Difference spectra

In vitro rumen digestion studies combined with NIR, using difference spectra, have been used to predict diet quality and monitor the time course of rumen fermentation in cattle (Deville and Givens, 1998). Spectra of samples that were not digested (washed with water only) were subtracted from spectra digested at various incubation times. Deville and Givens (1998) showed that NIRS can be successfully used to predict *in vitro* digestibility of fresh grass, grass silage and maize silage within sheep. Standard normal variate (SNV) and detrend (SDT) data was compared to degradability data. Spectral regions 1620 to 1690 nm and 2170 to 2290 nm were associated with low degradability, with the spectral data corresponding well to the spectra of extracted lignin. Regions 1430 to 1630 nm and 2020 to 2190 nm were associated with high degradability, with values in grasses ranging up to 2230 nm. Givens *et al.*, (1992) noted that SDT spectral regions at 1672 and 2254 nm became

increasingly positive as digestion incubation time increased, these regions are associated with indigestible cell wall material. SDT spectral regions 1498 to 2086 nm became increasingly negative as digestion incubation time increased, and these are associated with glucose and xylose. Whole tract digestibility can be approximated by using difference spectra between feed and faeces. Coleman and Murray (1993) used difference spectra between hay and faeces of steers fed on a diet of hay, and noted spectral regions above and below average digestion; 1450-1620, 2100-2200 nm and 1714, 2256, 2306, 2346, 2382 nm respectively.

2.6.7 Faecal NIR

Faecal NIRS can be said to have three main areas of usage (Dixon and Coates, 2009); estimating the species composition of the diet, estimating the chemical composition of faeces, and estimating animal physiological state.

Faecal NIRS has proved useful to determine physiological states of animals, including age, sex, species, reproductive state and parasite burden (Tolleson *et al.*, 2007; Weidower *et al.*, 2012). Tolleson *et al.*, (2005) was able to determine both sex and species of red and fallow deer, maintained on the same diet. A R^2 value of 0.87 was observed when samples collected over two years were combined. Another study (Walker *et al.*, 2007) found that spectral data was affected by diet, sex, breed and age. Age was shown to have a more profound effect on spectra than sex in Angora goats including both castrated and intact goats.

Chemical composition of faeces and nutritional value of diets have been analysed using faecal NIRS. Several studies have shown the accuracy of faecal NIRS to determine digestibility and diet quality. A study conducted between 2002 and 2003 showed that digestible organic matter (DOM) ranged from 52.4 to 75.8 % in sheep on various diets. Results were compared to laboratory data and R^2 of 0.78 and R^2 of 0.80 were determined for both stepwise regression and partial least squares regression respectively (Tolleson *et al.*, 2007). Decandia *et al.*, (2009) also showed strong R^2 values of 0.90 when determining organic matter digestibility (OMD) of dairy sheep diets. Huntington showed that DMD could be precisely predicted in bulls and steers (Huntington *et al.*, 2011), with no difference shown between the two groups, Lyons and Stuth (1992) previously reported similar results.

2.7 CONCLUSIONS

Currently existing techniques to determine between-animal variation in digestion are laborious, expensive and impractical to characterise large numbers of animals. From this review, it is clear to see that NIRS and LD have huge potential for describing chemical and physical breakdown of feed, and ultimately differences in digestion. Currently the use of NIRS requires large datasets for predictions and calibrations of chemical components so the use of NIRS without calibration will be sought. Next generation sequencing using 16S barcoded techniques shows potential as a rapid technique to assess the microbial communities, with studies suggesting that differences in microbial communities are associated with efficiency of cattle.

CHAPTER 3

GENERAL METHODS

3.1 LASER DIFFRACTION

3.1.1 Preparation of Samples

Faecal grab samples were collected and stored at -20°C until further analysis. Samples stored frozen (-20°C) were defrosted at 4°C for 72 h and hand mixed prior to sampling. Ten grams of faecal sample was weighed into a tea strainer (Le'Xpress, Deluxe Stainless Tea Infuser), with 1.8 mm holes. With the exception of Chapter 7, particles retained in the tea infuser were discarded. The sample was subsequently mixed in 200 ml deionized water (DI) water to give a target obscuration (sample concentration) of between 10 and 20 %, with particles larger than 1.8 mm remaining within the tea infuser. The sample was stirred constantly using a magnetic stirrer and a 40 ml aliquot was taken using a 50 ml pipette (Sparks Lab Supplies, Cat. #DP050), and transferred to a 50 ml barcoded screw top bottle. Samples were stored at 4°C . Screw cap tops were removed and samples placed on the autosampler prior to analysis using Mastersizer 2000G with autosampler (Malvern Instruments, Malvern, UK). The autosampler mixed the sample for 20s to ensure homogeneity and a sub-sample was transferred to the dispersion unit for analysis. A stirrer speed of 800 RPM and pump speed of 2000 RPM were used. Two lasers at 632.8 nm and 470 nm made measurements between 0.01 and 2000.00 μm , in 27 pre-set size categories (Table 3.1). Each sample was measured 3 times, the average of the 3 scans were automatically recorded. Three measurements were taken per sub-sample, over 27 size categories ranging from 0.01 μm to 2000 μm (Table 3.1), the average of the 3 scans was also reported. A self-cleaning cycle was automatically carried out between samples.

Table 3.1 details of the 27 different size categories for LD analysis

Fraction	Size Category (μm)
A	0.01-25.00
B	25.00-50.00
C	50.00-75.00
D	75.00-100.00
E	100.00-150.00
F	150.00-200.00
G	200.00-250.00
H	250.00-300.00
I	300.00-350.00
J	350.00-400.00
K	400.00-450.00
L	450.00-500.00
M	500.00-600.00
N	600.00-700.00
O	700.00-800.00
P	800.00-900.00
Q	900.00-1000.00
R	1000.00-1100.00
S	1100.00-1200.00
T	1200.00-1300.00
U	1300.00-1400.00
V	1400.00-1500.00
W	1500.00-1600.00
X	1600.00-1700.00
Y	1700.00-1800.00
Z	1800.00-1900.00
AA	1900.00-2000.00

3.1.2 Analysis of LD Results

Averaged scans were used in principal component analysis (PCA). PCA was performed in Unscrambler X (Camo Software, Norway) using the non-linear iterative partial least squared (NIPALS) algorithm, with all data being mean centred and all values having a weighting of 1.00. Statistical analysis of LD results is described in subsequent chapters.

3.2 NIRS

3.2.1 Sample Preparation for NIRS

Faecal grab samples were collected and stored at -20°C until further analysis. Defrosted samples were dried (temperature and times given in subsequent chapters; Sections 5.2.4, 5.2.4 and 7.2.4. Residues from in vitro digestion (used in Chapter 5) were dried at 40°C for 72 h. Samples were subsequently milled through a 0.75 mm sieve (Cyclotec Mill). Samples were further dried (as above, temperature and times given in subsequent chapters) before being scanned at 2 nm intervals between 400 – 2500 nm using NIRSystems 6500 (Chapter 5 and 7)/(FOSS, Warrington, UK) or 1100 – 2500 nm using NIRSystems 5000 monochromator (Chapter 4 and 6). Samples were scanned in duplicate (with the exception of Chapter 7) using WinISI V1.02a Software (FOSS, Warrington, UK) using NIRSystems 6500 and WinISI V1.50a (FOSS, Warrington, UK) using NIRSystems 5000. With the exception of samples carried out in Chapter 7, samples were scanned, repacked and rescanned. Spectra were stored as Log 1/R (R = reflectance). Spectra scanned using NIRSystems 6500 were trimmed and only 1100 to 2500 nm used.

3.2.2 NIRS: PCA analysis

Spectra were transformed using standard normal variate (SNV) and de-trending (DT) (Barnes *et al.*, 1989), when combined it will be referred to as SDT. PCA was performed (Unscrambler) using the Singular Value Decomposition (SVD) algorithm, with data being mean centred and all values having a weighting of 1.00. PC scores

plots and associated loadings were examined. Statistical analysis of PC scores is described in subsequent chapters.

3.2.3 NIRS: Difference spectra

Difference spectra was calculated for use in Chapter 5 and Chapter 7. SDT spectra from 0 hr residues (Chapter 5) were subtracted from the spectra of residues from different incubation time points. SDT spectra from median feed efficiency value (Chapter 7; FCR or RFI) were subtracted from spectra for each steer.

3.3 DNA EXTRACTION AND 16S NEXT GENERATION SEQUENCING

3.3.1 DNA Extractions

Lysis buffer (quantity described in Section 4.4.3.2 or 7.2.6.1) was added to each rumen sample before being homogenised for 3 minutes (3 x 60 second intervals at 6.0 M/s; FastPrep, MP Biomedicals LLC, California, USA) and incubated at 70°C for 15 minutes. Samples were centrifuged at 10°C for 5 minutes at 16,000 x g (all subsequent centrifugation steps were carried out at 16,000 x g unless otherwise stated) and the supernatant retained. Fresh lysis buffer (100 µl) was added and the homogenization, incubation and centrifugation steps were repeated and remaining supernatant retained. Ammonium acetate (10 M) was added to the supernatant to give a final concentration of 2.3 M ammonium acetate. Samples were incubated on ice for 5 min before being centrifuged at 4°C for 10 min. The supernatant was removed, whilst avoiding disturbance of the pellet: an equal volume of isopropanol was added and samples incubated at -20°C for at least 2 h. Samples were centrifuged at 4°C for 15 min. The supernatant was removed, samples centrifuged for a further 1 min and remaining supernatant removed. Eight hundred microliters of 70 % ethanol was added to each tube and samples centrifuged for 5 min. The supernatant was removed, samples were centrifuged for a further 30 s and remaining supernatant removed. Samples were left to air dry before 100 µl of TE (10 mM Tris Cl pH 8.0, 1 mM EDTA) was added to resuspend the pellet. Samples were incubated at -20°C overnight before 5 µl of DNase-free RNase (4 mg/ml; Promega cat. # A7973) was

added. Samples were incubated for 15 min at 37°C. Fifteen microliters of proteinase K and 200 µl Buffer AL (from QIAamp DNA Stool Mini Kit) were added to the tube and mixed thoroughly by inverting several times. Samples were then incubated for 10 min at 70°C. Two hundred milliliters of 100 % ethanol was added, tubes were inverted to mix, and contents transferred to a QIAamp column. Columns were centrifuged at 16,000 x g for 1 min. The flow through was discarded and 500 ml Buffer AW1 (Qiagen) was added. Samples were centrifuged for 1 min, and flow through discarded. Buffer AW2 (500 ml) was added to the column and samples centrifuged for 1 min. The flow through was discarded and samples centrifuged for 1 min to ensure no Buffer AW2 remains. The column was placed in a clean Eppendorf and 50 ml Buffer AE added. Columns were incubated for 2 mins at room temperature and centrifuged for 1 min to elute the DNA. Extracted DNA samples were assessed for quality on a 1% agarose gel and for quantity using the NanoDrop 1000.

3.3.2 Library Preparation

Extracted DNA, diluted to 10 ng/µl, was added as template to amplify the V4 region of the 16S rRNA gene. Amplification was performed using 10 µl Reaction Buffer, 1 µl dNTP, 2.5 µl Forward Primer, 2.5 µl Reverse Primer (details of primers can be found in “16S Primer Details” in Appendix; Caporaso *et al.*, 2012), 0.5 µl Taq Polymerase, 10 µl High GC Content Enhancer, 21.5 µl Molecular Grade Water and 2 µl DNA. For each positive reaction, a negative control was also amplified, using molecular grade water in the place of DNA and half the quantities of the positive reactions. This was carried out to check for contamination of each primer set. PCR reactions were carried out as follows: 94°C for 2 min, 94°C for 10 s, followed by 30 cycles of 94°C for 10 s, 68°C for 20 s, 72°C for 1 min, and a final hold at 4°C step. PCR amplicons were purified immediately using PCR Purification Kit (Qiagen; Hilden, Germany) as per the manufacturer’s guidelines. The quality of the amplified DNA was checked using gel electrophoresis and quantified using Nanodrop 1000, before being stored at -80°C. Samples were pooled to give an equimolar solution. Pooled libraries were gel purified (Qiagen) and checked for size with a DNA100 chip on an Aligent 2100 Bioanalyser (Aligent Technologies; CA, USA). The pooled

libraries were then quantified by qPCR on an ABI7500 FAST real time qPCR machine (Life Technologies, CA, USA) using the Universal qPCR master mix from the Kapa library quantification kit for Illumina platforms (Kapa Biosystems; MA, USA) according to the manufacturer's instructions. They were then diluted to 2 nM, denatured with sodium hydroxide, spiked with denatured PhiX V3 library (Illumina CA, USA) (6:4 volume:volume, pooled libraries:PhiX V3 library) and loaded into a 300 cycle V2 MiSeq (Illumina) reagent cartridge which was run on an Illumina Miseq according to the manufacturer's instructions.

3.3.3 Analysis of Sequence Data***

Data was analysed using QIIME software (Quantitative Insights Into Microbial Ecology; Caporaso *et al.*, 2010b; qiime.org). Quality control (QC) was performed to remove low quality bases before reads were merged to create a single sequence for each amplicon. Reads were split by individual sample indexes and converted to FASTA format. Chimeric OTUs were identified using usearch61 (Edgar, 2010) against the GreenGenes database (<http://greengenes.lbl.gov>; DeSantis *et al.*, 2006). Chimeric sequences were subsequently removed before subsequent analysis. OTUs were assembled using the open reference method (a combination of reference based and de novo methodologies) using QIIME. A 97% similarity was used to cluster reads into individual OTUs. Taxonomy was assigned to these OTUs using the RDP classifier (v2.2; Wang *et al.*, 2007) and the GreenGenes database. The UClust aligner Edgar 2010 was used for the reference based part.

Alpha diversity indexes were estimated based on repeated multiple rarefactions (10) with a sequence number cut off equal to the smallest number of reads in any one of the samples. Observed species, PD (phylogenetic diversity) whole tree and Shannon diversity indexes were calculated (QIIME). Beta diversity estimates were generated, as before with a sequence cut off equal to the smallest number of reads in any one of the samples. UniFrac Distances (Lozupone and Knight, 2005) were calculated for all samples and both weighted and unweighted PCA was carried out.

Filtered relative abundances (Chapter 4 and Chapter 7) were exported to Unscrambler and PCA calculated using the NIPALS algorithm, data was mean centred with all samples and variables having equal weighting. Differences between

treatment groups were assessed by ANOVA using STAMP software (v. 2.1.3) and multiple test correction using the Benjamini-Hochberg false discovery rate. Unclassified reads and Cyanobacteria (at any taxonomic level) were removed prior to running the ANOVA.

*** All bioinformatics, in this Chapter and Chapter 6, with the exception of analysis using GenStat, Unscrambler X or STAMP, was carried out in-house by Paul Cormican, Teagasc Bioinformatician.

CHAPTER 4

METHOD DEVELOPMENT

4.1 INTRODUCTION

The aim of the series of studies reported in this chapter was to develop and refine rapid techniques to characterise between-animal variation in digestion. The focus of the work is on rapid, high-throughput and low-cost methods since application in breeding programmes would require measurements from large numbers of animals. There are two main components to this study; (i) the development of laser diffraction (LD) and near infrared spectroscopy (NIRS) as rapid screening techniques to determine between-animal variation in digestion and (ii) the development of a rapid approach to characterise the rumen microbiome. Techniques developed and refined through these studies will be used in the studies reported in subsequent chapters.

4.2 DEVELOPMENT OF LASER DIFFRACTION METHOD

4.2.1 Introduction

Particle size analysis is able to assess animal effects on digestion in situations where the diet is constant. A higher proportion of smaller particles in faeces may indicate longer retention time within the rumen, or increased digestion for some other reason (e.g. an increased intensity of rumen fermentation). Traditional techniques to assess particle size distribution, such as wet and dry sieving, are time consuming and laborious. Consequently, they are impractical for use with large numbers of animals. A further limitation is that these techniques assess particle size based on the smallest dimension able to pass through the sieve (Schadt *et al.*, 2012) meaning that assessment of elongated particles may be misleading.

Faecal particle size distribution is dependent on both feed and attributes of the feed e.g. diet content and particle size. Smaller feed particle sizes provide greater opportunity for digestion as a consequence of the greater surface area/volume ratio and this may have consequences for faecal particle size distribution. Maulfair *et al.*, (2011) reported that DM digestibility increased with decreasing particle size.

Olaisen *et al.*, (2001) demonstrated that laser diffraction could be used as a rapid technique to assess particle size distribution of duodenal digesta samples, with no difference in median particle size when laser diffraction was compared to wet sieving techniques ($P = 0.98$). Okine and Mathison (1991) reported little difference in

particle size distributions measured using wet sieving of duodenal and faecal samples.

4.2.1.2 Laser diffraction as a particle sizing technique

Laser diffraction is a rapid technique used to assess particle size distributions of both wet and dry samples (with the aid of a dispersal agent) measured for materials ranging from 0.01 μm to 2000 μm . The laser diffraction (LD) technique is based on light scattering by particles that pass by a He-Ne (Helium-Neon) laser beam. The angle at which the light is scattered depends on the size of the particles: smaller particles give a wider and larger light scatter. The LD technique has been compared to wet sieving. Particle size distribution of duodenal samples from 3 lactating dairy cows were assessed using both techniques (Olaisen *et al.*, 2001) and gave comparable results.

4.2.2 Aims

The overall aims of these studies was to (a) provide a preliminary assessment of the suitability of LD a rapid technique to determine differences in faecal particle sizes and (b) to set up a standard operating procedure for future use.

4.2.3 Proof of principle

4.2.3.1 Materials and methods

4.2.3.1.1 Animal study

Faecal grab samples were collected from 5 mature multiparous Simmental cows at Teagasc Animal and Grassland Research and Innovation Centre, Grange, Dunsany, Co. Meath, Ireland during January 2013. Samples were stored at -20°C prior to subsequent analysis.

4.2.3.1.2 Laser Diffraction

Samples were defrosted at 4°C for 72 h, and mixed by hand within each sample pot. Samples of faeces were diluted in deionised (DI) water to give a target obscuration (sample concentration) of between 10 and 20 %. The LD instrument could not measure particles larger than 2 mm, therefore a pre-sieving step was carried out. Faecal sample (10 g) was placed into a stainless steel tea infuser (Le’Xpress, Deluxe Stainless Tea Infuser) and agitated in 200 ml deionised water so that faecal matter smaller than 1.8 mm washed out. This ratio of sample to water gave sufficient dilution to achieve the target obscuration level. Sample retained in the tea infuser was discarded. The sample was mixed with a magnetic stirrer for 1 min and a 40 ml aliquot taken by pouring. Diluted samples were scanned on the Mastersizer Hydro 2000G with Autosampler (Malvern Instruments, Malvern, UK) as described in Section 3.1.1.

4.2.3.1.3 Statistical Analysis

Distribution of the 27 particle size categories, and subsequently principal component (PC) scores (PC-1 and PC-2), was assessed using a Shapiro-Wilk test (Genstat v14; VSNi, Hemel Hempstead, UK). Data was not transformed for further analysis. Data was exported to Unscrambler (V10.3; Camo Software, Oslo, Norway) and principal component analysis (PCA) was performed using each of the 3 individual scans for each animal. PCA was carried out using the NIPALS algorithm, with data being mean centred and all values having a weighting of 1.00.

4.2.3.2 Results

Raw data were normally distributed, with the exception of size categories 1800.00 – 1900.00 µm and 1900.00 to 2000.00 µm; PC scores (PC-1 and PC-2) were also found to be normally distributed. Grouping by faecal sample (animal) was observed in the PCA plot (Figure 4.1). However, there was also variation between scans for the same sample. Variation between-animals was observed in the PC-1 direction, with ‘animal’ having a highly significant effect on PC-1 ($P < 0.001$), but not PC-2 ($P > 0.05$). In the PCA scores plot (Figure 4.1), a large amount of variation was observed for animal 162 compared to 1675 therefore the distributions plots for these

were examined. Greatest variation can be seen in the smallest fractions in Figure 4.2a and Figure 4.2b, in which distribution of all samples were assessed.

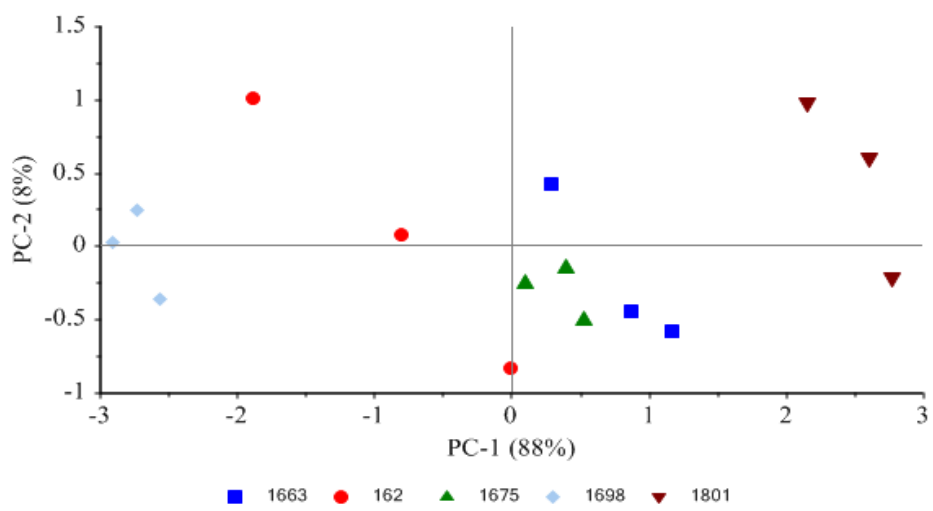


Figure 4.1 – PCA scores plot of the LD data showing the three scans from the 5 faecal samples. Samples are colour coded by animal.

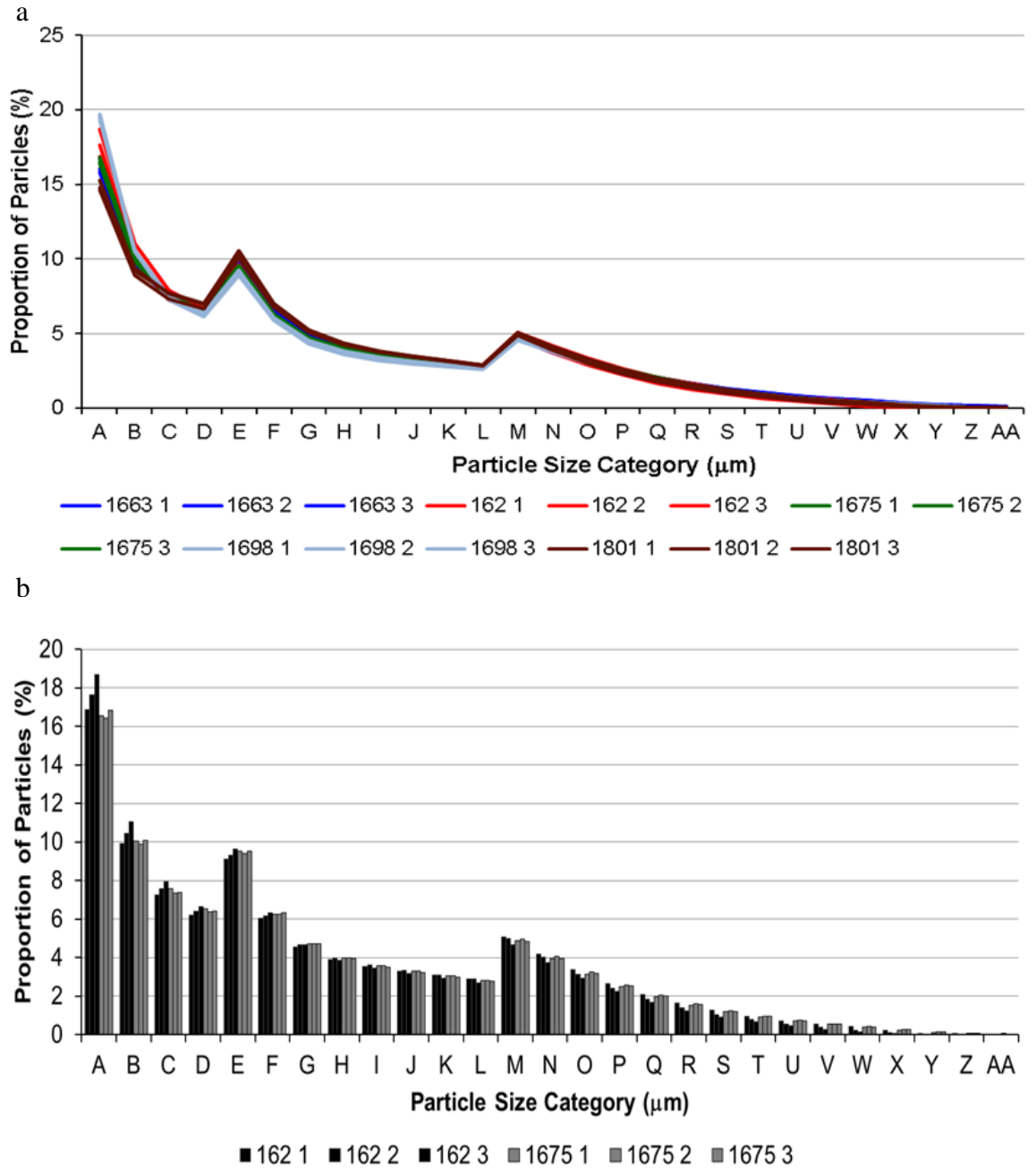


Figure 4.2 – (a) Particle size distribution plot from the proof of principle study (3 scans for each of 5 samples). 1, 2 and 3 after each sample number represents each of the individual scans from the LD instrument. (b) Distribution plot of samples 162 and 1675. Note – the particle size ranges are not equal across categories (A - AA).

4.2.3.3 Conclusion

LD is able to distinguish differences in faecal particle size from different cattle. However variation was observed between scans. This will be investigated further below.

4.2.4 Examination of variability between repeated sub-samples

The proof of principle study showed that variation existed between the 3 scans taken for each sample so a further study was carried out to assess sources of variation in measurements made with the same sample. This was achieved using repeated sampling from the same faecal grab sample (sub-sample) and repeated sampling from each of the sub-samples after preparation (aliquot). The aim of this study was to assess the repeatability of sampling both between sub-samples and aliquots.

4.2.4.1 *Materials and methods*

4.2.4.1.1 *Animal study and LD scans*

Cattle were fed on a constant diet of grass silage (GS; n=2), or a fattening diet (FD; n=1) consisting of 10 kg meal (86 % barley, 6 % soyabean meal, 6 % molasses, 2 % minerals) and *ad libitum* first cut grass silage, dietary information can be found in McGee and Earley (2013). Faecal grab samples from three mature multiparous cows were collected at Teagasc Grange (1663 (FD), 1698 (GS), 1801(GS)) during March 2013, before being stored at -20°C. Samples were defrosted, mixed and 3 sub-samples were taken for each of the samples and processed as described in Section 3.2.3.1.2. Two aliquots were then taken from each of the sub-samples. This gave a total of 3 sub-samples and 6 aliquots from each faecal sample. A summary of this process is shown in Figure 4.3. All statistical analysis was carried out using the average of 3 individual scans for sub-samples and aliquots.

4.2.4.1.2 *Statistical Analysis on LD Scans*

Distribution of the LD scans, derived PC scores and raw data were assessed for normality as described in Section 4.2.3.1.3. A general ANOVA was performed on LD scans for each of the 27 pre-set particle size categories; initially using ‘animal’ as factor (to assess the repeatability of sub-samples) and subsequently using ‘sub-sample’ as factor (to assess the repeatability of aliquots). When ANOVA showed significant effects ($P < 0.05$), the AMCOMPARISON procedure in GenStat was used to compare individual values for sub-samples and aliquots. PCA was carried out as

described in Section 3.1.2. One way ANOVA was performed on PC scores (PC-1 and PC-2), firstly using ‘animal’ as a factor and subsequently using ‘sub-sample’ as a factor. When ANOVA showed significant effects ($P < 0.05$), AMCOMPARISON (GenStat) was used to compare values for sub-samples and aliquots (analysis carried out on 5 size categories which were found to be significant when assessed by ANOVA).

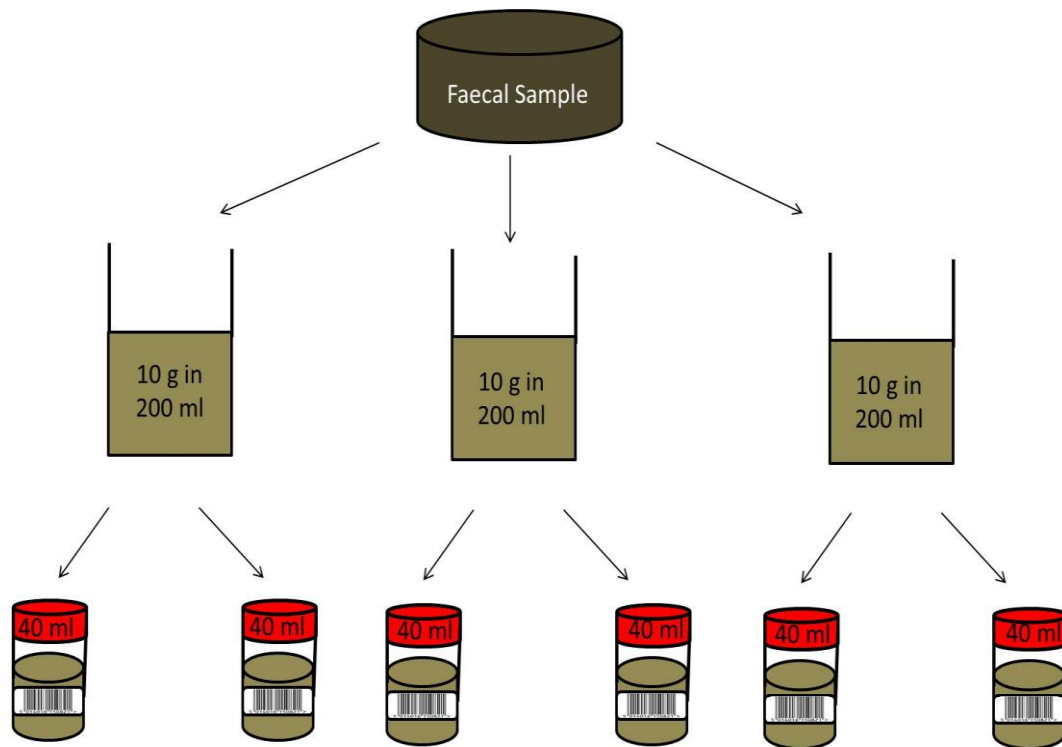


Figure 4.3 – Schematic representation of sample preparation technique for repeatability of samples and sub-samples. Each faecal sample had 3 sub-samples ($n=3$) and 2 aliquots ($n=6$ for each faecal sample).

4.2.4.2 Results

4.2.4.2.1 LD scans

LD scans were not normally distributed for fractions G – M, S – U and W – AA. Fractions A – F, N – R and V were found to be normally distributed

For the smallest particle size categories (A - C), statistically significant differences were found between-animals, as well as between sub-samples from the same

animals. In addition, statistically significant differences were found between sub-samples from the same animals for particle size categories G and N – O, and between aliquots taken from the same sub-sample for particle size categories O and P. Significantly different samples at the sub-sample level were assessed using the AMCOMPARISON procedure in GenStat (Table 4.1). Although shown to be significant overall using ANOVA, samples associated with particle size category A showed no significant differences when individual means were compared (AMCOMPARISON). Animal 1663 showed no significant differences across any of the size categories analysed. This sample also showed less variation in PCA scores plot (Figure 4.4). Sample 1698 showed significant differences between sub-samples across 4 of the 5 particle size categories used for this analysis, whilst 1801 showed significant differences across 3 particle size categories (Table 4.2).

Table 4.1 – Statistical significance of particle size distributions estimated on 3 sub-samples taken from 3 faecal samples. Sub-samples with different letters within column are significantly different at $P < 0.05$ within animal.

Cow # / sub-sample	Particle size fraction (μm)				
	0.01 – 25.00	25.00 – 50.00	50.00 – 75.00	700.00- 800.00	800.00 – 900.00
1663 A	cd	def	bc	bc	bcd
1663 B	bc	bcd	abc	cd	cd
1663 C	cd	cd	bcd	bc	bc
1698 A	cd	cde	bc	c	cd
1698 B	d	ef	cd	ab	ab
1698 C	d	f	d	a	a
1801 A	a	ab	ab	c	bcd
1801 B	abc	ac	bc	bc	bc
1801 C	ab	a	a	d	d

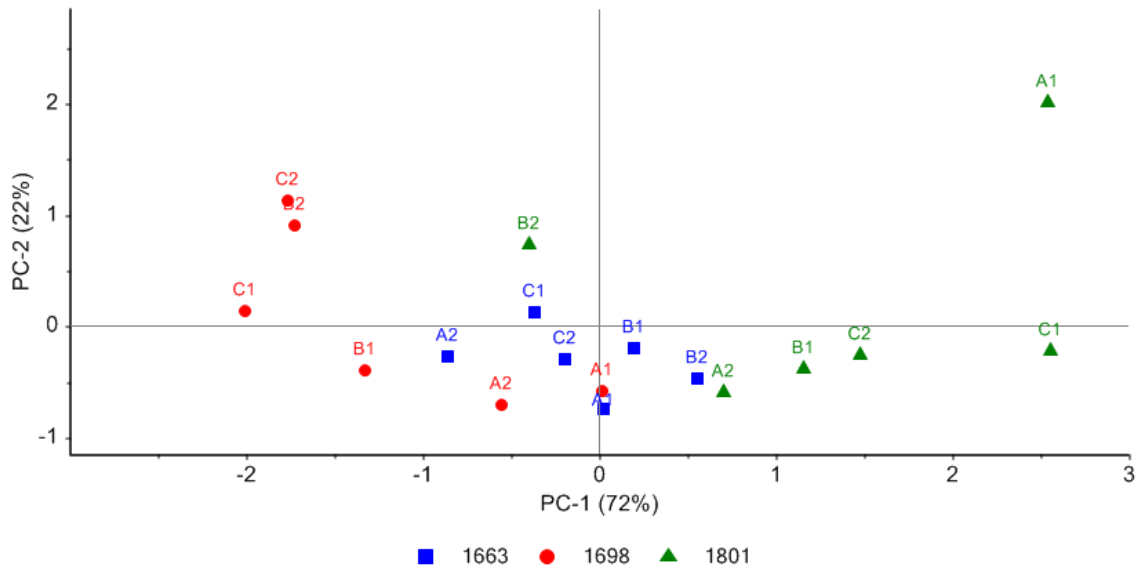


Figure 4.4 – PCA score plot of 3 faecal samples to assess repeatability of sub-samples and aliquots. Samples are labelled in pairs to represent two aliquots taken from the same sub-samples (A1/A2, B1/B2, C1/C2), with colours representing faecal samples from each of the animals.

4.2.4.2.2 PCA

PC-1 values were found to be normally distributed, but not values for PC-2. PCA scores plot showed separation when assessed by animal (Figure 4.4). PC scores (PC-1 and PC-2) were assessed using one way ANOVA at both animal and sub-sample level. Animal was found to have a highly significant effect on PC-1 ($P < .001$), but not on PC-2 ($P = 0.469$). Sub-sample also had a significant effect on PC-1 ($P = 0.004$) but not on PC-2 ($P = 0.657$). As sub-sample had a significant effect on PC-1, AMCOMPARISON was carried out on PC-1 scores (Table 4.2). As noted with the LD scans, Animal 1663 showed no significant differences for PC-1 between the 3 sub-samples (A, B, C), whilst animals 1698 and 1801 showed significant differences.

Table 4.2 – Comparison of PC-1 values for 3 sub-samples of faecal samples from each of the 3 animals (values with different letters are significantly different at $P < 0.05$).

Cow # / sub-sample	PC-1
1663 A	abc
1663 B	cd
1663 C	bc
1698 A	bc
1698 B	ab
1698 C	a
1801 A	de
1801 B	cd
1801 C	e

4.2.4.3 Conclusion

This study showed that variation existed between sub-samples and aliquots, possibly due to fractionation of samples during the preparation stage. This will be investigated further below.

4.2.5 Preparation technique

As described in the section above, variation was observed within sub-samples and within aliquots. The preparation of samples will be examined in this section.

4.2.5.1 Materials and methods

4.2.5.1.1 Animal study and LD scans

Faecal grab samples were collected from 10 adult Simmental cows at Teagasc Grange during May 2013 and stored at -20°C prior to analysis. Cattle were fed on a high concentrate diet consisting of 86 % barley, 6 % soyabean meal, 6 % molasses and 2 % vitamins and minerals. Samples were defrosted and a 200 ml sample

prepared as described in Section 3.1.1. Retained particles in the tea infuser were discarded. Aliquots (40 ml) were taken using either the pouring technique (see Section 4.2.3.1.3) or pipetting technique using 50 ml serological pipette (Sparks Lab Supplies, Cat. #DP050). This procedure was repeated to give 2 aliquots from the pouring technique and two from the pipetting technique. All samples were taken from the same initial dilution of 10 g faecal matter in 200 ml DI water. Samples were scanned using Mastersizer and data analysis carried out on the mean of 3 scans.

4.2.5.1.2 Statistical analysis on LD scans

Assessment of distribution was carried out as described in Section 4.2.3.1.3. A one way ANOVA was carried out on individual LD fractions using ‘animal’ as factor. A second ANOVA was carried out using ‘technique’ as factor and ‘animal’ as block. Regression was carried out (GenStat) on preparation technique within fractions that were significantly different after ANOVA (using ‘technique’ as a factor). A simple linear regression was carried out using ‘pipetting technique’ as response variable and ‘pouring technique’ as explanatory variable for each significantly different particle size fraction.

4.2.5.1.3 Analysis using PCA

PCA was carried out as described in Section 3.1.2. One way ANOVA was carried out on PC scores using ‘animal’ as a factor. One way ANOVA was repeated using ‘preparation technique’ as a factor and ‘animal’ as a block. Distribution of the individual LD particle size fractions were assessed using a Shapiro-Wilk test (GenStat).

4.2.5.2 Results

4.2.5.2.1 LD scans

Shapiro-Wilk test showed that data was not normally distributed for fractions A – B, J – L and X – AA. All fractions were found to be significantly different ($P < 0.001$) when ANOVA was performed using ‘animal’ as a factor. When assessed by

‘preparation technique’ (using ‘animal’ as block) particle size fractions A – F and M – V were significantly different ($P < 0.005$) with particle size fractions B – E and N – P being highly significant ($P < 0.001$). Significantly different fractions were assessed using regression (pipette vs. pouring technique) with R^2 values ranging from 41.9 (particle size fraction V) to 87.6 (particle size fraction A) when assessed using regression. A distribution plot of the average values for the pipetting or pouring techniques for each of the 27 particle size fractions is shown in Figure 4.5. For the pouring technique, a higher proportion of particles were found in particle size fractions A – G. Whereas the highest proportion of particles were found in particle size fractions L - V for the pipetting technique. Both particle size fractions A – G and L - V showed significant differences when compared using ANOVA for ‘preparation technique’.

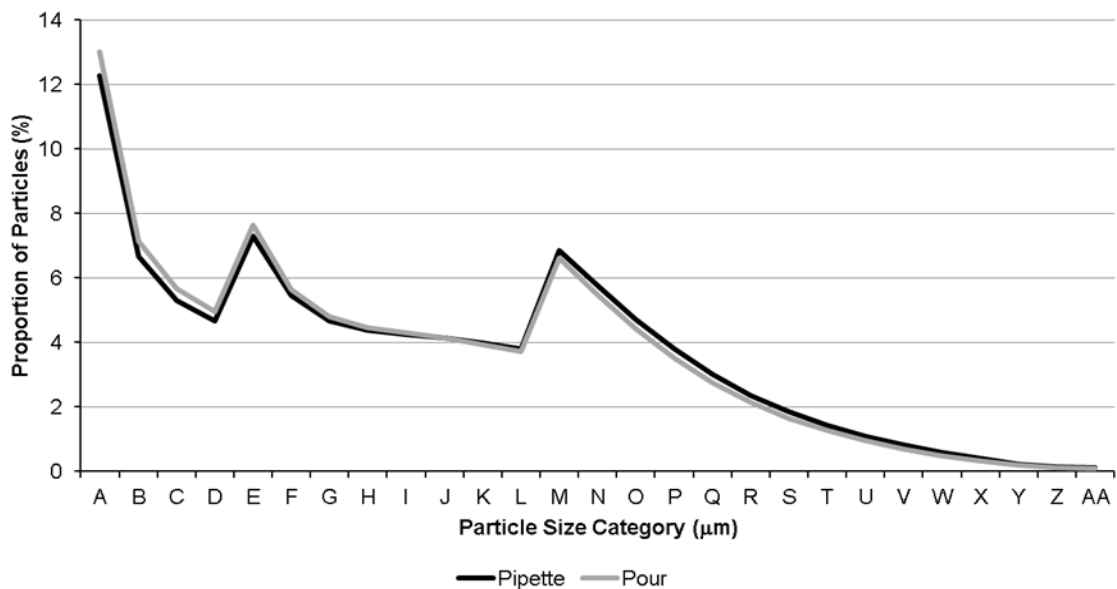


Figure 4.5 – Average particle size distributions for samples prepared using pipetting or pouring technique. See Table 3.1 for particle size categories.

3.2.5.2.2 PCA

Values for both PC-1 and PC-2 scores were found to be normally distributed. PCA plots showed clustering by animal, but grouping by preparation technique was seen within each cluster (Figure 4.6). Effects of ‘animal’ were highly significant for both PC-1 and PC-2 ($P < .001$). Preparation technique had a significant effect on PC-1 ($P < 0.001$) and on PC-2 ($P = 0.019$). Variation between preparation techniques was observed within the PC-1 direction.

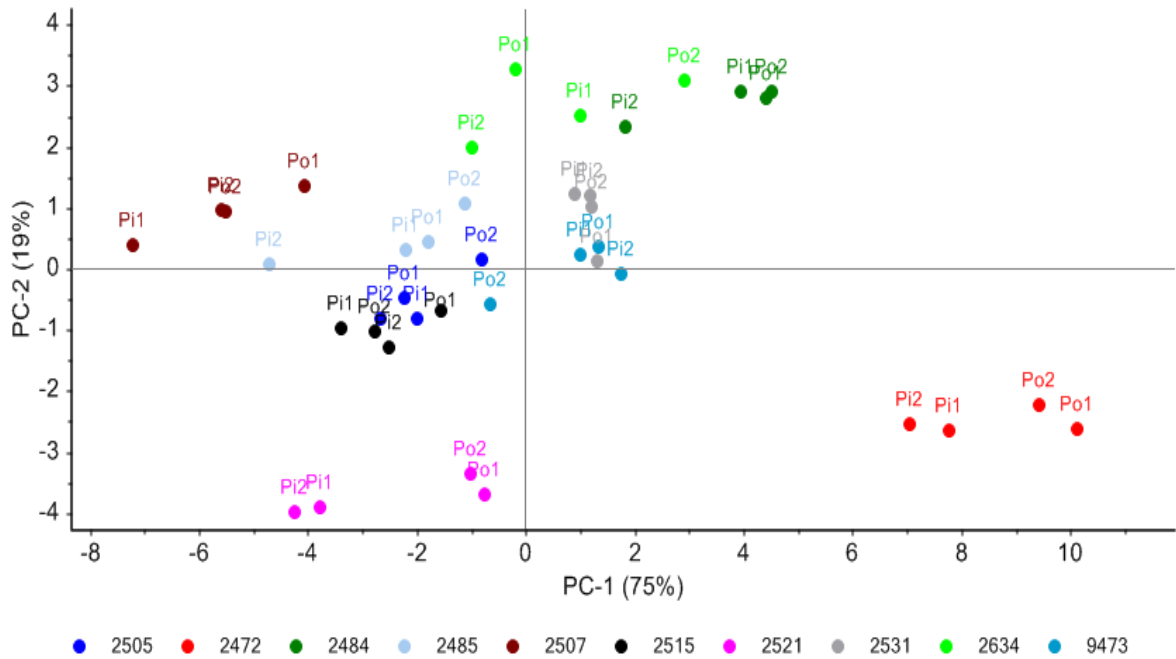


Figure 4.6 – PCA plot showing all 10 samples used to assess preparation technique. Samples are colour coded by animal and labelled by preparation technique (pipetting (Pi) or pouring (Po) and 1 or 2 for first and second sample taken respectively).

4.2.6 Discussion

During the three studies described, samples were mixed thoroughly to ensure a representative sample was used, however variation between both sub-samples and aliquots was observed in addition to the variation between samples, and between pipetting and pouring techniques. But it must be remembered that samples will not be completely homogenous due to the nature of the samples which include a range of particle sizes. The LD technique is traditionally used in soil science for determining differences in particle sizes of soils, sands and sediments. The influence of particle shape on LD measurements in sand has been previously shown (Polakowski *et al.*, 2014). Faeces consist of undigested matter which are typically long and spherical in shape, therefore differences in shape of faecal particles are likely contributing to some of the variation observed in studies described in this chapter. One further constraint is the LD instrument used in these studies is limited to a maximum particle size of 2 mm. Whilst Poppi *et al.*, (1980) suggested that particles no larger than 1.8

mm escape the rumen, Cardoza (1985) and Welch (1986) noted particles as large as 3.6 mm and 5 mm respectively have been reported in the faeces of cattle. The potential of missed information between 2 mm and 5 mm must not be ignored.

The proof of principle study showed variation between instrument scans, which led to investigation of the repeatability of samples. The averages of the three scans were used in subsequent studies to overcome some of the issues associated with variability between scans. The repeatability was assessed within faecal samples, therefore the fact that the samples came from animals offered different diets was not relevant to this part of the analysis. The Proof of Principle and Repeatability studies suggested problems with fractionation of particles associated with sample preparation technique. Hence a third study was carried out to assess differences in preparation technique using either a 'pouring' or a 'pipetting' technique. For the pouring technique, a higher proportion of particles were found in fractions A – G, whereas the highest proportion of particles were found in fractions L – V. Differences observed between these categories are likely to be due to settling of larger particles when the pouring technique was used. When this process was used, diluted faecal samples were removed from the magnetic stirrer in order to pour off the aliquot and this allowed some settling of particles. However samples were stirred continuously when the aliquot was taken using a pipette thus overcoming issues associated with buoyancy and sedimentation of particles and giving a more representative sample. As a consequence of these observations, the pipette technique was chosen as the standard sample preparation technique for use in subsequent work, however it must be noted that variation was also noted between first and second aliquots taken using either the pipette or pouring technique.

Despite difficulties associated with repeatability of results between repeated subsampling, differences between-animals were observed, confirming the potential use of LD as a rapid technique to assess differences in faecal particle size distribution between-animals. Ollaisen *et al.*, (2001) showed that LD could be used to assess particle sizes of duodenal digesta samples, with similar results to wet sieving ($P = 0.98$). Little particle breakdown occurs after feed has left the rumen (Okine and Mathison, 1991) so it is not surprising that our results with faeces are consistent with Ollaisen's results using duodenal digesta. However no phenotypic data, such as intake

and feed efficiency, was available for the current study and therefore no link between digestion processes and feed efficiency could be determined. This aspect will be further investigated in Chapter 7 will assess the use of LD to explore differences between-animals differing in feed efficiency, using both residual feed intake (RFI) and feed conversion ratio (FCR) values.

4.3 THE USE OF LASER DIFFRACTION AND FAECAL NIRS TO DETECT PHYSICAL AND CHEMICAL DIFFERENCES OF CATTLE OFFERED TWO DIFFERENT DIETS

4.3.1 Introduction

The LD method has shown potential as a rapid technique to assess faecal particle size distribution. LD was successfully used to identify differences in faecal particle size distribution between 10 cattle.

Faecal NIRS is a rapid technique that has been used to assess chemical composition of faeces through prediction of chemical constituents (Dixon and Coates, 2009). However this approach requires large data sets in order to develop calibrations for such dietary attributions.

4.3.2 Aims

The aim was to use NIRS, without calibrations, to examine between-animal variation in the NIR spectra of faeces, as well as between-diet variation

4.3.3 Materials and Methods

4.3.3.1 Sample Collection

Faecal grab samples were collected from 18 mature multiparous Simmental cows at Teagasc Grange on two occasions during December 2012, six days apart. Cows were fed on either a grass silage based diet (n=8) or total mixed ration (TMR; n=10). Full dietary details can be found in McGee & Earley (2013), in brief, the TMR diet consisted of 70 % grass silage (~65 % DMD) and 30% straw (~45 % DMD) on a dry

matter basis. All cows were also given 60 g of dry cow mineral daily (Calcium, 46 g/kg; Sodium, 200g/kg; Magnesium, 165 g/kg; Copper, 90mg/kg; I, 300mg/kg; Manganese, 6670 mg/kg; Zinc, 5200 mg/kg; vitamin A, 600,000 IU/kg; vitamin D3, 100,000 IU/kg; and vitamin E, 5000 IU/kg). TMR and grass silage diets were prepared using silage from the same silage pit. A sample of feed was collected on the first day of sampling and stored at -20°C. Faecal samples were stored at -20°C, and defrosted at 4°C for 96 h prior to analysis by LD and NIRS

4.3.3.2 Laser Diffraction

LD was carried out as described in Section 3.1.1. and analysis using PCA as described in Section 3.1.2 to assess all samples to assess differences between sampling times and diets.

4.3.3.3 NIRS

The remaining sample was dried at 40°C for 80 h, before being ground through a 0.75 mm sieve (Tecator 1093 Cyclotec Mill, FOSS, Warrington, UK). Ground samples were scanned at 2 nm as described in Section 3.2.1. Data was pretreated using SDT. PCA was carried out on transformed NIR scans as described in Section 3.2.2, with all samples, and subsequently with spectra from the two days averaged. Loadings associated with each PC were used to assess spectral regions of interest.

4.3.3.4 Statistical Analysis

The effects of diet and sampling day, on PC score (PC-1 or PC-2) were assessed for both laser diffraction and NIRS using one way ANOVA in GenStat with ‘diet’ and ‘sampling day’ used as factors.

4.3.4 Results

4.3.4.1 Laser Diffraction

PCA of the LD data for the 36 samples (18 animals over 2 days) was performed. PC-1 accounted for 89 % of the variation and PC-2 accounted for 7 % of the variation. Figure 4.7a shows the first and second PC’s and shows that there is an overlap

between the two sample days but no clear difference was seen for either PC-1 or PC-2 ($P = 0.972$ and $P = 0.742$ respectively). LD data from the two sample days were averaged and PC's derived. Diet (TMR or Silage) had no effect on PC-1 ($P = 0.985$) or PC-2 ($P = 0.881$) and therefore no effect of PCA plot overall (Figure 4.7b). LD results were not influenced by either sample day or by diet.

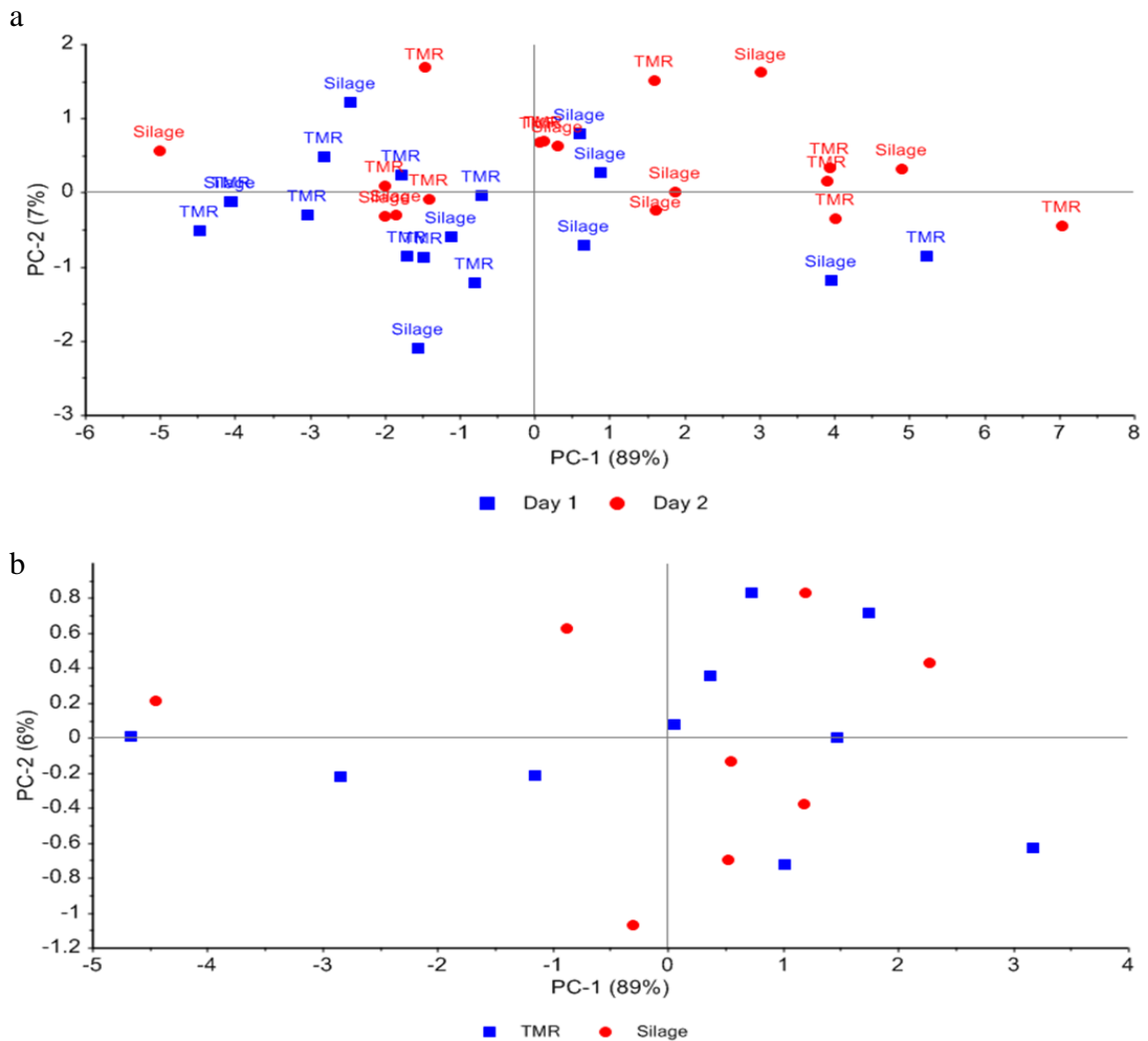


Figure 4.7 – PCA score plot of PC 1 and PC-2 for laser diffraction results (a) from all samples over both sample days, samples coded by diet. (b) averaged samples across both days coded by diet.

4.3.4.2 Faecal NIRS

PCA of the NIRS spectral data from the 36 animals (18 animals over 2 days) was performed. PC-1 accounted for 70 % of the variation, and PC-2 for 20 % of the variation. When assessing the corresponding scores plot PC-1 against PC-2,

separation was observed (Figure 4.8a). Sampling day had a significant effect on PC-1 ($P < 0.001$) but no significant effect on PC-2 ($P = 0.111$). Loadings plots associated with this PC scores plot can be seen in Figure 4.8b. Wavelengths associated with moisture (1940 nm) showed the largest loadings, with regions 1420 – 1600, 2020 – 2190, 2310 and 2350 nm showing differences but at a lower magnitude than seen in the classic moisture peak. Spectral regions 1420 – 1600 and 2020 – 2190 nm are associated with high degradability (Deaville and Givens, 1998), and spectral regions 2310 and 2350 nm associated with lipids (Workman and Weyer, 2012).

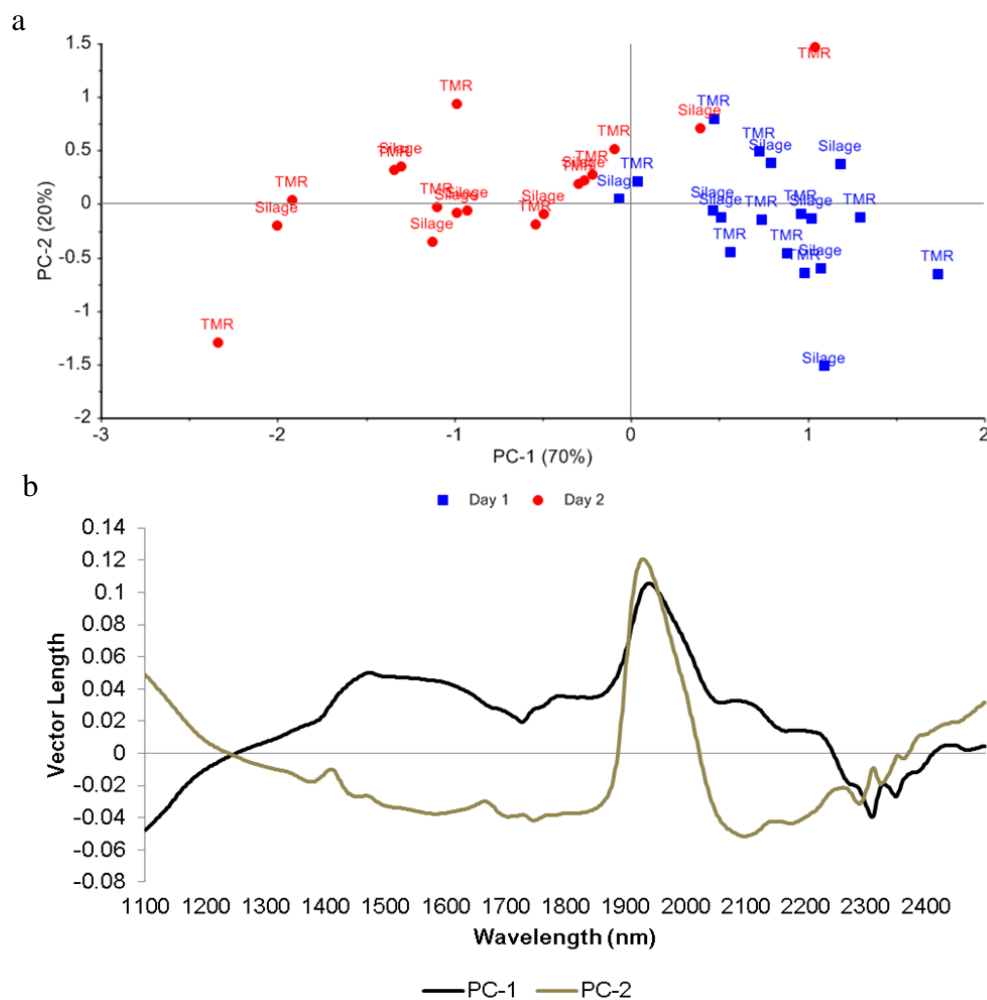


Figure 4.8 – PCA scores plot of NIR spectra (SDT transformed) for both sample time points (a) PC-1 and PC-2 (b) associated loadings plot of PC-1 and PC-2

NIR spectra from the two days were averaged to give one spectrum per cow and the data re-analysed (Figure 4.9a), and ANOVA conducted for the effect of ‘diet’. No

separation of samples between diets was observed. There was no significant difference between TMR or Silage diets (PC-1: $P = 0.489$; PC-2: $P = 0.471$). Peaks associated with lipids/oils showed highest loadings for PC-1 in the positive direction (Figure 4.9b), observed at 2310 and 2350 nm and repeated in the overtone region at 1724 nm. Water/moisture, seen as the classic moisture peak at 1940 nm, had a large influence on PC-1 in the negative direction, and to a lesser extent PC-2 in the positive direction.

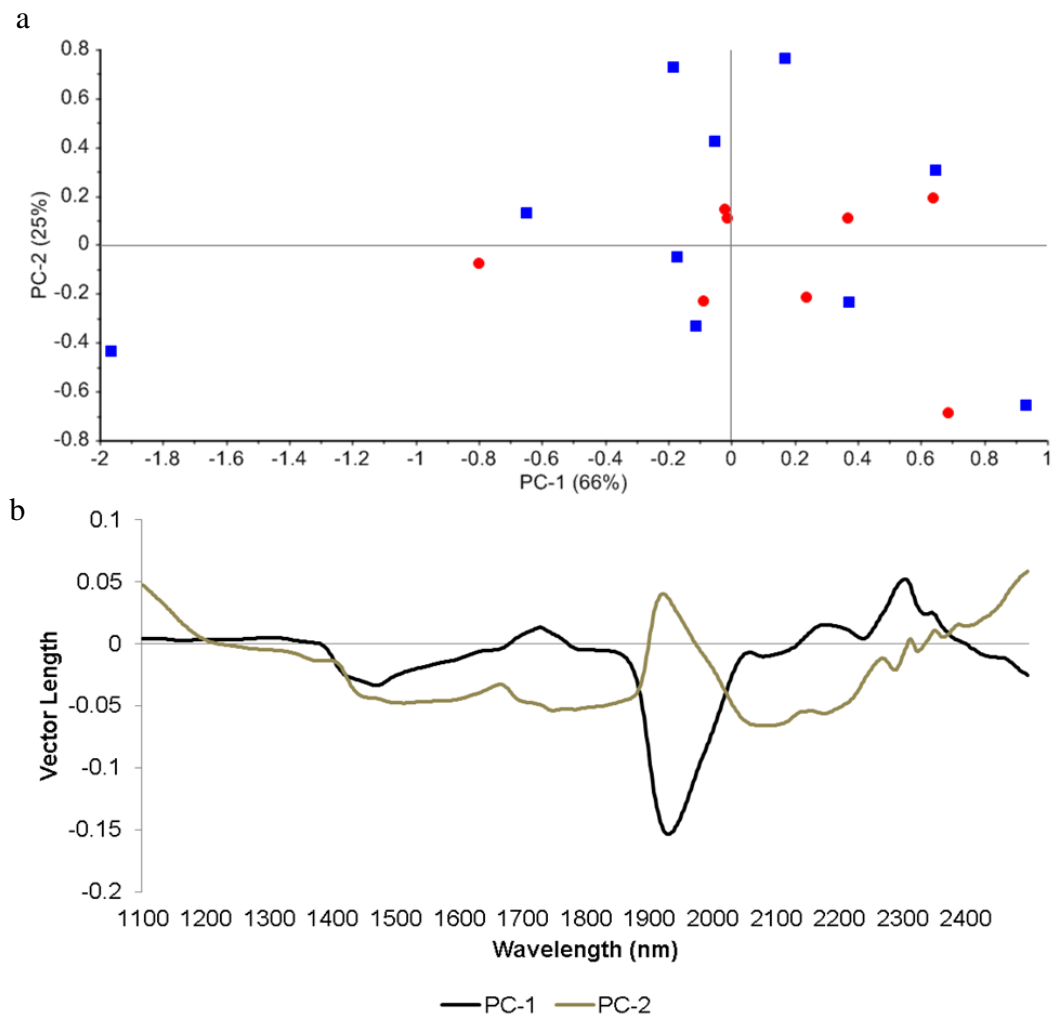


Figure 4.9 – PCA score plot of NIR spectra (SDT transformed) for averages of the time points (a) PC-1 and PC-2 (b) associated loadings of PC-1 and PC-2.

4.3.5 Discussion

No differences were seen when comparing faeces from different sampling dates for LD, however NIR spectra showed differences between samples collected on different days. Moisture was the main driving force of differences seen, spectral regions associated with lipids and high degradability as described by Deaville and Givens (1998) also had an effect on differences observed in PCA scores plot. Samples were not re-dried before they were scanned therefore it is possible that samples absorbed moisture. However, neither LD nor faecal NIRS was able to discriminate between faeces from animals on TMR or silage based diets. The TMR diet consisted 70 % grass silage and 30 % straw both diets receiving the same mineral supplement. The grass silage used for the TMR diet was the same as that used for the grass silage diet. The fact that 70 % of the diet was the same meant that diets were too similar for either LD or NIRS to find physical or chemical differences in the faeces. No efficiency data (e.g. RFI/FCR etc.) was available for this study, therefore it was not possible determine if between-animal variation was linked to differences in feed efficiency. A future study (Chapter 7) will assess the use of both LD and NIRS to detect variation between-animals and relate these to feed efficiency (RFI and FCR) measurements.

4.4 EVALUATION OF MICROBIAL COMMUNITIES ASSOCIATED WITH THE LIQUID AND SOLID PHASES OF THE RUMEN OF CATTLE OFFERED A DIET OF PERENNIAL RYEGRASS OR WHITE CLOVER

4.4.1 Introduction

Microorganisms are an essential part of rumen function, with the diverse microbiota responsible for degradation of complex carbohydrates, production of volatile fatty acids (VFA) and synthesis of microbial protein. The rumen microbiome plays an important role in animal physiology and a key role in productivity.

4.4.2 Aims

The work reported here aimed to establish methods for library preparation, sequencing and data analysis for the rumen microbiome and to assess the suitability of a stomach tube to collect rumen samples. Therefore the aim of this study was to assess differences in microbial communities (i) between solid- and liquid-phases of the rumen, and (ii) associated with diet offered either white clover or perennial ryegrass.

4.4.3 Materials and Methods

4.4.3.1 Animal Study

Full details of the animal study can be found in McCartney *et al.*, (2014). In brief, 4 lactating Holstein-Friesian cows fitted with rumen cannulae were used in this study. Animals were strip grazed on pure stands of either perennial ryegrass (*Lolium perenne* cv. Fennema; PRG) or white clover (*Trifolium repens* cv. AberHerald; WC) in a changeover design with 3 periods (Table 4.3), each period consisting of 3 weeks. Cows were milked twice daily (8 am and 4 pm) and received 2 kg/head of a proprietary concentrate feed at each milking. Rumen contents were collected by total rumen evacuation at 9am on the penultimate day of each period. Ruminal pH was measured morning and evening on the final 2 days of each of the experimental periods.

Table 4.3 – Diet each animal received during each of the experimental periods

Cow #	Period 1	Period 2	Period 3
4371	Grass	Grass	White Clover
1566	Grass	White Clover	Grass
852	White Clover	White Clover	Grass
9248	White Clover	Grass	White Clover

4.4.3.2 Isolating liquid and solid phase associated microbes

Rumen contents were hand squeezed through 4 layers of cheesecloth to obtain approximately 1 L of liquid. Liquid associated microbes were obtained from this liquid fraction sample. Solid associated microbes were obtained from material obtained by gently washing 500 g of rumen contents that had previously been retained within the cheesecloth with physiological saline and hand squeezed twice. The solid samples were then processed with physiological saline (320 ml) in a Stomacher 400 Circulator (Steward UK Ltd, Worthing, UK) to detach microbes from the solids. Differential centrifugation was used to remove feed particles at a low speed (10 min at 500 x g) and then a higher speed to spin down microbes (25,000 x g for 25 min) for both liquid and solid samples. The microbial pellets from solid and liquid samples were washed twice with physiological saline, freeze dried and stored at -20°C. DNA extractions were carried out using an adapted version of Yu and Morrison's (2004b) repeated bead beating plus column method (as described in Section 3.3.1. ***

4.4.3.3 Next Generation Sequencing of Rumen Samples

Extracted DNA samples were diluted to 10 ng/µl and libraries subsequently prepared for Illumina MiSeq sequencing as described in Section 3.3.2. In brief, extracted DNA was amplified by PCR, amplifying the V4 regions of the 16S rRNA, before being purified and amplicon fragments checked for size using gel electrophoresis. Amplified DNA was pooled and checked for size, and libraries quantified using quantitative PCR (qPCR). Pooled samples were diluted, denatured and spiked with PhiX, and subsequently sequenced using a V2 MiSeq reagent cartridge (Illumina, CA, USA).

4.4.3.4 Analysis of Sequencing Data

Data was analysed using QIIME software (qiime.org; Caporaso *et al.*, 2010b) as described in Section 3.3.3. Filtered relative abundance data was exported to Unscrambler and PCA calculated. In order to assess the effect of diet on phase and microbial populations a one-way ANOVA was performed in GenStat on PC scores,

*** Sample collection and sample preparation to this stage was completed prior to start of PhD. ***

using either phase (liquid vs. solid) or diet (PRG vs. WC) as a factor. Differences between fractions and diet were assessed by ANOVA using STAMP software (v. 2.1.3) and multiple test correction using the Benjamini-Hochberg false discovery rate. Unclassified reads were removed prior to running the ANOVA.

Samples were assessed by a presence or absence method, in which two groups were compared, one having OTUs present in ≥ 4 samples and the other having 0 OTUs present, e.g. ≥ 4 OTUs in 'liquid clover' and 0 in 'solid clover' using an in-house script. Shannon diversity indices were calculated in QIIME for each of the samples to assess both species evenness and richness. Chi-squared testing (GenStat) was used to compare the frequency of OTUs in ≥ 4 or 0 samples between diets or fractions. Chi-squared tables were generated within GenStat in order to determine whether there were significantly more unique OTUs in one or the other of samples being tested. The null hypothesis was that there was no difference between treatments (or fractions) in numbers of OTUs in ≥ 4 or 0 samples respectively.

4.4.4 Results

4.4.4.1 *16S sequencing data*

Overall 2,077,290 reads were generated after sequencing, giving an average of $86,553 \pm 12,797$ reads per sample. Overall 1,997,399 counts were generated post quality filtering. The average number of counts per sample assigned to an OTU (post filtering) was $83,224 \pm 12,620$. Rarefaction plots started to plateau at 35,500 reads per sample, indicating that a sufficient level of depth of sequencing was achieved.

4.4.4.2 *PCA Analysis*

Separation of solid and liquid associated samples for both WC and PRG samples was seen (Figure 4.10). Greater variation was observed in the PCA scores plot for cows grazing WC. There were significant differences in the microbes associated with the different phases (solid vs. liquid) for PC-1 ($P < 0.001$), but not for PC-2 ($P = 0.287$). Diet (PRG vs. WC) had a significant effect on PC-2 ($P = 0.008$) but not PC-1 ($P = 0.494$). Circled samples, (Figure 4.10) showed lower ruminal pH values compared to

other cows grazing the clover diet (Table 4.4). These samples had lower pH for the first exposure to WC (i.e. during the first period only), but the low pH did not explain why these samples were outliers within the WC samples.

Table 4.4 – pH values for each cow during each period. Highlighted cells show consistently low values over the two sample points. Values with * represent missing values

Diet	Period	Cow #	pH (mean am/pm)
WC	1	852	5.51/5.64
WC	2	852	6.18/6.29
PRG	3	852	6.04/6.00
PRG	1	1566	6.46/6.54
WC	2	1566	6.08/5.97
PRG	3	1566	6.22/6.33
PRG	1	4371	5.92/6.28
PRG	2	4371	5.81/6.27
WC	3	4371	*/*
WC	1	9249	5.5/5.74
PRG	2	9249	6.02/6.32
WC	3	9249	6.08/6.25

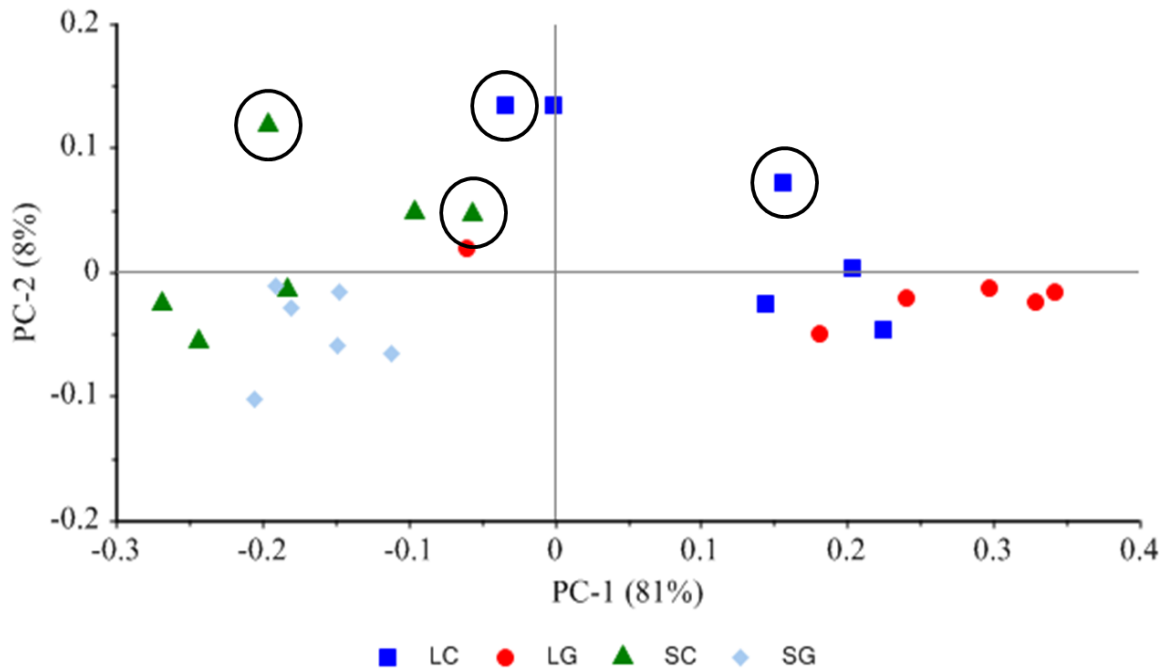


Figure 4.10 – PCA plot based on microbial community analysis for all liquid clover (LC), liquid grass (LG), solid clover (SC) and solid grass (SG).

4.4.4.3 Microbial community structure

Bacteroidetes were the most abundant phylum in liquid samples (53.1 % (s.d. 12.7) for liquid-phase/WC and 61.5 % (s.d. 15.4) for liquid-phase/PRG). The next most abundant phylum was Firmicutes, which accounted for 38.1 % (s.d. 15.8) and 27.9 % (s.d. 11.4) of OTUs in the liquid-phase/WC and liquid-phase/PRG respectively. The reverse was seen in solid-phase samples: Firmicutes was the most abundant taxonomic group at the phylum level (59.8 % (s.d. 6.0) and 49.8 % (s.d. 5.7) for solid-phase/WC and solid-phase/PRG respectively). The next most abundant taxonomic group in the solid phase were Bacteroidetes, accounting for 25.9 % (s.d. 6.5) in solid-phase/WC and 27.9 % (s.d. 3.2) in solid-phase/PRG. Other phyla present, although at lower relative abundances (liquid; solid), include Actinobacteria (4.0 %; 3.4 %), Fibrobacteres (1.8 %; 5.2 %), Tenericutes (1.2 %; 1.6 %), Spirochaetes (1.1 %; 4.4) and Euryarchaeota (0.2%; 2.2%). Other phyla were present at < 1 %.

Prevotella were the most abundant taxonomic group (genus level) within liquid-phase samples, 58.6 % (s.d. 15.1) and 48.0 % (s.d. 10.6) for liquid-phase/PRG and liquid-phase/WC respectively. *Prevotella* were also the most abundant genus within solid-phase grass and clover samples, although at a lower level; 21.3 % (s.d. 3.5) and 19.8 % (s.d. 8.1) respectively (Figure 4.11).

When ANOVA was performed using STAMP, the abundance of 24 genera were significantly different ($P < 0.05$) between liquid-phase/PRG and solid-phase/PRG. Of these, 20 were higher in solid-phase/PRG compared to liquid-phase/PRG. Six significantly different genera were found when comparing microbes associated with liquid-phase/WC and solid-phase/WC; the lower number of significant differences may be due to the larger variation between samples (Figure 4.10). Five of the six significantly different genera were found to be higher in solid-phase/WC than liquid-phase/WC. No significant differences in abundance of genera were found when comparing liquid-phase/WC to liquid-phase/PRG, or comparing solid-phase/WC to solid-phase/PRG. Twenty seven genera were found to have significantly different abundance when comparing all four groups (solid-phase/PRG, liquid-phase/PRG, solid-phase/WC, liquid-phase/WC). Two Families were found to be significantly different in abundance ($P < 0.05$) when comparing PRG to WC diets at genus level - *Comamonadaceae* and *Succinivibrionaceae*.

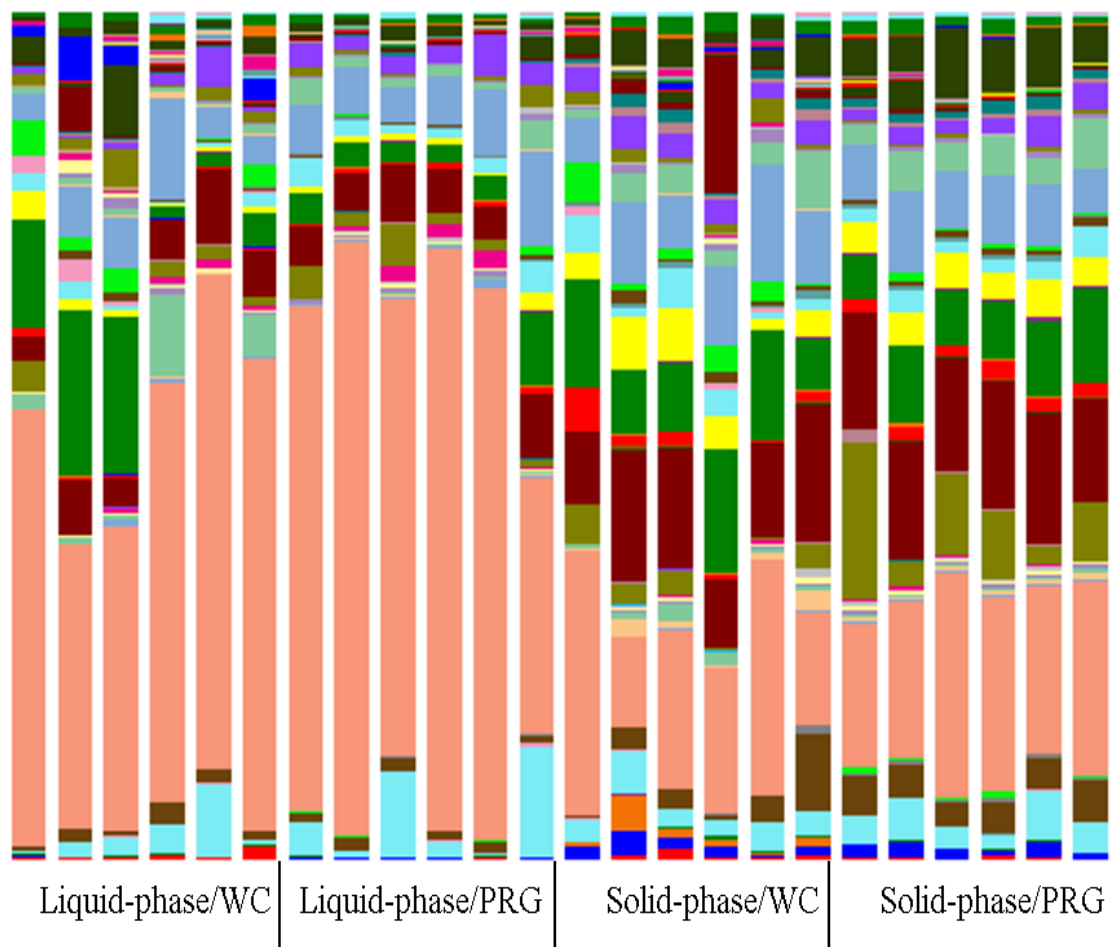


Figure 4.11 – Taxa summary plot of all diets and phases at the genus level (Page 68) and accompanying legend (Page 69 and Page 70)

■ Unassigned;Other;Other;Other;Other;Other
■ k_Archaea;p_Euryarchaeota;c_Methanobacteria;o_Methanobacteriales;f_Methanobacteriaceae;g_Methanobrevibacter
■ k_Archaea;p_Euryarchaeota;c_Methanobacteria;o_Methanobacteriales;f_Methanobacteriaceae;g_Methanosphaera
■ k_Archaea;p_Euryarchaeota;c_Thermoplasmata;o_E2;f_[Methanomassiliococcaceae];g_vadinCA11
■ k_Bacteria;p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Cryocolla
■ k_Bacteria;p_Actinobacteria;c_Actinobacteria;o_Actinomycetales;f_Microbacteriaceae;g_Curtobacterium
■ k_Bacteria;p_Actinobacteria;c_Coriobacteriia;o_Coriobacteriales;f_Coriobacteriaceae;g__
■ k_Bacteria;p_Actinobacteria;c_Coriobacteriia;o_Coriobacteriales;f_Coriobacteriaceae;g_Adlercreutzia
■ k_Bacteria;p_Actinobacteria;c_Coriobacteriia;o_Coriobacteriales;f_Coriobacteriaceae;g_Atopobium
■ k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f__;g__
■ k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_BS11;g__
■ k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Bacteroidaceae;g_BF311
■ k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_Prevotellaceae;g_Prevotella
■ k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_RF16;g__
■ k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_S24-7;g__
■ k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Paraprevotellaceae];g__
■ k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Paraprevotellaceae];g_CF231
■ k_Bacteria;p_Bacteroidetes;c_Bacteroidia;o_Bacteroidales;f_[Paraprevotellaceae];g_YRC22
■ k_Bacteria;p_Chloroflexi;c_Anaerolineae;o_Anaerolineales;f_Anaerolinaceae;g_SHD-231
■ k_Bacteria;p_Elusimicrobia;c_Elusimicrobia;o_Elusimicrobiales;f_Elusimicrobiaceae;g__
■ k_Bacteria;p_Fibrobacteres;c_Fibrobacteria;o_Fibrobacteriales;f_Fibrobacteraceae;g_Fibrobacter
■ k_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Lactobacillaceae;g_Lactobacillus
■ k_Bacteria;p_Firmicutes;c_Bacilli;o_Lactobacillales;f_Streptococcaceae;g_Streptococcus
■ k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;Other;Other
■ k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f__;g__
■ k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Christensenellaceae;g__
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k_Bacteria;p_Firmicutes;c_Clostridia;o_Clostridiales;f_Veillonellaceae;g_Dialister
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 k_Bacteria;p_Proteobacteria;c_Betaproteobacteria;o_Burkholderiales;f_Comamonadaceae;g_
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 k_Bacteria;p_Proteobacteria;c_Gammaproteobacteria;o_Aeromonadales;f_Succinivibrionaceae;g_
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 k_Bacteria;p_Spirochaetes;c_Spirochaetes;o_Spirochaetales;f_Spirochaetaceae;g_Treponema
 k_Bacteria;p_Synergistetes;c_Synergistia;o_Synergistales;f_Dethiosulfovibrionaceae;g_Pyramidobacter
 k_Bacteria;p_Tenericutes;c_Mollicutes;o_;;f_;g_
 k_Bacteria;p_Tenericutes;c_Mollicutes;o_Acholeplasmatales;f_;g_
 k_Bacteria;p_Tenericutes;c_Mollicutes;o_Anaeroplasmatales;f_Anaeroplasmataceae;g_Anaeroplasma
 k_Bacteria;p_Tenericutes;c_Mollicutes;o_RF39;f_;g_
 k_Bacteria;p_Tenericutes;c_RF3;o_ML615J-28;f_;g_
 k_Bacteria;p_Verrucomicrobia;c_Verruco-5;o_WCHB1-41;f_;g_
 k_Bacteria;p_Verrucomicrobia;c_Verruco-5;o_WCHB1-41;f_RF12;g_
 k_Bacteria;p_Verrucomicrobia;c_Verruco-5;o_WCHB1-41;f_WCHB1-25;g_

4.4.4.4 Unique OTUs

A 'presence vs. absence' approach was taken to further explore the distribution of unique OTUs within the different sample types (liquid-phase/PRG vs. liquid-phase/WC; solid-phase/PRG vs. solid-phase/WC). The largest differences were seen for OTUs present in ≥ 4 liquid-phase/PRG diet samples and absent from all liquid-phase/WC diet samples. Comparing the reverse of this situation (OTUs present in ≥ 4 liquid phase/WC samples and absent from all liquid-phase/PRG samples), only 17 OTUs were identified. The differences between these two groups in the number of unique OTUs were found to be highly significant (Table 4.5; $P < 0.001$). Forty three unique OTUs were observed when comparing OTUs present in ≥ 4 solid-phase/PRG and 0 solid-phase/WC samples, the reverse (≥ 4 solid-phase/WC; 0 solid-phase/PRG) had 12 unique OTUs, with differences between these groups also being highly significant (Table 4.5; $P < 0.001$). 'Presence vs. absence' analysis was repeated for each diet (solid-phase/PRG vs. liquid-phase/PRG; solid-phase/WC vs. liquid-phase/WC). Only 2 OTUs were found to be unique between liquid-phase/PRG (≥ 4 OTUs) and solid phase-PRG (0 OTUs) samples; when considering the reverse situation (≥ 4 OTUs in solid-phase/PRG; 0 OTUs liquid-phase/PRG samples), 6 were found to be unique; there were no significant difference when comparing these groups. No unique OTUs were found when comparing ≥ 4 OTUs in liquid-phase/WC and 0 OTUs in solid-phase/WC samples, the reverse analysis found 21 unique OTUs between, with differences between these groups being highly significant ($P < 0.001$; Table 4.5). The order Clostridiales, in particular families *Lachnospiraceae* and *Ruminococcaceae*, and family *Prevotellaceae* contained the majority of the unique OTUs, 61 and 39 OTUs respectively out of a total of 170 OTUs. Clostridiales predominated amongst OTUs identified in ≥ 4 liquid-phase/PRG and 0 liquid-phase/WC samples, the reverse of this (≥ 4 liquid/phase/WC and 0 liquid-phase/PRG samples) and a few were involved in the ≥ 4 solid-phase/WC and 0 liquid-phase/WC comparison. When comparing significant differences in OTUs present in ≥ 4 solid-phase/PRG and 0 liquid-phase/WC samples, the family *Prevotellaceae* dominated.

Table 4.5 – Summary of presence vs. absence analysis. Samples were assessed by having OTUs present in ≥ 4 samples and the other having 0. Table also shows Chi-Squared results, all 1 degree of freedom. NS, non-significant.

Present ≥ 4	Present 0	Number of OTUs	Chi-Squared	Significance (Chi-Squared)
Liquid-phase/PRG	Liquid-phase/WC	66	28.93	P < 0.001
Liquid-phase/WC	Liquid-phase/PRG	17		
Solid-phase/PRG	Solid-phase/WC	43	17.47	P < 0.001
Solid-phase/WC	Solid-phase/PRG	12		
Liquid-phase/PRG	Solid-phase/PRG	2	1.07	NS
Solid-phase/PRG	Liquid-phase/PRG	6		
Liquid-phase/WC	Solid-phase/WC	0	14.00	P < 0.001
Solid-phase/WC	Liquid-phase/WC	21		

4.4.4.5 Diversity indices

Diversity within treatment (liquid-phase/WC, solid-phase/WC, liquid-phase/PRG, solid-phase/PRG), period (1, 2, 3), diet (WC, PRG) and phase (Liquid, Solid) were calculated using the Shannon Index (H). H values levelled off above 5,500 sequences per sample (Figure 4.12). Therefore all H values are reported at 5500 sequences per sample with a corresponding Shannon index error value. Highest H values (s.d.) were observed for solid fractions 7.571 (0.604) compared to liquid fractions 6.083 (0.687). The same trend was also observed when comparing samples individually; solid-phase/WC (7.314 (0.236)), solid-phase/PRG (7.827 (0.236)), liquid-phase/WC (5.739 (0.340)) and liquid-phase/PRG (6.427 (0.769)). PRG samples had higher H values compared to WC, 7.127 (0.902) versus 6.526 (0.974) respectively. Diversity

(measured with H) increased over the course of the experiment: 6.428 (0.868), 6.823 (1.026) and 7.228 (0.888) for periods 1, 2 and 3 respectively.

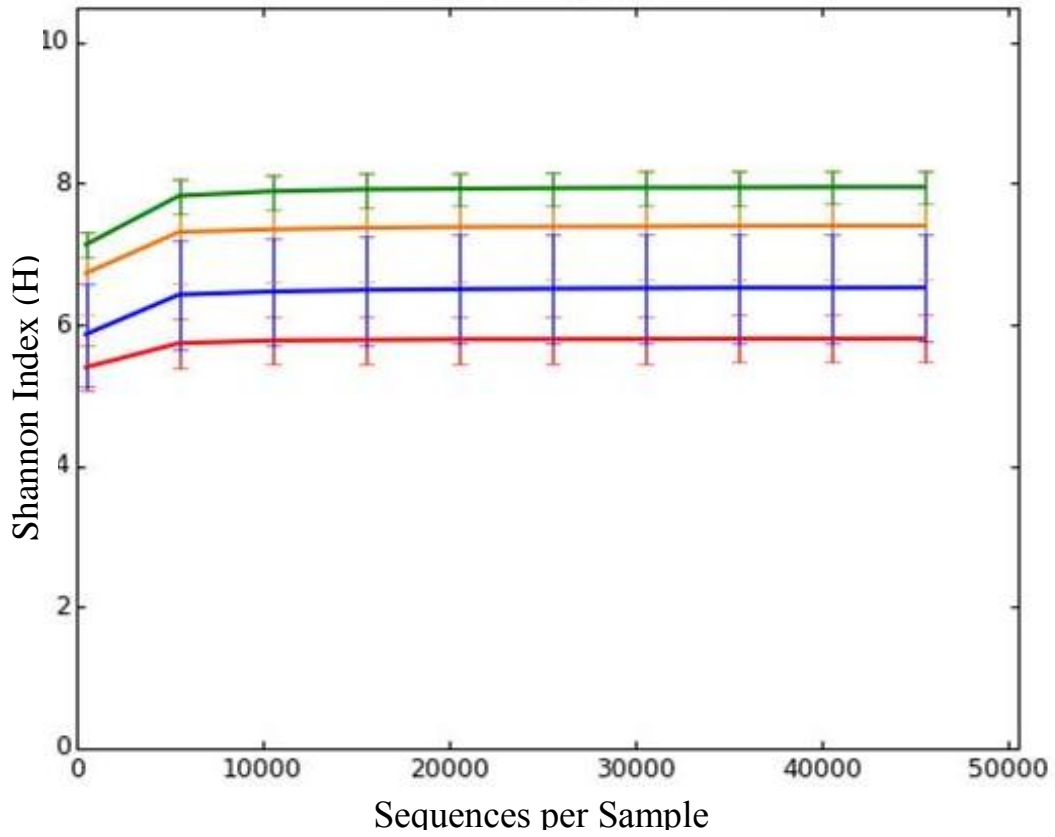


Figure 4.12 – Rarefaction plot showing Shannon Indexes for liquid-phase/PRG (blue), liquid-phase/WC (red), solid-phase/PRG (green) and solid-phase/WC (orange).

4.4.5 Discussion

Differences between both diets and phases were observed in the PCA scores plot based on the 16S microbial community analysis; however, more variation was observed for the WC diet compared to the PRG diet. Low ruminal pH levels did not explain this greater variation seen for the WC diet. Petri *et al.*, (2013) reported a shift in rumen microbiome in acidotic heifers, in particular increases in relative abundances of *Acetivomaculum*, *Lactobacillus*, *Prevotella* and *Streptococcus*.

Differences in microbial communities at the genus level were assessed, along with identification of unique OTUs between diet and phase, and diversity using Shannon Index. Shannon index takes account of species evenness and richness (Whittaker, 1972).

4.4.5.1 Differences between diets

4.4.5.1.1 Microbial communities and diversity

Greater diversity was found in PRG samples compared to WC samples. Two genera were found to be significantly different between PRG and WC diets, *Comamonadaceae* and *Succinivibrionaceae*, both of which had higher relative abundances in WC diet. Diversity was shown to be higher in PRG. However no significant differences were found when comparing diets within the liquid and solid phase separately. Khan *et al.*, (2002) reported that family *Comamonadaceae* included denitrifiers in activated sludge. *Succinivibrio* have been reported to be predominantly amylolytic (Huws *et al.*, 2016), the authors also reported that *Succinivibrio* (and *Comamonadaceae*) increase during the first 1-2 h after ingestion of feed (simulated by incubation in the rumen); they are said to be primary colonisers.

4.4.5.1.2 Unique OTUs

When assessed using presence vs. absence analysis, a greater number of OTUs were identified as being unique between diets rather than between phases. Differences for both liquid-phase/WC and liquid-phase/PRG, and solid-phase/WC and solid-phase/PRG were found to be highly significant when assessed using Chi-squared.

The main unique OTUs between diet were found from the order Clostridiales, and in particular families *Lachnospiraceae* and *Ruminococcaceae*, with higher numbers being found in the PRG diet, and in liquid samples found in samples from the PRG diet. In contrast, de Menezes *et al.*, (2011) reported that *Lachnospiraceae* are prevalent in solid rumen samples.

4.4.5.2 Differences between phases

4.4.5.2.1 Microbial communities and diversity

Prevotella was the most abundant genus in both liquid and solid samples, but was present at higher relative abundances in the liquid phase. This is in agreement with other studies (de Menezes *et al.*, 2011; Patel *et al.*, 2014; Pitta *et al.*, 2010) which also found Prevotella to be abundant at higher levels in the liquid fraction. This is to be expected as *Prevotellaceae* have been noted as being polysaccharide metabolisers (de Menezes *et al.*, 2011; Matsui *et al.*, 2000). The liquid phase of the rumen includes dissolved sugars and other easily digestible feed. The action of rumen contraction means that the liquid phase of the rumen inoculates newly-ingested solid material in the rumen mat. Of the significantly different genera (ANOVA using STAMP) all except Prevotella were found to be at higher abundances in the solid-phase samples. Diversity of the microbiome was higher in solid-phase samples compared to those from the liquid phase. Prevotella predominated the liquid phase, reducing richness of the overall community. These results indicate that differences in the microbiome exist between solid and liquid samples. Tapio *et al.*, (2016) reported that buccal swabs could be used to obtain a representative sample of the rumen microbial community. They compared buccal swabs of the mouth, liquid rumen samples and strained bolus samples but found no difference between the different samples, concluding that buccal swabs contained a representative study of the rumen microbiome. However it should be noted that the strained bolus sample was also effectively a liquid sample as no attempt was made to detach solid-associated microbes.

4.4.5.2.2 Unique OTUs

No significant differences were found between liquid-phase/PRG and solid-phase/PRG, or the reverse, was detected when numbers of unique OTUs were compared using Chi-squared. However significant differences were found when comparing liquid-phase/WC to solid-phase/WC samples. Microbes present at low levels (below detectable limit at this sequencing depth) are likely to be present within the rumen, but are only able to proliferate and increase in numbers when a more suitable substrate is introduced (e.g. PRG compared to WC diet). The majority of unique OTUs seen within diets (e.g. solid-phase/WC vs. liquid-phase/WC) were found from the order Clostridiales, in particular *Lachnospiraceae*. A second family, *Coriobacteriaceae*, was also found to be unique in solid samples but not liquid samples. *Ruminococcaceae* and *Lachnospiraceae* have been previously reported for their fibrolytic activity (Biddle *et al.*, 2013; Gardner *et al.*, 1995; Kong *et al.*, 2010; Krause, 2003). Both *Ruminococcaceae* and *Lachnospiraceae* have been reported to be secondary colonisers of ingested feed (Huws *et al.*, 2016; Piao *et al.*, 2014), meaning that the relative abundances increase 4-8 h after ingestion.

In conclusion, whilst unique OTUs were identified, differences between diet (WC or PRG) and sampling technique (liquid or solid) were found to have an effect on the rumen microbiome.

CHAPTER 5

USE OF NIRS TO DESCRIBE THE TIME- COURSE OF RUMEN FERMENTATION OF FRESH HERBAGE

5.1 INTRODUCTION

Once ingested, feed is chewed, mixed with saliva and passed into the rumen. Fermentation by rumen microbial action breaks down feed components, including those that are not susceptible to mammalian enzymes, to produce end products that can be used by the host ruminant. Retention time within the rumen (Aikman *et al.*, 2008) and selectivity of diet components (Lambert and Litherland, 2000) are both important factors in determining extent of digestion and, ultimately, animal performance.

5.1.1 Selectivity of diet

Selectivity is an important factor in determining the potential digestibility of ingested pasture, with animals generally selecting higher quality material, if available. Leaf fractions and younger pastures contain lower levels of cellulose and hemicellulose than more mature or stem fractions of grass (van Soest, 1994). Consequently, leaf fractions have higher nutritional value than stem fractions (Lambert and Litherland, 2000). These authors also noted the lower nutritional value of pastures with a 6 week regrowth period compared to 3 week regrowth period and this is related to an increased proportion of stem material. The maturity of plants has an important effect on digestibility. Mature plants contain more lignin which reduces the availability of structural carbohydrates such as cellulose and hemicellulose (Morrison, 1975) for digestion within the rumen.

5.1.2 Digestion of feed in the rumen

Feed leaves the rumen either through absorption of small molecules (notably volatile fatty acids (VFAs)) across the rumen wall or passage of small particles along the gastrointestinal (GI) tract. Waghorn and Barry (1987) noted that approximately 60 % of organic matter digestion (OMD) takes place in the rumen. There are strong interactions between particle size reduction and fermentation in the rumen, with smaller particles being more susceptible to fermentation because of their higher surface area/volume ration and fermentation leading to loss of structural integrity,

and so particle size reduction. The rate of digestion within the rumen is mainly dependent on quality of the feed, though there can be interactions between feed ingredients, for example reduced fermentation due to the lack of rumen degradable protein in the diet (Gressley and Armentano, 2007). Rumen retention time also affects the extent of rumen digestion so the longer that feed is retained in the rumen the greater chance of further digestion.

5.1.2.1 Carbohydrates

Cellulose, hemicellulose and a small amount of starch are the main carbohydrate sources found in grass. The rumen is the site of digestion of all soluble carbohydrates and the majority of complex carbohydrates, with one estimation given at 85 % (Lambert and Litherland, 2000).

Complex carbohydrates are broken down by microbial fermentation in the rumen to simple sugars which are then converted to the final end products: VFAs and gases, including carbon dioxide and methane. VFAs are absorbed across the rumen wall where they are transported in the blood stream to the liver, which is the primary source of gluconeogenesis.

5.1.2.2 Lipids

Phospholipids and glycolipids are the two main lipid-containing membrane groups in grasses. The thylakoid membrane, where photosynthesis takes place, contains approximately 400 g of lipids/kg dry matter (DM). Lipids in leaf chloroplasts account for 200 – 250 g/kg DM (Roughan and Batt, 1969; Heinz and Siefermann-Harms, 1981). Fatty acids (FA) that leave the reticulorumen are either of dietary origin or the result of microbial activity. Free FAs (FFA) are released in the rumen by the action of microbial lipolysis. FFAs have a short half-life as they are rapidly biohydrogenated to become saturated (Buccioni *et al.*, 2012). The main fatty acids in grass are polyunsaturated fatty acids (α -linolenic acid (C18:3) and linoleic acid (C18:2)), biohydrogenation results in a series of intermediates such as conjugated linoleic acid (e.g. C18:2 cis-9, trans-12) and vaccenic acid (C18:1 trans-11) (Kim *et*

al., 2005). Microbial cells contain high levels of both protein and lipids, so microbial growth has important effects on both the protein and lipid composition of the rumen contents and digesta leaving the rumen. Colonisation of grass in the rumen results in increases in levels of odd- and branched-chain fatty acids (a series of C15 and C17 fatty acids) that are distinctive components of microbial cells (Kim *et al.*, 2005).

5.1.2.3 Protein

Microbial action breaks down plant protein to amino acids (AAs) and eventually ammonia. Non-protein nitrogen sources, such as nitrate, can also be converted into ammonia. A portion of the AAs and ammonia is converted into microbial protein. Undigested plant proteins, as well as microbial protein, leave the rumen and move down the GI tract, so that the supply of protein to the animal depends both on the level and type of dietary protein as well as the extent of rumen microbial protein synthesis. Further digestion of protein, including digestion of microbial protein, is carried out in the abomasum, which connects the omasum to the small intestine. Digestion in the abomasum is carried out by proteolytic enzymes called pepsins rather than by microbial fermentation found in the reticulorumen. A high proportion of AA absorption occurs in the small intestine.

5.1.2.4 Dry Matter Digestibility

Dry matter digestibility can be measured by two main techniques: (i) *in vivo* (*in sacco*) studies and (ii) *in vitro* studies. *In vivo* studies to assess digestibility involve placing dried feed samples into nylon/Dacron bags and incubating within the rumen itself (*in sacco*). Determining digestibility *in vitro* can be carried out by using rumen liquor from a donor animal, or by using enzymes. Enzymatic digestion is commonly carried out using cellulase-pepsin digestion (Jones and Hayward, 1973). Tilley and Terry (1963) demonstrated the use of rumen fluid from a donor animal followed by an acid-pepsin digestion stage to estimate whole tract dry matter digestibility of dried samples. These techniques generally use dried and milled samples to determine digestibility.

5.1.3 NIRS and difference spectra

Deaville and Givens (1998) used NIRS to examine the time-course of fermentation in the rumen. By comparing NIRS difference spectra of feeds following different periods of incubation in dacron bags in the rumen they identified regions that were associated with low and high degradability of fresh grass, grass silage and maize silage. Coleman and Murray (1993) showed that differences between the spectra of paired samples of hay and faeces can provide useful information about the diet components affecting whole tract digestibility.

5.1.4 Aims

The aim of this work was to assess the use of NIRS to monitor digestion of different components of fresh grass incubated *in vitro* with buffered rumen fluid. Different incubation times were used to simulate the effects of different retention times within the rumen and different sections of grass plants were used to simulate the effects of differences in diet selectivity.

5.2 MATERIALS AND METHODS

5.2.1 Sample collection and preparation

Grass samples were collected from two different paddocks (paddock 177: Grass A; paddock 147; Grass B) at Teagasc Grange on 26th May 2014. Both plots were a permanent pasture with perennial ryegrass being the dominant species present; both plots were last reseeded over 10 years prior to the sampling date. Plots were intensively grazed throughout this time and received 150-180 kg nitrogen (N) and 1 application of cattle slurry per year. Plots had been grazed 21 and 14 days prior to sample collection, for paddock 177 and 147 respectively. Samples were collected between 7:50 and 8:20 am. Grass was cut 5 cm above the ground using scissors, with approximately 2 kg of grass being collected from each paddock. The length of the grass was measured for height in bundles and cut in half to give nominal fractions in which stem and leaf fractions predominated. These fractions will be referred to as

Stem A and Leaf A for Grass A and Stem B and Leaf B for Grass B. Each fraction was chopped at high speed in a mixing bowl (Muller MTK20h Spiral; Saarbrucken, Germany) for 1 minute. A sub-sample of each fraction was taken both before and after chopping and used to determine dry matter and for chemical analysis.

5.2.2 *In vitro* digestion

Rumen fluid was collected on the morning of the trial from 4 sheep fitted with a rumen cannula and stored in 2 pre-warmed Thermos flasks for transport back to the laboratory. Donor sheep had been kept on pasture but were adapted to *ad libitum* hay and 1 kg of sheep nuts (a mixture of grass nut pellets) per day for 1 week prior to sampling. Rumen fluid was strained through 4 layers of cheesecloth to remove large feed particles. Thirty grams of chopped fresh grass was added to each 250 ml Duran bottle (Sigma-Aldrich, St Louis, MO, USA). Strained rumen fluid (40 ml) and 160 ml artificial saliva (see Appendix) were added to each bottle using Varispenser 50 ml pipettes (Eppendorf, Stevenage, UK). The space above the fluid was flushed with CO₂ and a rubber bung containing a gas release valve fitted. This process was repeated for each grass sample and each time point in duplicate, giving 64 samples in total. Two incubators were used in this study and one set of replicates were incubated in each (Rep 1/Rep 2). Samples were incubated at 37°C and agitated at a constant speed of 80 RPM for the following time periods: 0, 1, 2, 6, 12, 24, 48 h. The remaining bottles were subjected to a further incubation step in pepsin for 48 h (48+p). For the 0 h time point, grass samples were prepared using the same process but were filtered immediately, meaning samples were “washed” in the rumen fluid and artificial saliva. At each time point, the relevant bottles were removed from the incubator and the contents strained through Dacron material (bags) with 40 µm pore size (10 cm x 20 cm Dacron bags, ANKOM Technology, NY, USA) by hand. Residues and bags were dried at 40°C for 72 h then weighed. For the additional 48+p time point, undigested grass samples were removed from each bottle and the remaining liquid sample transferred to three large test tubes (125 ml) and centrifuged at 1600 RPM for 10 min. The supernatant was discarded and the pellet resuspended in pepsin (200 ml) which was prepared the previous day (see Appendix). The undigested grass and resuspended pellet in pepsin were transferred back to the

corresponding bottle, rubber bung with gas release valve refitted, and samples were incubated at 37°C and shaken at 80 RPM for a further 48 h. At the end of the pepsin incubation, the samples were strained through dacron bags and dried for 72 h at 40°C. Chopped (100 g) and unchopped (20 g) grass samples were also dried for 72 h at 40°C to determine DM contents. Results were reported as DM disappearance as follows:

$$DM\ Initial\ (g) = Fresh\ Weight\ (30\ g) \times \left(\frac{DM\ (\%) \ of\ Grass\ Section}{100} \right)$$

$$DM\ Disappearance\ (\%) = \frac{DM\ initial\ (g) - DM\ (residue)(g)}{DM\ initial\ (g)} \times 100$$

5.2.3 Statistical Analysis

GenStat (V14; VSNI, Hemel Hempstead, UK) was used for statistical analysis described here. Differences in DM disappearance of residues between replicates were assessed using a paired t-Test. Analysis of variance (ANOVA) was carried out to assess differences in DM disappearance at each time point, using ‘Grass’ and ‘Fraction’ as treatment factors. The ANOVA was repeated to assess differences between Fractions and Grass.

5.2.4 NIRS

After drying, samples were scanned at 2 nm intervals as described in Section 3.2.1. NIR spectrum for the two replicates for each sample and time point were averaged, data transformed using SDT and difference spectra calculated as described in section 3.2.3. In brief, SDT spectra from the 0 h residue were subtracted from the spectra of residues from the different time points for Stem A, Leaf A, Stem B and Leaf B. Spectral changes due to the pepsin stage were assessed by calculating difference spectra between the 48+p residue and residues from 48 h and 0 h. Difference spectra were calculated between Grass A and Grass B for the leaf and stem fractions and

between the leaf and stem fractions for Grass A and Grass B for the chopped starting material and the 48+p residues. The standard deviation (SD) at each wavelength was calculated for the time course difference spectra for Stem A, Leaf A, Stem B and Leaf B (Excel, 2010).

5.2.5 Chemical analysis ***

Prior to wet chemical analysis of the grasses, NIR predictions of the chopped grass were made using calibrations developed by IBERS (Sue Lister, personal communication) to estimate chemical composition. The predictions included water soluble carbohydrate (WSC), nitrogen (N), neutral detergent fibre (NDF), acid detergent fibre (ADF), dry matter digestibility (DMD) and dry matter (DM). Chopped and unchopped grass were subjected to chemical analysis to assess N, WSC, Ash, NDF, ADF and digestible organic matter content (DOMD).

5.3 RESULTS

5.3.1 Grass Composition

A shorter regrowth interval for Grass B resulted in differences in the height of the grass swards. Grass A samples had an average height of 37.5 cm (s.d. 2.9) whereas Grass B samples were 22.6 cm (s.d. 3.2) from cut material. DM was determined for both unchopped and chopped samples of Stem and Leaf fractions. Stem A had a DM content of 13.4 % and 12.5 % and Leaf A had a DM content of 8.4 % and 13.7 % for unchopped and chopped samples respectively. Stem B had DM content of 11.7 % and 12.1 %, and Leaf B had a DM content of 15.1 % and 12.8 % for unchopped and chopped grass samples respectively. Chemical analysis of both unchopped and chopped grasses can be found in Table 5.1. There was insufficient sample to determine ADF in unchopped Stem B and Leaf B. Predicted chemical composition of each of the grasses and fractions, using IBERS NIRS equations, can be found in Table 5.2. Leaf B had a higher N content than other fractions. WSC was higher in

Chemical analysis, and NIRS scans, were carried out in Aberystwyth by Sue Lister

Stem A compared to other fractions. ADF and NDF values were higher in stem fractions compared to relevant leaf fraction.

Table 5.1 – Chemical composition (wet chemistry) of dried and milled unchopped and chopped grass samples. IS denotes insufficient sample

Form	Grass	Nominal Fraction	Residual DM (g/kg)	Constituent (g/kg DM)					
				N	WSC	Ash	NDF	ADF	DOMD
Unchopped	A	Stem	933	32.9	137.5	85.0	522.6	293.9	703.2
		Leaf	934	41.5	103.9	81.2	478.5	243.9	765.7
	B	Stem	940	41.9	72.4	105.4	582.2	IS	698.4
		Leaf	942	48.1	86.1	95.3	454.5	IS	767.6
Chopped	A	Stem	933	29.0	160.0	87.3	492.0	261.9	700.6
		Leaf	933	38.3	92.4	85.9	489.9	245.1	694.9
	B	Stem	933	37.8	74.4	102.8	516.9	275.3	674.9
		Leaf	936	50.9	91.2	94.0	467.0	222.4	728.6

Table 5.2 – NIR predicted composition of dried and milled chopped grass samples (IBERS calibration equations; Sue Lister, personal communication).

Grass	Nominal Fraction	Residual DM (g/kg)	NIR predicted constituent (g/kg DM)				
			N	WSC	NDF	ADF	DMD
A	Stem	907	30.9	168.7	538.0	267.9	803.7
	Leaf	909	40.7	95.7	518.2	240.9	805.3
B	Stem	907	39.7	77.2	566.7	266.8	772.9
	Leaf	902	53.2	58.8	511.1	212.9	821.5

5.3.2 NIR spectra

Log 1/R and SDT transformed spectra of the starting material, chopped Stem A, Leaf A, Stem B and Leaf B samples are shown in Figure 5.1. Log 1/R spectra are generally featureless but contain a vast amount of information of spectra arising from broad overlapping peaks which are overtones and combinations of the mid-IR

fundamental bands. Log 1/R spectra are difficult to interpret and are affected by particle size and path-length effects. These effects were reduced by applying SDT transformations to the spectra to highlight the chemical differences. Differences between the fractions are more pronounced between SDT transformed spectra than Log 1/R spectra (Figure 5.1).

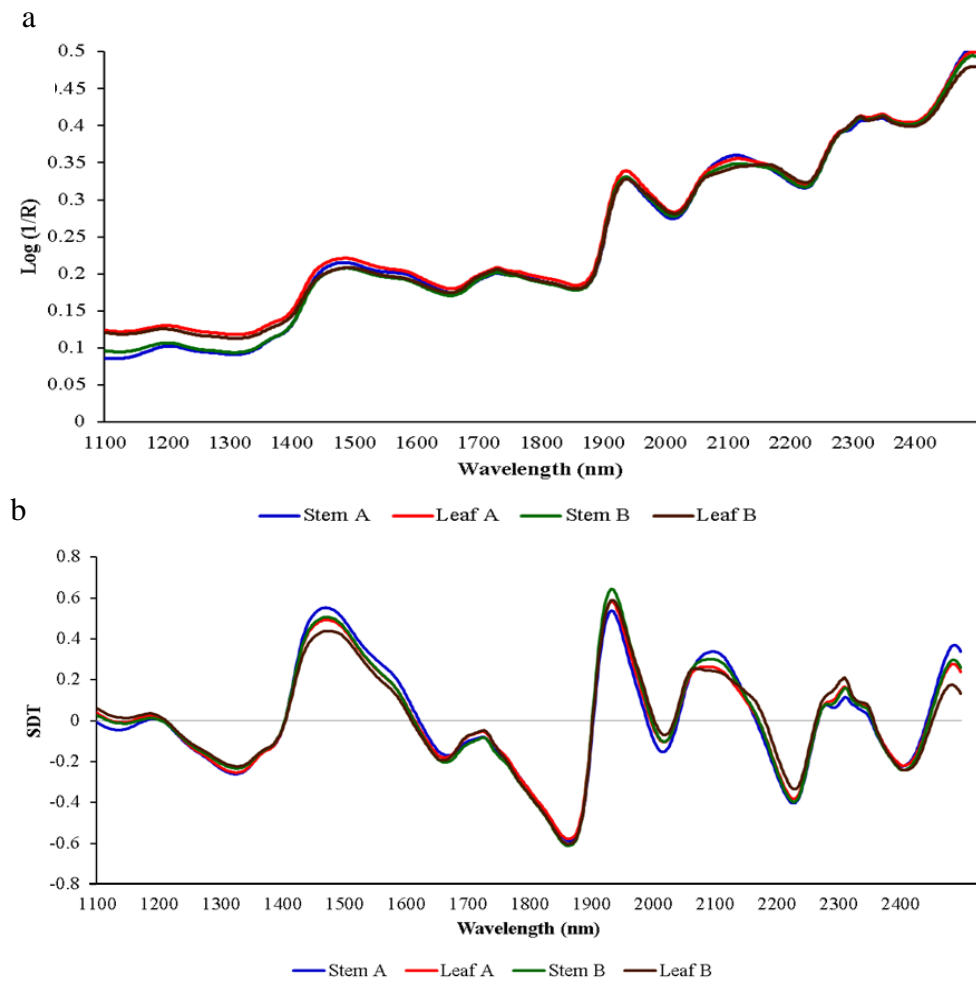


Figure 5.1 – (a) Log 1/R spectra of the four chopped grass samples/fractions and (b) SDT transformed Log 1/R spectra of the four grass samples/fractions

Differences were evident between the samples/fractions in the wavelength regions 1326, 1460, 1684-1730, 1926, 2000, 2056 – 2100, 2200, 2270 -2340 and 2410 nm. To look more closely at these differences spectral regions between Grass A and Grass B, and nominal stem and leaf sections of chopped samples were assessed (Figure 5.2). Positive regions relate to compounds that are higher in concentration in

Grass B than Grass A, and negative regions relate to compounds that are lower in concentration. The main difference between grasses (Figure 5.2a) was seen between 1900 – 2000 nm with a peak at 1936 nm, which is the O-H bend in the 2nd overtone for water (Shenk *et al.*, 1992). A shoulder is observed to this peak at 2040 nm, which along with the peak at 2164 nm is associated with N-H stretch due to Amide II and Amide III respectively (protein) (Murray, 1987). Higher N was observed in Stem B compared to Stem A (Table 5.1 and Table 5.2). The spectral region 1684 – 1730 nm is dominated by C-H bands in the 1st overtone region. A number of absorptions relate to aliphatic C-H absorptions, with the 1st overtone at 1728 nm and the combination bands at 2310 and 2350 nm. These have been associated with lipids (Bertrand, 2002; Decruyenaere *et al.*, 2009; Murray, 1987; Wetzels, 1983; Workman and Weyer, 2012). Spectral region 2244 nm is associated with indigestible cell wall (Deville and Givens, 1998), a small peak was observed in this region for the Stem nominal fraction. Lower DMD and DOMD values were observed in Stem B compared to Stem A (Table 5.1 and Table 5.2). A broad trough was observed with minima at 1594 nm and a sharp trough at 2090 nm, these correspond to the O-H bond in the 1st overtone (1580 nm) and combination (2100 nm) bands respectively for sugars (Murray, 1987). This is reflected in Table 5.1 and Table 5.2 which show that Stem B had a lower concentration of WSC than Stem A. Similar differences were seen between the leaf fractions for Grass B and Grass A, with the exception of spectral region 1900 – 2100 nm. Peaks at 1988 and 2050 nm were apparent, both of these relate to N-H absorptions. These wavelengths along with the peak centred at 2180 nm reflect higher N concentrations seen in Leaf B compared to Leaf A. The spectral region 2282 nm has been associated with cellulose (Workman and Weyer, 2012), and region 2346 nm associated with lipids (Murray, 1987).

The two nominal fractions were compared within each Grass and calculated as Leaf-Stem (Figure 5.2b). The most noticeable differences were observed in spectral region 1900 – 2100 nm and 2180 – 2190 nm. Spectral regions between 1900 – 2000 nm are likely due to differences in moisture. The trough observed at 2090 nm corresponds to various sugars, Leaf A had lower concentrations of WSC than Stem A (Table 5.1 and Table 5.2). Spectral differences 2180 nm showed differences between grasses, this region relates to N-H absorptions. Higher N concentrations were observed in Leaf A and Leaf B relative to their respective Stem fractions (Table 5.1 and Table 5.2).

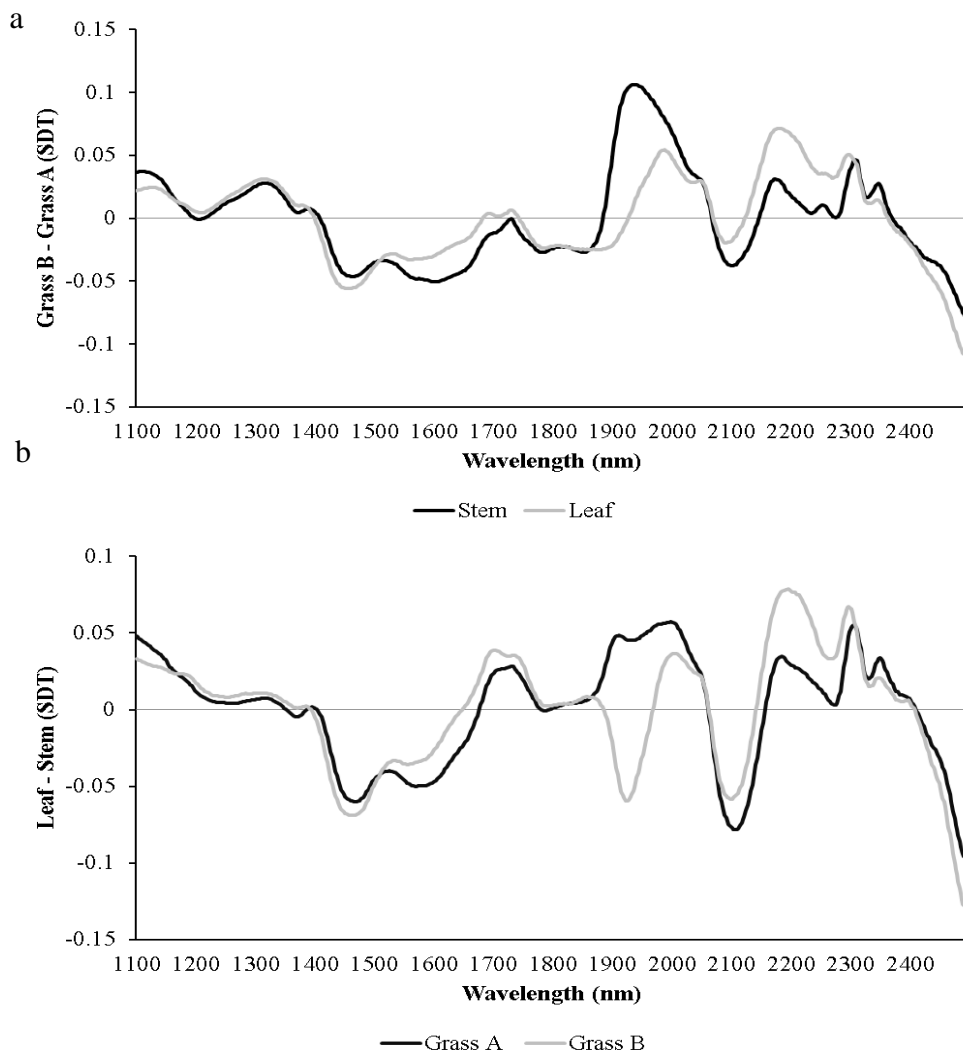


Figure 5.2 – SDT spectra for chopped (a) Grass B minus Grass A for the nominal Leaf and Stem sections, (b) nominal Leaf-Stem sections for Grass A and Grass B

5.3.3 DM disappearance

Samples were lost for one of the replicates (Rep 2) of the 2 h and 6 h incubations of Stem A owing to a defective rubber bung. DM disappearance increased as incubation time increased for each of the grasses and fractions (Figure 5.3). The Leaf fraction from Grass A showed higher DM disappearance between 12 and 24 h, followed by a slower DM disappearance over the following 24 h. DM disappearance did not differ between the grasses and fractions for the final time point (48+p; $P > 0.05$).

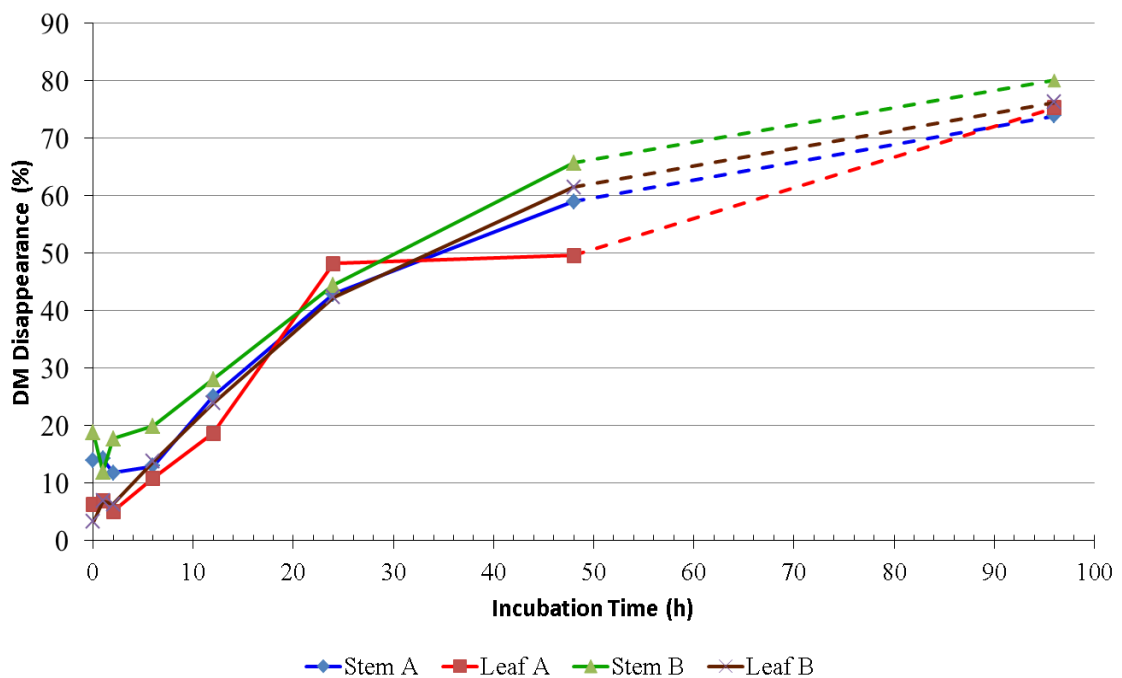
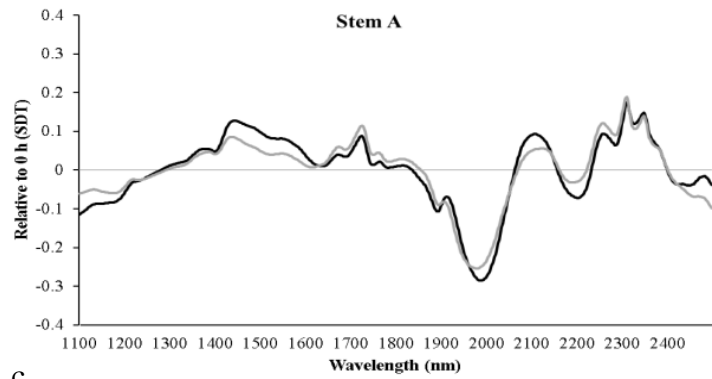


Figure 5.3 – DM disappearance % of Leaf and Stem fractions for Grass A and Grass B for each incubation time point; 0, 1, 2, 6, 12, 24, 48 h, 48+p (48+p represented as 96 h). The broken line represents the addition of the pepsin incubation step.

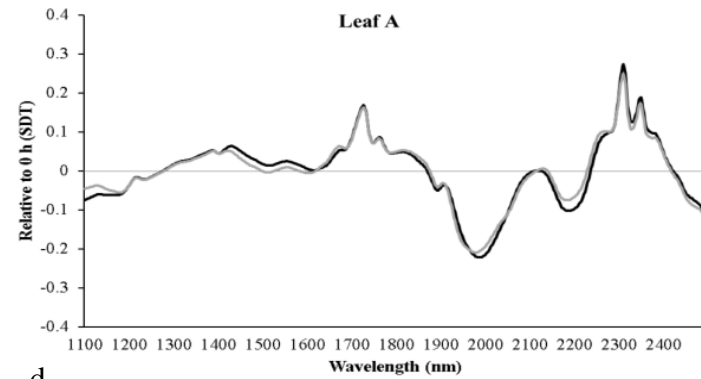
5.3.4 NIR difference spectra (0 – 48 h)

Differences were noted between difference spectra of residues from the two incubators. However no difference was found between the replicates for DM disappearance of residues ($P = 0.071$), therefore the replicate scans were averaged and difference spectra calculated. For each fraction, the 0 h spectrum was subtracted from all other time point spectra. Figure 5.4 shows differences between replicates for difference spectra of 48+p and 0 h, and therefore differences in overall digestion between the two incubators (Rep 1 and Rep 2). The main differences were observed in spectral regions 1900 – 2000 nm, in particular for Grass B. These spectral regions are associated with moisture.

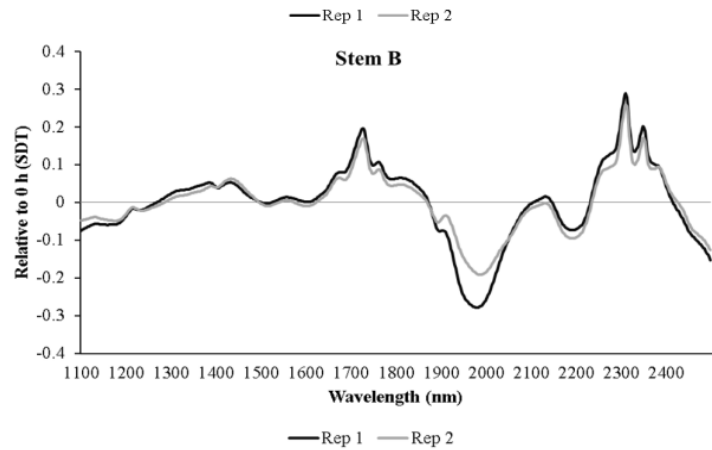
a



b



c



d

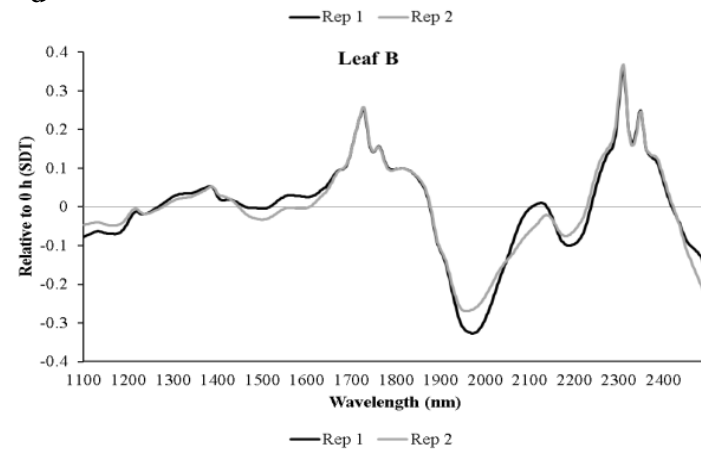


Figure 5.4 - SDT difference of pepsin relative to 0h of both replicates for (a) Stem A, (b) Leaf A, (c) Stem B, (d) Leaf B.

The difference spectra represent changes in the composition of the residues associated with the time course of rumen digestion are shown in Figure 5.5. Spectra show spectral regions that become increasingly positive with incubation time relate to compounds that increased in concentration and regions which become increasingly negative relate to compounds that were lost during the course of incubation (i.e. degradable compounds). Both grasses and the two fractions showed differences in the same spectral regions but they varied in amplitude scale. Spectral differences were greater for the Leaf fractions than Stem fractions and spectral differences were greater for Grass B than Grass A. Differences in residual moisture between the residues were apparent as changes in the spectral regions associated with water at 1940 nm (2nd overtone region) and 1450 nm (1st overtone region) (Shenk *et al.*, 1992).

Negative regions associated with high degradability were seen as a broad trough between approximately 1440 and 1620 nm. Minima were centred near 1460 - 1470 nm and 1570 - 1580 nm. The 1470 nm spectral region is associated with the first overtone of the N-H stretch (amide) (Murray, 1987; Workman and Weyer, 2012). The 1580 nm spectral region has been assigned to O-H bonds in the first overtone related to starch/glucose (Osborne and Fearn, 1986). The spectral region 1900 – 2000 nm is dominated by water (moisture). The peak centred at 2100 nm is characteristic of alcoholic –OH groups in compounds such as cellulose (Workman and Weyer, 2012). This appears as quite a broad peak in the Figure 5.4 so it is likely that the shape is affected by underlying shoulders near 2040 and 2170 nm which represent N-H stretch with Amide II and Amide III combinations respectively, from proteins (Murray, 1987).

Positive regions are associated with compounds of low degradability. The main spectral regions were dominated by sharp bands in the 1700 - 1770 and 2300 – 2400 nm regions. Both of these regions relate to aliphatic C-H absorptions. These are observed as 1st overtone bands at 1728 and 1762 nm and combination bands at 2310, 2350 and 2390 nm associated with lipids (Murray, 1987; Workman and Weyer, 2012). A small shoulder around 2260 nm was observed for Leaf A, Stem B and Leaf B. This spectral region has been assigned to the O-H stretch/C-O stretch combination in cellulose (Shenk *et al.*, 1992; Workman and Weyer, 2012) and has also related to

digestibility and lignin (Coleman and Murray, 1993; Workman and Weyer, 2012). A small peak centred at 1892-1898 nm increased with incubation time for Leaf A and Stem A. There appeared to be no peak in this region for Leaf B. This region is apparent as a shoulder on the broad peak centred at 1950 nm for Stem B.

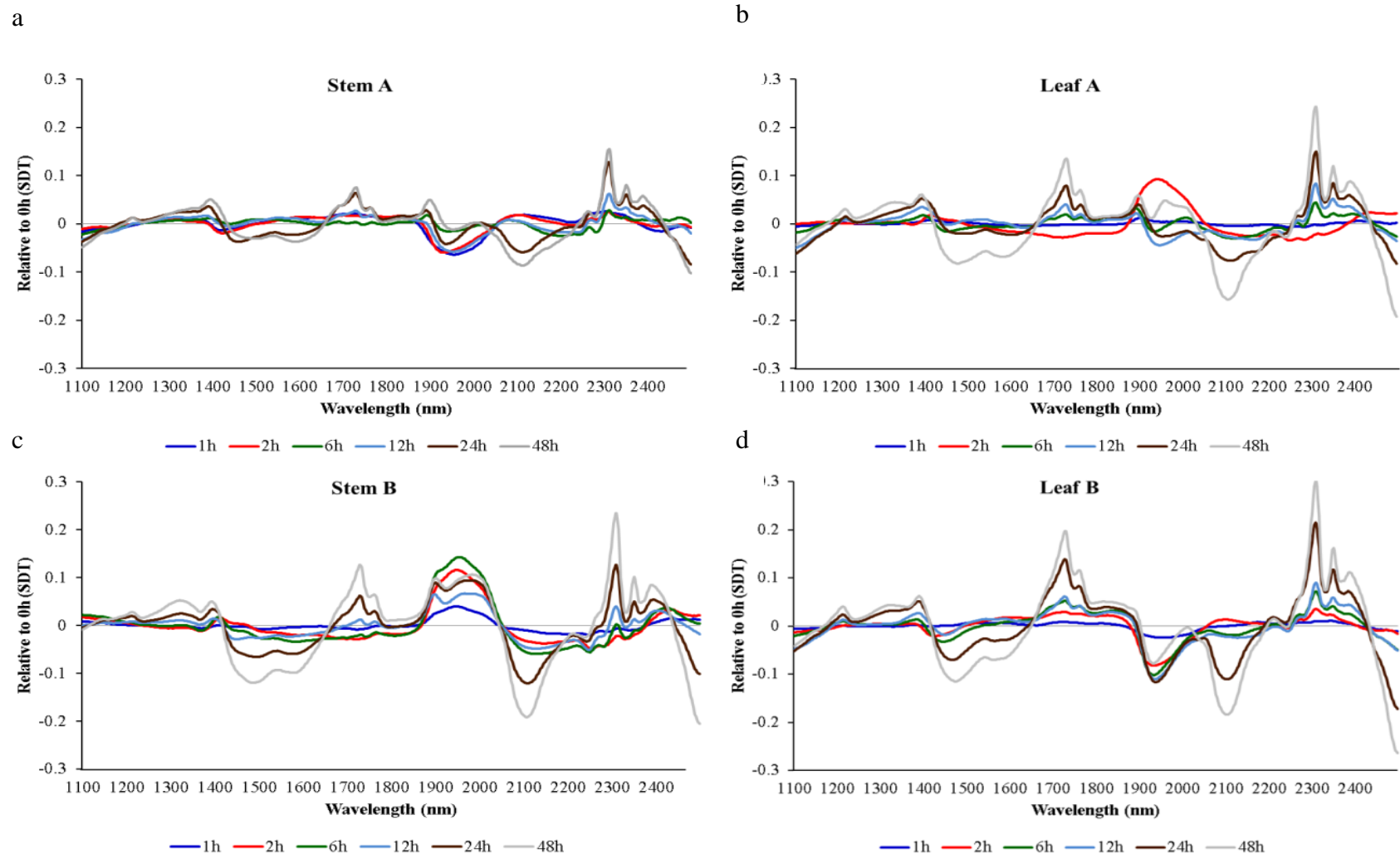


Figure 5.5 – (a) SDT difference spectra of 1, 2, 6, 12, 24 and 48 h spectrum relative to 0 h for (a) Stem A, (b) Leaf A, (c) Stem B, (d) Leaf B.

5.3.4.1 Deviation between residues

Standard deviation (SD) plots were compiled to assess variation between residues incubated at (i) 0 to 48 h and (ii) 0 to 48+p. SD plots show differences seen in Figure 5.5 but to a more condensed format.

Figure 5.6a shows variation between 0 and 48 h incubation times. Spectral variation occurred in similar regions over the two fractions and grasses up to 48 h. Stem B and Leaf B had similar amplitude for 1100 – 1900 nm but Leaf B had higher amplitude than Stem B in regions 1990 – 2500 nm. Leaf A showed similar patterns to Stem B and Leaf B but at lower amplitudes. Stem A showed lower amplitude, and therefore lower variation across the whole spectral region – 1100 – 2500 nm. Stem A showed distinct peaks at 1906 and 1990 nm, whilst other fractions showed a broad peak which centred at 1940 nm. Other differences were observed as a small peak for spectral region 2200 nm for Leaf B and a peak at 2256 nm for Stem A.

Figure 5.6b shows variation observed between 0 and 48+p residues. Similar regions were noted as described for the differences between 0 and 48 h residues, however a less distinct difference was observed between Stem A and Leaf A, and Stem B and Leaf B. A shoulder was observed at 1660 nm, this spectral region was more apparent for Stem A than other fractions. All fractions showed variation around 1970 nm and a shoulder was observed around 1890 nm which again is most pronounced for Stem A. Increased variation in regions 2180 – 2200 nm was seen in all fractions compared to that seen in Figure 4.10a. Stem A showed a distinct peak at 2256 nm, however this only appeared as a shoulder for Stem B.

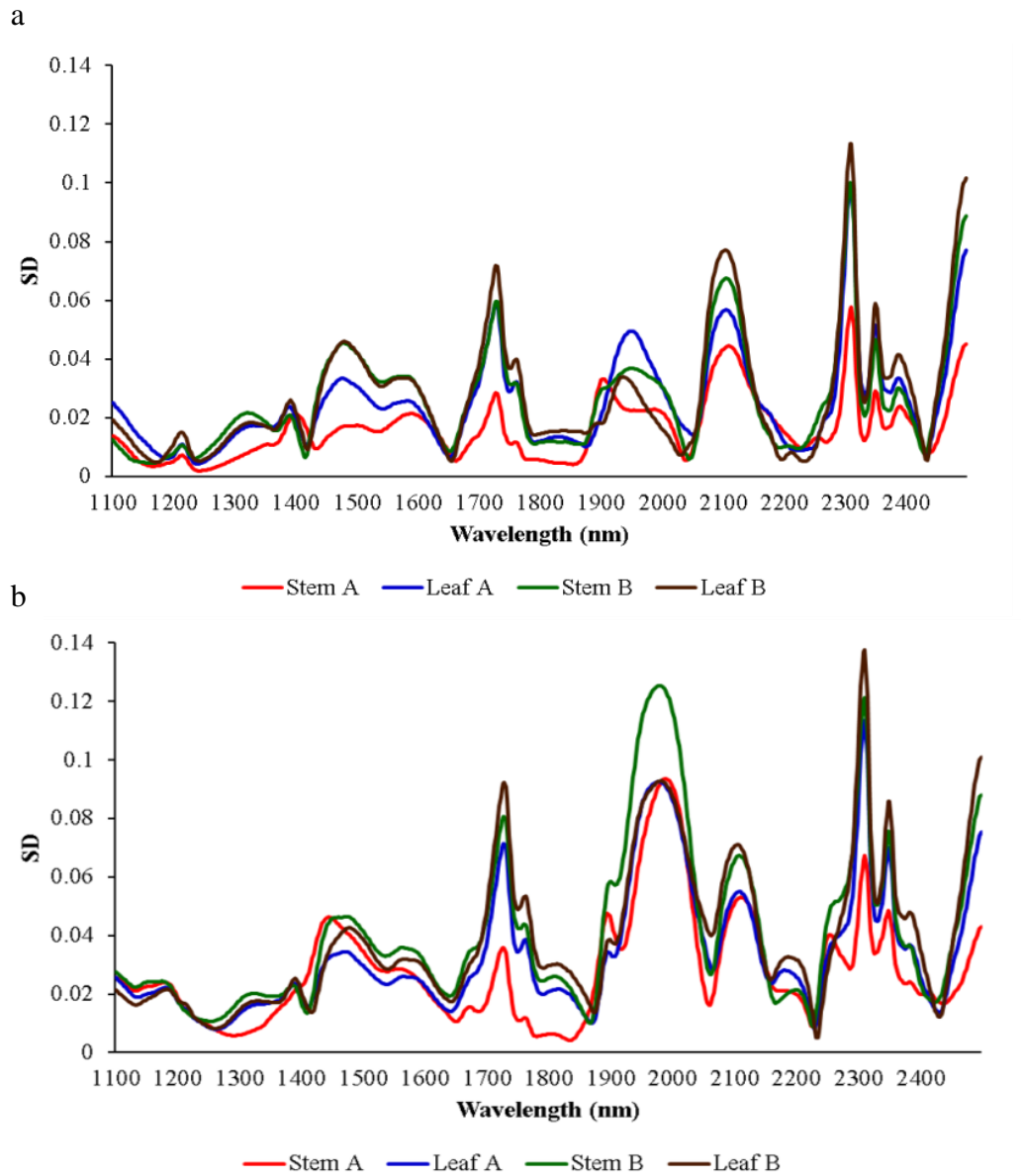


Figure 5.6 – SD plots for (a) 0 – 48 h residues and (b) 0 – 48+p residues

5.3.4.2 Pepsin relative to 48 h spectra

The addition of the pepsin stage was carried out to represent protein digestion post rumen. The difference between pepsin residue and the 48 h time point was calculated for all fractions (Figure 5.7). Increases in concentrations were observed at two main spectral regions: (i) 1400 – 1820 nm with underlying peaks at 1440, 1550 and 1710 nm and (ii) 2060 – 2380 nm with peaks at 2100, 2240 and 2330 nm. Spectral region 2090 – 2100 nm is associated with cellulose. Spectral region 1440 nm is due to moisture, 1550 nm due to N-H bonds, 1719 nm due to aliphatic C-H bonds (lipids),

2240 nm is associated with CHO bonds likely from lignin (Workman and Weyer, 2012) and 2330 nm due to cellulose (Workman and Weyer, 2012).

The negative spectral region was dominated by one trough centred at 1970 nm, with a shoulder at 1890 nm. Spectral region 1970 nm is associated with N-H bands, or moisture, and 1890 nm associated with C=O stretch of the 2nd overtone region. Troughs of smaller magnitude were noted at 2190 and 2404 nm. Spectral region 2180 nm has been associated with Amide III and 2404 is due to C-H combinations (Murray, 1987).

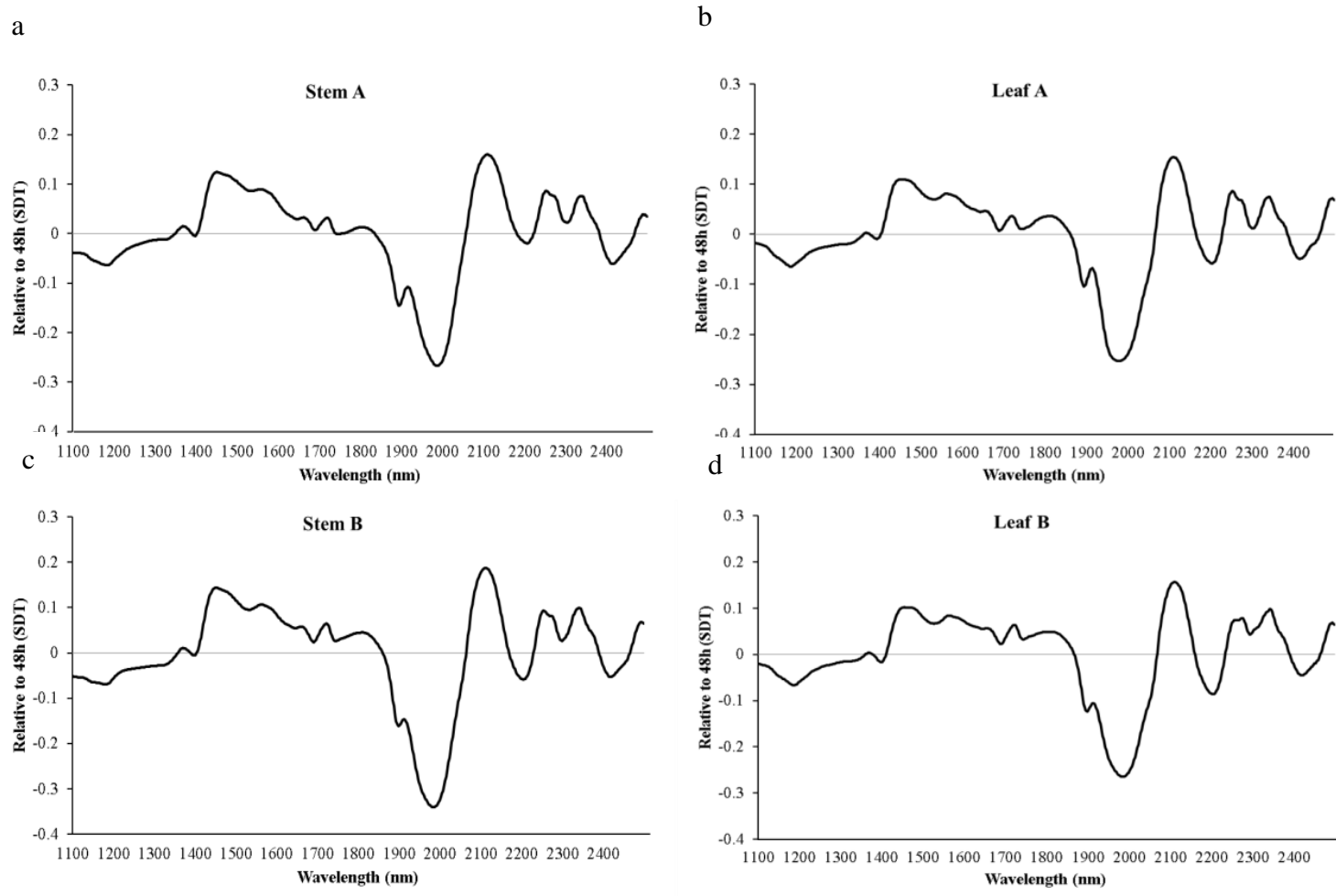


Figure 5.7 – SDT difference spectra of pepsin (48+p) relative to 48 h for (a) Stem A, (b) Leaf A, (c) Stem B, (d) Leaf B.

5.3.5 NIRS difference spectra (addition of pepsin)

The addition of the pepsin stage was carried out to look at the spectral differences due to the addition of pepsin relative to the 0 to 48 h differences. The differences between each pepsin residue and the 0 h time point was calculated (Figure 5.8). The addition of the pepsin stage resulted in differences in similar spectral regions as observed with the 1 to 48 h residues, however there were some noticeable differences. The main spectral regions which showed increase in concentration with time were dominated by sharp peaks in the 1700 - 1790 and 2300 - 2400 nm regions. Both of these regions relate to aliphatic C-H absorptions, in particular lipids.

Similar spectral regions were observed as noted in Figures 5.4 and Figure 5.5, for positive regions. Increases in concentration in the 1728, 1762, 2310 and 2350 nm regions were observed. A shoulder at 2370 nm was only observed after the addition of pepsin, this was previously observed as a peak. This region is associated with lipids. The spectral region 1450 nm was positive for all fractions except for Leaf B, however this region was found to be negative for difference spectra (0 – 48 h; Figure 4.8), this is likely due to moisture. Negative regions observed were very different to those seen for 0 – 48 h time points. The negative region was dominated by one large trough centred at 1980 nm, which had an addition of a shoulder at 1890 nm, however this shoulder was not as pronounced for Leaf B. The spectral region 1970 nm is due to the N-H stretch/bend combination and spectral region 1900 nm is caused by C=O stretch in the 2nd overtone region (Shenk *et al.*, 1992), however spectral region 1980 nm could also be due to water (Workman and Weyer, 2012). A smaller trough was also observed at 2160 – 2180 nm which is due to N-H stretch with amide III combinations generally deriving from proteins (Murray, 1987).

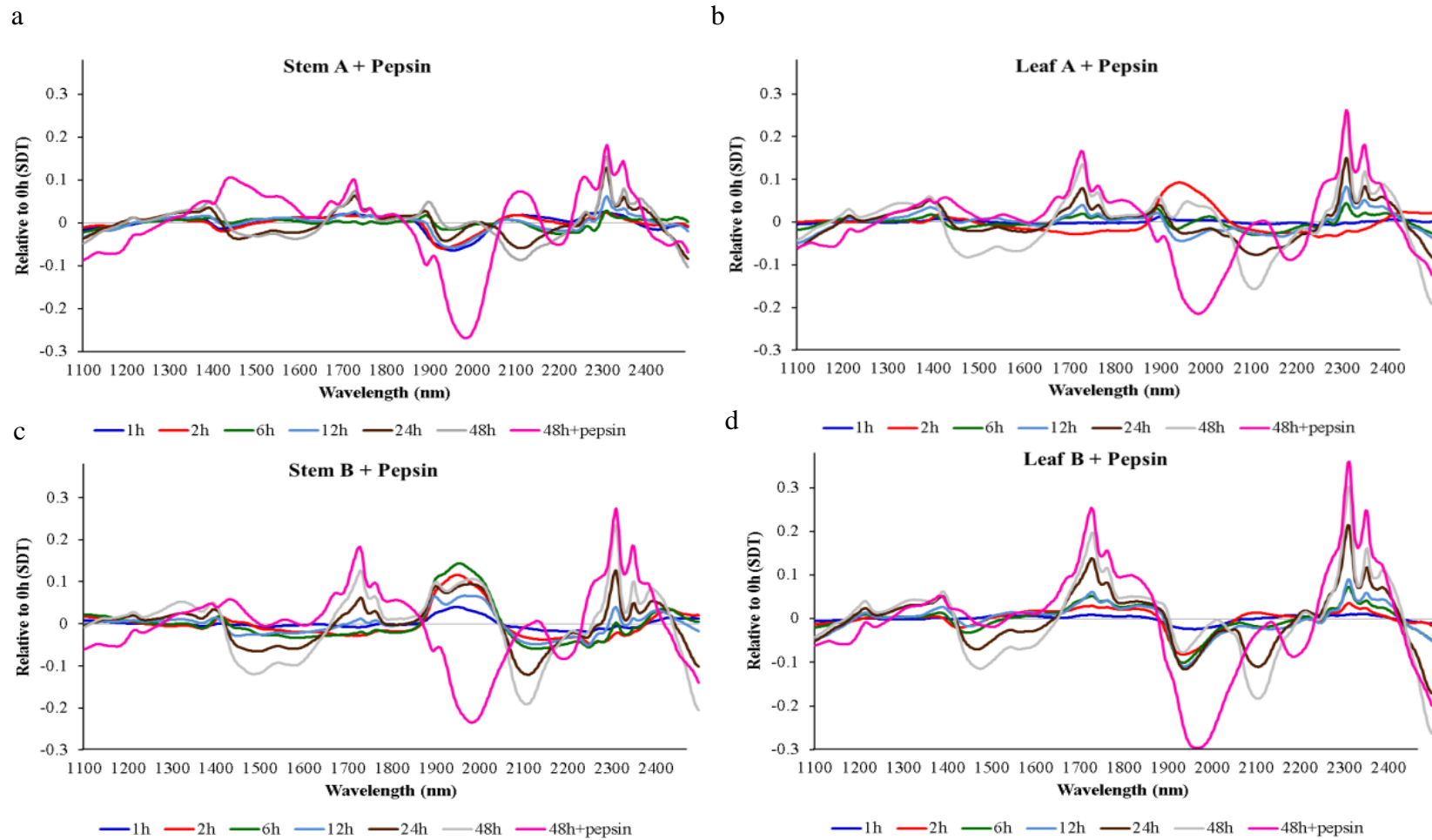


Figure 5.8 – SDT difference spectra, including additional pepsin stage for (a) Stem A, (b) Leaf A, (c) Stem B, (d) Leaf B.

Spectral differences between Grass A and Grass B and nominal stem and leaf sections were assessed after addition of pepsin to evaluate total tract digestion (Figure 5.9). Larger differences in peak amplitude were observed between Grass B and Grass A for Stem compared to Leaf fractions (Figure 5.9a). Spectral regions 1312, 1700 – 1880, 2300 – 2400 nm showed greatest differences in positive regions. These regions are associated with lipids. Spectral regions 1400 - 1600 and 1900 - 2120 nm showed greatest differences in negative regions. A shift in the water peak of Leaf nominal fractions was noted i.e. 1920 nm compared to 1934 nm in Stem nominal fractions. Negative regions at 1400 – 1600 nm had a broad trough at 1450 nm, likely caused by differences in residual moisture. A shoulder was observed at 1560 nm, this region is associated with protein. Larger differences in peak amplitude were observed between Leaf and Stem nominal sections for Grass A compared to Grass B (Figure 5.9b). Similar differences were observed between the fraction and grass pepsin residues.

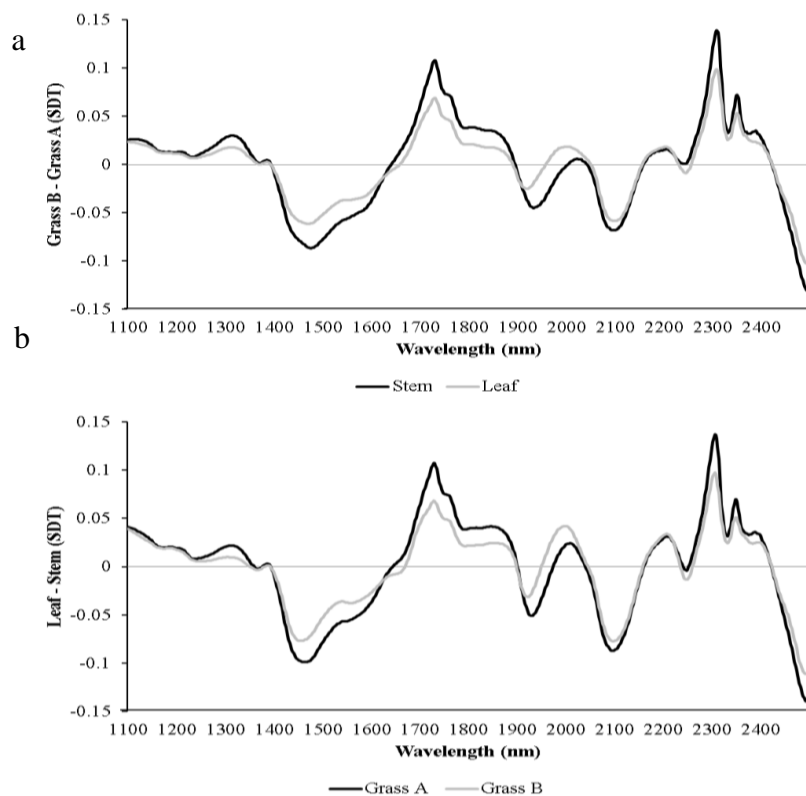


Figure 5.9 – SDT difference spectra of the pepsin residues (a) Grass B minus Grass A for Leaf and Stem fractions and (b) Leaf minus Stem for Grass A and Grass.

5.4 DISCUSSION

5.4.1 Differences between Replicates

Differences were observed between NIRS spectra for samples incubated in each of the incubators. Two incubators were used, with a sample of each substrate (grass and fraction) for each time point incubated in each. The temperature of each incubator was set using an independent external thermometer. Despite every effort being taken to match the incubator conditions, small differences in temperatures and shaking speeds could have led to differences in the rate of fermentation and hence the observed differences in spectra. DM disappearance was also numerically different between replicates, although this not statistically significant. Spectra were subsequently averaged to give one spectrum per grass and fraction per incubation time. It is important to note that 2 bottles were lost during the experiment owing to a defective bung these were from the same incubator (Rep 2) and both were from Stem fraction of Grass A (2 h and 6 h).

5.4.2 Differences between fractions and grasses

The stem fractions of Grass A showed consistently lower standard deviations across the NIR spectrum. Two samples were lost from this sample and it is likely this caused the lower standard deviations, especially as variation between replicates was observed. Leaf fractions showed highest standard deviations, indicating highest variation between time points and therefore greatest differences in degradable and non-degradable fractions. Leaf fractions are generally more digestible due to their higher levels of cellulose and hemicellulose and lower levels of lignin compared to stem fractions (Lambert and Litherland, 2000; Van Soest, 1994). Grass B showed highest standard deviations, Grass B was the shorter and younger of the two grasses. Largest standard deviations were observed for Leaf fraction of Grass B, indicating that this was the most digestible sample. This sample also had highest DOMD (Table 5.1) and DMD (Table 5.2) values. It has previously been shown that few spectral differences can be seen between Log 1/R spectra of young and old grass (Paul, 1991), as Log 1/R is generally featureless.

Difference spectra for regions 1870 – 2040 nm, with a peak at 1950 nm, were in the positive direction (Figure 5.5 and 5.8) for Grass B Stem fraction, whilst these regions were negative for the remaining grasses and fractions. It is unknown why this was observed but spectral regions 1940 nm is associated with moisture (Shenk *et al.*, 1992), therefore positive peaks for these samples could be due to differences in moisture content. Samples were dried at 40°C for 72 h and it is possible that low levels of moisture remained after the low temperature drying. It has previously been reported that small differences in residual moisture of silage causes large spectral differences between regions 1900 – 2000 nm (Baker *et al.*, 1994) as water may interact with other compounds such as protein and starch (Coleman and Murray, 1993).

5.4.3 Time Course Study (0 – 48 h)

As described above regions that became increasingly negative as incubation time increased are related to highly digestible compounds, whilst regions becoming increasingly positive relate to less digestible components.

Coleman and Murray (1993) used difference spectra between faeces and hay to determine spectral regions associated with digestion. They found that spectral regions 1450 – 1620 nm and 2100 – 2200 nm represented above average digestion whereas spectral regions 1714, 2256, 2306, 2436 and 2382 nm represented below average digestion. Results from their study showed that effects seen are the same but their spectra were calculated in reverse. Deaville and Givens (1998) assessed the time course of fermentation of fresh grass, grass silage and maize silage and calculated difference spectra on residues. These authors found that spectral regions 1430 – 1630 nm and 2020 – 2230 nm were associated with high degradability (decreasing troughs as incubation time increased), and spectral regions 1620 – 1690 nm and 2170 – 2290 nm were associated with low degradability (increasing peaks as incubation time increased). With the exception of spectral regions 2170 – 2290 nm these regions described correspond well to results seen in this experiment (Figure

5.8). Givens *et al.*, (1992) used NIRS to assess the time course of rumen digestion of ammonia treated and untreated straws. These authors also noted that spectral regions 1672 and 2254 nm were associated with indigestible cell walls, these regions were associated with positive regions in this study and therefore associated with less degradable fractions. These authors also noted spectral regions 1498 and 2086 nm to be associated with highly degradable components such as glucose and xylose, this also corresponds with results described in this study.

Difference spectra were dominated by spectral regions 2310, 2350 and 2388 nm. These have been previously associated with lipids (Betrand 2002; Decruyenaere *et al.*, 2009; Wetzel 1983), these regions were also repeated in the overtone regions at 1724 and 1766 nm. Lebzien and Paul (1997) determined spectral regions 2310 – 2348 nm were due to lipids in duodenal samples from dairy cows. Deaville and Givens (1998) did not report increases in the combination regions in the fresh grass difference spectra of their study, however they did report an increasingly positive spectral peak at 1720 - 1730 nm. Flinn *et al.*, (1992) reported that region is likely due to the presence of cuticular waxes. A study comparing NIR feed and faecal spectra (Coleman and Murray, 1993) suggested that aliphatic C-H stretch bands were likely due to sloughed cells or animal tissue, or cuticular waxes, however the study described here was carried out *in vitro* therefore peaks are not due to these compounds but likely due to cuticular waxes.

In this study, regions increased in magnitude as incubation time increased, therefore increased in concentration, increases were only seen after the 6 h incubation time points. Spectral differences of ammoniated and untreated straws digested *in sacco* were only evident after 48 h, as very little difference was observed at 12 and 24 h time points (Barton *et al.*, 1986)

Little DM disappearance was noted in the first 6 h. It is possible that this phenomenon was caused by one of two mechanisms (i) colonisation of microbes on the feed or (ii) increase in lipids within “the rumen”, or even a combination of both (i) and (ii). Proliferation of microbes during colonisation and digestion of feed increases the amount of lipids present. Goldfine, (1984) reported that microbial cells

contain 25 % lipids by weight. As plant matter is digested, lipids contained within the chloroplasts are released and digested. Kim *et al.*, (2005) incubated grass samples in nylon bags in the rumen of cows and showed an increase in biohydrogenation intermediates (particularly C18:1 trans-11) and odd-chain (microbial) fatty acids over the time-course of incubation. At the same time, they reported substantial reductions in concentrations of the fatty acids that were present in the original grass (C18:3 and C18:2). Spectral regions reported here that are associated with lipids correspond well to NIRS spectral scans of C18:2 and C18:3 (Murray, 1987). However, it must be noted that fatty acids are higher in concentration within the liquid phase (Dijkstra *et al.*, 1993), and therefore lost during the filtering step. Less defined peaks were noted for spectral regions associated with lipids when comparing 48+p to 48h spectra, therefore peaks are not due to hindgut fermentation, and likely not due to lipids present in microbial cells. Cuticular waxes are indigestible (Dove and Mayes, 1996), so would therefore increase in concentration as incubation time increased. Waxes would be bound closer to the solid sample and therefore little would be lost during the filtering stage, these would therefore be increasing in concentration as incubation time increased.

5.4.4 Time course study (addition of pepsin)

The addition of pepsin caused changes in the difference spectra, most notably troughs in regions 1890 and 1980 nm, associated with protein. A second smaller trough was observed at 2160 - 2180 nm. Formation of these troughs indicates that protein was decreasing in concentration as a consequence of incubation with pepsin. Pepsin is a well-documented proteolytic enzyme (Knowles, 1970; Tilley and Terry, 1963). *In vitro* digestibility is commonly carried out using the two step method described by Tilley and Terry (1963). Using this method samples are incubated in rumen fluid for 48 h with a second pepsin digestion stage to simulate digestion that would occur in the abomasum. A peak shoulder associated with C=O from carboxylic acids (Workman and Weyer, 2012) appeared with the addition of the pepsin digestion stage. Pepsin contains carboxylic acid groups that become protonated at low pH, e.g. with addition of hydrochloric acid (Delpierre and Fruton,

1965; Knowles, 1970). It is plausible that residual pepsin was present on the residues.

Lyons and Stuth (1992) used faecal NIRS using the second derivative to predict diet quality. These authors found that greater spectral absorption in high quality forage at 2297 nm. This region could indicate a microbial response, in response to quality of feed. These authors suggested the spectral region could be due to microbial cell walls, microbial by-products or microbial cells from the lower gastrointestinal. This spectral region was increased after the addition of pepsin, especially for Stem A. Stem A contained a higher level of WSC than other fractions. It is possible that microbial populations increased in response to the increased amounts of rapidly digestible feed. The action of pepsin would have lysed open cells therefore increasing concentrations of compounds associated with microbial cell walls. These authors also found that spectral region 2107 nm showed a greater absorbance in faecal samples from cattle fed low quality forage and suggested that regions were caused by increased proportions of cell wall carbohydrates. Lebzian and Paul (1997) also noted that spectral region centred at 2110 nm in duodenal samples was due to carbohydrates from microbial cells. Lister *et al.*, (1997) also noted a peak at 2120 nm in duodenal samples from steers fed a high DM silage and noted that this was likely due to sugars starch and cellulose. In the study described in this chapter an increase in concentration (relative to 0 h) was observed in Stem A. However, DOMD and DMD values for Stem A were similar to Leaf A and Stem B, where no peak was observed.

5.5 CONCLUSION

This study has proved useful to determine regions of the NIR spectra associated with degradable and non-degradable feed components. Regions of the spectra that disappeared most rapidly corresponded with those identified with high degradability in previous studies. Regardless of grass or fraction used, spectral regions associated with lipids increased over the course of incubations. The addition of pepsin showed disappearance in spectral regions associated with proteins.

CHAPTER 6

EVALUATION OF FAECAL NIRS FOR DESCRIBING DIFFERENCES IN FEED EFFICIENCY RESULTING FROM FEED RESTRICTION AND REALIMENATION

6.1 INTRODUCTION

Compensatory growth occurs when previously marginal- or under-fed animals are realimented on a higher nutritional level. Restricted feeding periods typically occur over the winter months, when feed quality is low, followed by an *ad libitum* (realimentation) feeding period once higher quality feed becomes available in spring. The latter period of greater or increased nutrient availability allows for live weight gains that are greater than would be predicted from feed intake alone. Live weight may be almost fully recovered if the compensatory growth period is long enough (Hornick *et al.*, 2000). Several factors allow for accelerated growth during the realimentation period, including increased gut fill, a reduction in maintenance energy requirements and increased feed intake (compared to the restriction period). Mechanisms which affect digestion, such as passage rates and rumen retention time, may play a role also.

6.1.1 Compensatory growth at the animal level

Cattle are able to adapt to the period of feed restriction by lowering maintenance energy requirements. The reduction in maintenance energy requirements results in an overall increased efficiency of energy utilization during the realimentation period. A metabolic lag phase after realimentation begins is observed, in which maintenance energy requirements remain low, before returning to higher maintenance levels at the end of the realimentation period (Carsten, 1995). This lag phase, coupled with increased DM intake, increases the availability of energy for growth, allowing for previously restricted cattle to have higher weight gain during the compensatory period. During feed restriction the body becomes leaner, with fat deposition more affected than protein deposition. Visceral organs exhibit the most marked loss of weight, followed by subcutaneous fat. The efficiency of energy deposition increases during the realimentation period, causing rapid growth of the visceral organs and deposition of subcutaneous fat, more so than deposition of intramuscular fat (Hornick *et al.*, 1998; Yambayamba *et al.*, 1996). Fat has a lower water content than lean tissue, and combined with its higher energy content results in higher energy requirements per kg growth when fat predominates. Visceral organs, such as the liver and gastrointestinal tract decrease in size during feed restriction. Due to the liver

being a highly metabolically active organ and the primary site of gluconeogenesis, changes in energy metabolism have a large effect on the efficiency of energy use by animals (Hornick *et al.*, 2000).

Passage rates and retention time within the rumen have long been associated with digestibility of feed. A longer retention time within the rumen allows for further chemical and physical breakdown of the feed by rumen fermentation. This is particularly beneficial for less fermentable fractions, such as fibre. As intake increases, passage rates of the liquid and solid digesta also increase. Higher passage rates through the rumen leads to a reduction in particle breakdown and reduced fermentation of feed by the microbes. Previous studies have shown that restricted feeding results in improved digestion of feed. Faecal outputs of organic matter (OM), nitrogen (N), neutral detergent fibre (NDF) and acid detergent fibre (ADF) were found to be lower in dairy bucks when fed at 25 % maintenance level, however digestibility was similar to bucks fed at maintenance during the realimentation period (Karakou *et al.*, 2008). A decrease in N excretion during both the restriction and realimentation periods has been shown in pigs subjected to dietary amino acid restrictions (Fabian *et al.*, 2004) and in double-muscled Belgium Blue bulls, in which N retention was highest for the compensating group (Hornick *et al.*, 1998).

6.1.2 Compensatory growth at the cell and tissue level

Differences between-animals under restricted feeding and realimentation have also been observed at the cell and tissue levels. Firstly, protein turnover rates are altered during both the restriction and realimentation periods. Restrictive feeding results in a decrease in rates of muscle protein degradation. Compensatory growth has previously been found to enhance both protein synthesis and degradation rates in cattle (Hornick *et al.*, 2000; Therkildsen, 2005; van Eenaeme *et al.*, 1998). When dry matter intake (DMI) is increased during the realimentation period, there are increases in both synthesis and degradation of protein, with synthesis taking place at a faster rate than degradation giving rise to rapid growth.

Hormones, particularly insulin-like growth factor 1 (IGF-1), play a role in accelerated growth during the realimentation period. During compensatory growth

the production of growth hormone (GH) by the pituitary gland increases, whilst the number of GH receptors decreases (Breier *et al.*, 1988; Yambayamba *et al.*, 1996). This leads to GH resistance and reduces IGF-1 secretions. In addition fatty acid mobilisation is increased due to the high levels of growth hormone circulating in the plasma (Hornick *et al.*, 2000). During the realimentation period, more nutrients are able to be utilised as a result of the increased insulin levels and plasma GH levels, which remain high (Hornick *et al.*, 2000, Keogh *et al.*, 2015b, Keogh *et al.*, 2015c). Similarly, levels of blood metabolites are altered during the compensatory and realimentation periods. Fiems *et al.*, (2007) found that metabolic differences (plasma concentrations of non-esterified fatty acids and α -amino nitrogen) between restricted and non-restricted animals disappeared at the end of the realimentation period in double-muscled Belgium Blues. During the restriction period, plasma insulin levels decrease, reaching a more normal level, compared to cattle offered *ad libitum* feeding, within the first few days of the realimentation period. It has been suggested that the higher plasma insulin levels allow for improved fat deposition during the realimentation period (Blum *et al.*, 1985).

6.1.3 Components of between-animal variation in feed conversion ratio

Some differences in feed conversion ratio (FCR) may be explained by between-animal variation in rumen and hind gut digestion processes, as well as differences in host metabolism. However, little has been reported in the literature about the contribution of such mechanisms in the case of compensatory growth. Herd *et al.*, (2004) showed where the variation between-animals in feed efficiencies (residual feed intake) lies, this can be accounted for by differences in heat increment of feeding (9 %), differences in digestion (14 %), differences in body composition (5 %) and differences in activity (5 %). These authors suggested that the remaining 65 % of the variation is most likely accounted for by heat loss due to variation in protein transfer and ion transport.

6.1.4 Estimating Differences in Digestibility

The traditional ‘gold standard’ method for estimating digestibility, and differences in digestion between individual animals, involves full digestion experiments in which animals are adapted to diets for a period of up to 3 weeks, followed by recording of total intake and faecal output for a period of 5 to 10 days, as described by Thornton and Minson, (1973). This is a costly and laborious process and not suitable for characterising a large number of animals, especially large numbers of animals at the same point in time. A number of laboratory methods, including chemical analysis and *in vitro* digestion procedures with enzymes or rumen fluid, have been developed to predict digestibility values (e.g. two step digestion technique to determine dry matter digestibility (Tilley and Terry, 1963)). However, these laboratory methods are not suitable for use where variation in digestibility is related to animal factors. The need for more practical techniques to determine between-animal variation has resulted in the development of markers to determine digestion. Markers, either internal (already contained within the diet, e.g. acid insoluble ash and silica), or external (substance added to the diet and recovered from the faeces, e.g. chromic oxide (Cr₂O₃) or *n*-alkanes) are commonly used to determine faecal output, and ultimately intake and digestibility. McGeough *et al.*, (2010) showed that estimating digestibility using acid insoluble ash and total faecal collection showed good agreement when steers were fed whole-crop barley. The addition of grass silage to the diet weakened the agreement. Carruthers and Bryant, (1983) demonstrated that the use of Cr₂O₃ to assess intake was useful but they found that intake was overestimated by 14 %. Despite giving an estimation of digestibility, these techniques are laborious, often inaccurate, and unable to characterise a large number of animals; a more high-throughput method is required.

6.1.5 Feed Conversion Ratio

Live-weight gains and feed intakes are commonly recorded during feeding and digestion trials and can be used to measure feed conversion efficiency, commonly expressed as FCR:

$$\text{Feed Conversion Ratio} = \frac{\text{Feed intake}}{\text{Average Daily Gain}}$$

FCR is a method of measuring gross efficiency and commonly used to determine production efficiencies (Crews and Carstens, 2005) as the production efficiency of cattle is an important economic trait. Selection for lower FCR values is desirable, as lower values relate to less feed per unit of live-weight gain.

6.1.6 NIRS

Near infrared spectroscopy (NIRS) is a low-cost and rapid technique commonly used in agriculture and food science, which can be used to characterise chemical composition of a range of materials. Examples of this include assessment of meat quality (Prieto *et al.*, 2009), quality of and chemical properties of food and beverages (Woodcock *et al.*, 2008) and faeces. Previous studies have assessed the use of faecal NIRS to predict constituents, such as crude protein, fibre and digestibility of the diet, of the faeces (Dixon and Coates, 2009), however these require large data sets and associated laboratory data in order to form these predictions.

6.1.7 Aims

The aim of this study was to evaluate the use of faecal NIRS, without the use of calibrations, as a method for describing between-animal variation in digestibility as a result of feed restriction and realimentation. A secondary objective was to determine what residual effect, if any, the restricted period has on digestion processes.

6.2 MATERIALS AND METHOD

6.2.1 Experimental Design and Sample Collection

Samples collected were part of a wider trial to determine animal performance (Keogh *et al.*, 2015a, and mechanisms associated with compensatory growth (Keogh *et al.*, 2015b, c; Keogh *et al.*, 2016; McCabe *et al.*, 2015; O'Shea *et al.*, 2016). Full details of the experimental design can be found in Keogh *et al.*, (2015c). In brief, 30 Holstein-Friesian bulls with an age of 497 (s.e.m 15) days and weight of 370 (s.e.m

35) kg were assigned to either a low growth trajectory diet (Restricted; n=15) or a high growth trajectory diet (*ad libitum*; n=15) over 2 experimental periods (Period 1 and Period 2). The diet consisted of total mixed ration consisting of 70 % concentrate and 30 % grass silage, which was fed individually using Calan gates. Fresh weight of feed offered and feed refused was measured daily and sub-samples collected for dry matter determination and analysis of chemical composition. Period 1 consisted of 125 days in which bulls in the restricted group were fed to grow at 0.6 kg/day, whilst bulls on the high trajectory diet were offered feed *ad libitum*. Period 2 consisted of 55 days during which feeding groups were offered feed *ad libitum*. Where possible 8 faecal grab samples per bull were collected in total; one sample each in the morning (AM) and one in the afternoon (PM) of days 70 and 71, and AM and PM of days 43 and 44 during Period 1 and Period 2 respectively. Faecal samples were stored at -20°C until analysis. It should be noted that one bull, from the *ad libitum* group, was removed due to illness, all results have been analysed by removing data for that animal from all subsequent analysis. The illness was not related to the treatment (feed intake level).

6.2.2 Feed intake and analysis

As described by Keogh *et al.*, (2015a), fresh feed was offered daily, with feed intake also being recorded daily. Two samples of offered grass silage and concentrate sample were collected weekly and stored at -20°C prior to chemical composition analysis. Dry matter values were obtained on silage samples pooled on a weekly basis. Silage and concentrate samples, pooled on a 3 week basis were analysed for ADF, ash, crude protein (CP), dry matter digestibility (DMD), digestible organic matter content (DOMD), NDF, Oil A (concentrate only) and organic matter digestibility (OMD), as described by Keogh *et al.*, (2015a).

6.2.3 Live weight and FCR measurements

As described by Keogh *et al.*, (2015 c), bulls were weighed every two weeks during Period 1, and weekly during Period 2. In addition, bulls were weighed on 2 consecutive days at the start of Period 1 and at the end of Periods 1 and 2. FCR values were calculated for *ad libitum* and restriction groups separately during both Period 1 and Period 2. Data was transformed with square root transformation as

results were not normally distributed. Square root transformations were analysed in GenStat (v14; VSNI, Hemel Hempstead, UK) using one way ANOVA with transformed FCR values as Y-variate and diet (*ad libitum* vs. restriction) as Treatment. One way ANOVA was performed on Period 1 and Period 2 separately. Means on the square root scale were converted back to the original scale by squaring. FCR values were ranked (lowest to highest) and bulls subsequently assigned to either low (most efficient) or high (least efficient) groups. This was carried out within Period 1 and Period 2 separately. Due to unequal numbers within *ad libitum* and restricted groups, FCR levels were split as follows: *ad libitum* (Low, n=7; High, n=7), restriction (Low, n=7; High, n=8))

6.2.4 NIRS ***

Defrosted samples were dried at 65°C for 80 h before being ground through a 0.75 mm sieve. Dried and ground samples were dried for a further 16 h at 80°C and scanned on NIRSystems 5000 as described in Section 3.2.1.

6.2.5 Spectral Analysis

Data was transformed as described in Section 3.2.2 and subsequently averaged to give on spectral value per bull. Data was analysed separately for Periods 1 and 2. Mean transformed spectra were calculated for both Period 1 and Period 2. Standard deviation (SD) plots were also derived for these groups for the different periods to identify spectral regions of maximum variance (Excel, 2010). Within each period, the effect of feeding level on the first (PC-1) and second (PC-2) principal component scores was assessed by one-way ANOVA (GenStat), with PC-1 and PC-2 values as Y variate and diet (*ad libitum* or restriction) as factor. FCR values were assigned to either Low or High groups, as described in section 5.2.3. FCR groups were labelled within feeding level (restriction or *ad libitum*), principal component analysis (PCA) was performed and associated loadings assessed. Effects of FCR level was assessed for PC score within Period 1 and Period 2 separately using one way ANOVA (GenStat). In this PC score was set as the Y variate, FCR level (Low/High) set as

*** Preparation of faecal samples and NIRS scans were carried out by Lauren Van Rooyen at Teagasc Grange during summer 2012***

treatment group and treatment (restriction or *ad libitum*) as a block. The effect on individual FCR values on PC scores was assessed within Period 1 and Period 2 separately using a simple linear regression (GenStat).

6.2.6 Chemical analysis of feed

The mean chemical composition of both concentrate and silage samples is presented in Table 6.1. Diets were fed at 70:30 ratio (concentrate:silage) throughout the trial:

Table 6.1 – Chemical composition of concentrate and silage offered during each period. Full chemical analysis found in Keogh *et al.*, (2015a)

Feed	Constituent (g/kg)	Period 1		Period 2	
		Mean	s.d.	Mean	s.d.
Concentrate	ADF	46.9	3.16	49.4	2.62
	Ash	51.8	5.71	50.7	2.38
	CP	182.3	13.08	174.2	8.42
	DMD (g/kg DM)	887.8	10.90	881.4	9.91
	DOMD (g/kg DM)	889.1	11.97	884.1	8.89
	NDF	118.8	8.89	121.2	3.16
	Oil A	17.2	1.05	15.2	1.37
	OMD	882.2	13.82	874.3	14.02
Forage	ADF	360.7	30.15	266.7	15.34
	Ash	71.2	5.47	91.3	3.79
	CP	118.9	9.87	143.3	2.90
	DMD (g/kg DM)	647.5	52.18	807.2	33.65
	DOMD (g/kg DM)	585.4	55.16	723.2	33.45
	NDF	588.9	48.43	444.1	13.28
	OMD	630.1	56.91	795.9	36.74

6.3 RESULTS

6.3.1 Growth Rates and Intake Data

There was a significant difference ($P < 0.001$) in DMI between restricted and *ad libitum* groups during Period 1, 5.4 and 12.7 kg/day respectively. However, there was no significant difference ($P = 0.660$) between restricted or *ad libitum* groups in DM intakes during Period 2, 11.1 and 11.6 kg/day respectively. During period 1 restricted bulls grew at an average rate of 0.6 kg/day whilst *ad libitum* bulls grew at an average of 1.9 kg/day, giving an average live weight difference of 161 kg per bull between groups at the end of the first experimental period. At the end of the realimentation period (Period 2) an average live weight difference of 84 kg was found.

6.3.2 FCR

FCR values were calculated for individual bulls, separately for Periods 1 and 2. During Period 1 FCR values ranged from 5.59 to 8.84 for *ad libitum* bulls and 6.74 to 17.9 for restriction bulls. FCR values ranged between 7.43 – 28.83 for *ad libitum* bulls and 2.99 – 15.04 for restriction bulls. Averaged results for each period are shown in Table 6.2, and ranges within low and high groups in Table 6.3. For the restricted group FCR values reduced from 10.19 in Period 1 to 8.82 during the realimentation period (Period 2). The decrease in FCR allowed cattle to recover from a 161 kg difference per animal after Period 1 to 84 kg difference per animal at the end of Period 2. The opposite was seen for *ad libitum* bulls, where an increase in FCR, 6.89 to 14.21, from Period 1 to Period 2 was seen.

Table 6.2 – Mean values for FCR on square root scale and back-transformed to the original scale (kg/kg).

Period		Treatment Group		s.e.d	P value
		<i>Ad libitum</i>	Restriction		
Period 1	$\sqrt{\text{FCR}}$	2.624	3.19	0.138	<.001
	FCR	6.89	10.18		
Period 2	$\sqrt{\text{FCR}}$	3.77	2.97	0.215	<.001
	FCR	14.21	8.82		

Table 6.3 – Ranges of FCR values within low and high groups for *ad libitum* and restricted bulls

Period		Treatment Group	
		<i>Ad libitum</i>	Restriction
Period 1	Low	5.59 – 6.33	6.74 – 9.86
	High	6.44 – 8.84	10.16 – 17.90
Period 2	Low	7.43 – 12.96	2.99 – 8.14
	High	13.44 – 28.83	8.51 – 15.04

6.3.3 NIR Spectral Analysis

6.3.3.1 Differences between treatments

Log (1/R) spectra for faecal samples from the restricted and *ad libitum* groups during Period 1 and Period 2 are shown in Figure 6.1a. Transformations were carried out using SDT, plots for restricted and *ad libitum* groups can be found in Figure 6.1b. Differences between Period 1 and Period 2 was seen in spectral regions 1430 – 1480, 1640, 1720, 1860, 2220 and 2310. Spectral regions are associated with lipids (1720 and 2310 nm; Workman and Weyer, 2012), C-H in 1st overtone region (1640, 1860 nm) and N-H bonds (1470 and 2220 nm; Murray, 1987). Differences between restricted and *ad libitum* fed bulls in Period 1 were noted in spectral regions 1920, 2056 – 2170 and 2220 nm. These regions are associated with moisture (1920 nm; Shenk *et al.*, 1992), N-H bonds (2220 and 2056 - 2170 nm).

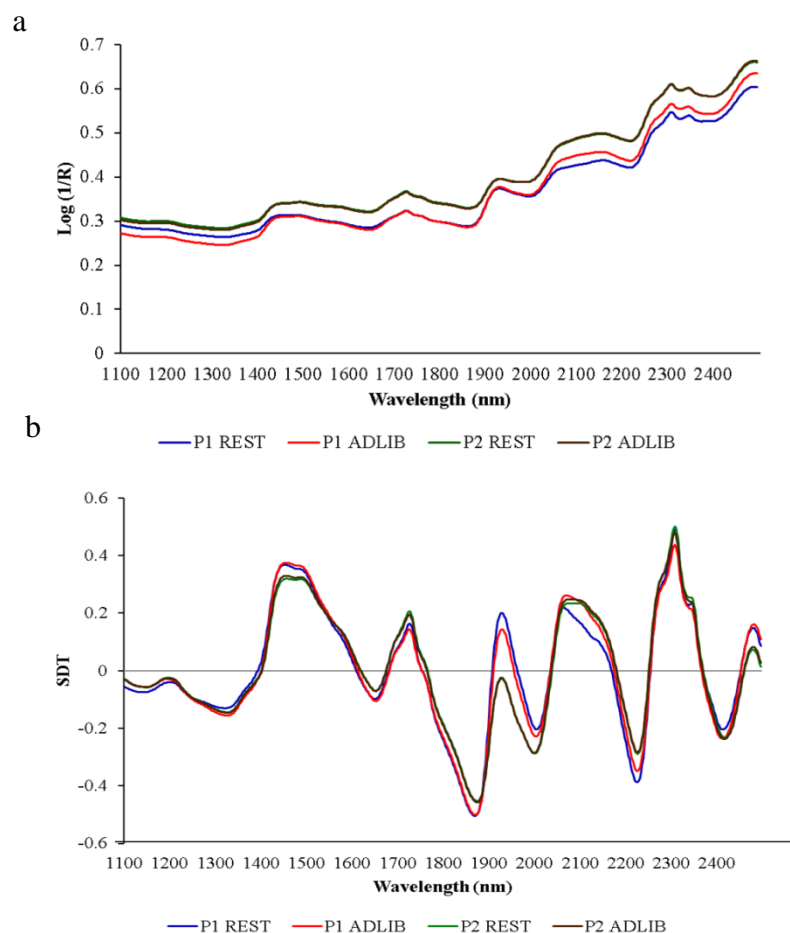


Figure 6.1 – Spectral of faecal samples from restricted and *ad libitum* bulls from Period 1 and Period 2 as (a) Log (1/R) and (b) transformed spectra using SDT.

Difference spectra were calculated between Period 1 and Period 2 and between restricted and *ad libitum* bulls. Differences between *ad libitum* and restricted bulls in Period 1 relative to Period 2 can be found in Figure 6.2a. Negative spectral regions indicate regions that are lower in concentration in Period 1 compared to Period 2, whilst positive regions relate to compounds increased in concentration. The main negative regions were noted as a broad trough between 1750 – 1880 nm and a trough at 2200 nm. The spectral region 1750 – 1880 nm is dominated by C-H absorptions (Workman and Weyer, 2012) and spectral region 2200 nm (Workman and Weyer, 2012) associated with carbohydrates. An additional trough associated with sugars and cellulose was noted at 2100 nm, however this was only present for restricted bulls. The main positive spectral region was noted at 1924 nm, which is due to moisture (Shenk *et al.*, 1992). A smaller peak was noted at 1440 and 1420 nm for *ad libitum* and restricted bulls respectively. This region is also caused by moisture.

Differences between Period 1 and Period 2 of *ad libitum* bulls relative to restricted can be found in Figure 6.2b. The main negative regions were noted in regions 1320, 1380, 1910, 2308, 2348 and 2378 nm for Period 1. These regions are associated with C-H bonds (1320 and 1380 nm), moisture (1920 nm) and lipids and cuticular waxes (2308, 2348 and 2378 nm; Flinn *et al.*, 1992). The main positive regions were noted at 2090 and 2200 nm. For Period 2, very little difference was observed between *ad libitum* and restricted bulls which resulted in a line very close to the origin.

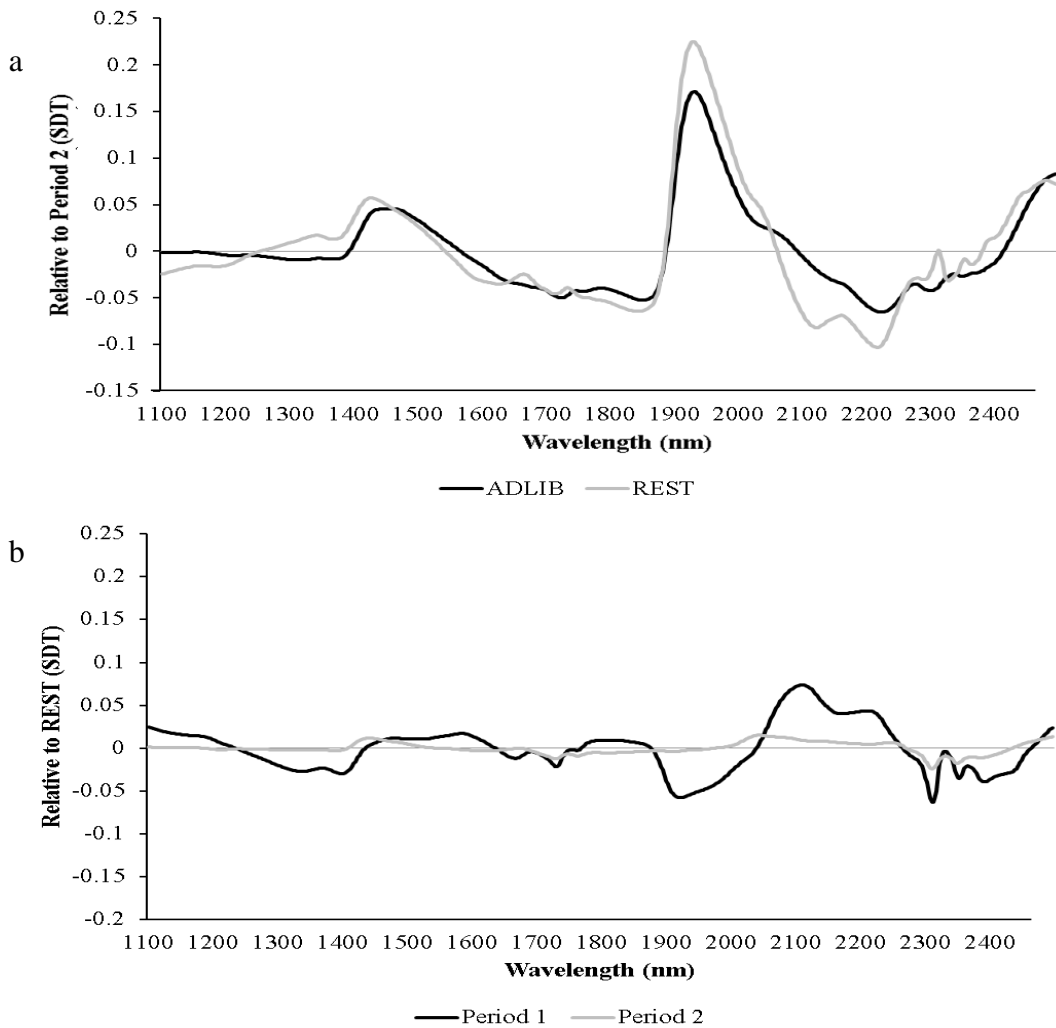


Figure 6.2 – Difference spectra (SDT transformed) between (a) Period 1 relative to Period 2 and (b) *ad libitum* relative to restricted.

PCA plots for Period 1 and Period 2 were calculated. A clear separation of diet in Period 1 was observed, with separate clusters for each intake level (restricted vs. *ad libitum*) clearly apparent (Figure 6.3a). Seventy six per cent of variation was described by the first two principal components. Feeding level (restricted or *ad libitum*) had a significant effect on PC-1 ($P < 0.001$) but not on PC-2 ($P = 0.180$)

during Period 1. The grouping associated with intake treatment group observed during Period 1 was not seen during Period 2 (Figure 6.3b), indicating that there was no residual effect of restricted feeding 43 days into the realimentation period. Feeding level had no effect on PC-1 ($P = 0.681$) or PC-2 ($P = 0.189$) during Period 2, with 74 % of variation being described by the first two principal components. One bull, circled in Figure 6.3 a and b, had consistently lower DMI during both periods. This bull, from the *ad libitum* group, clustered with bulls on the restricted diet during Period 1. There were no factors recorded, such as ill health, which appeared to be related to the low intakes of this animal.

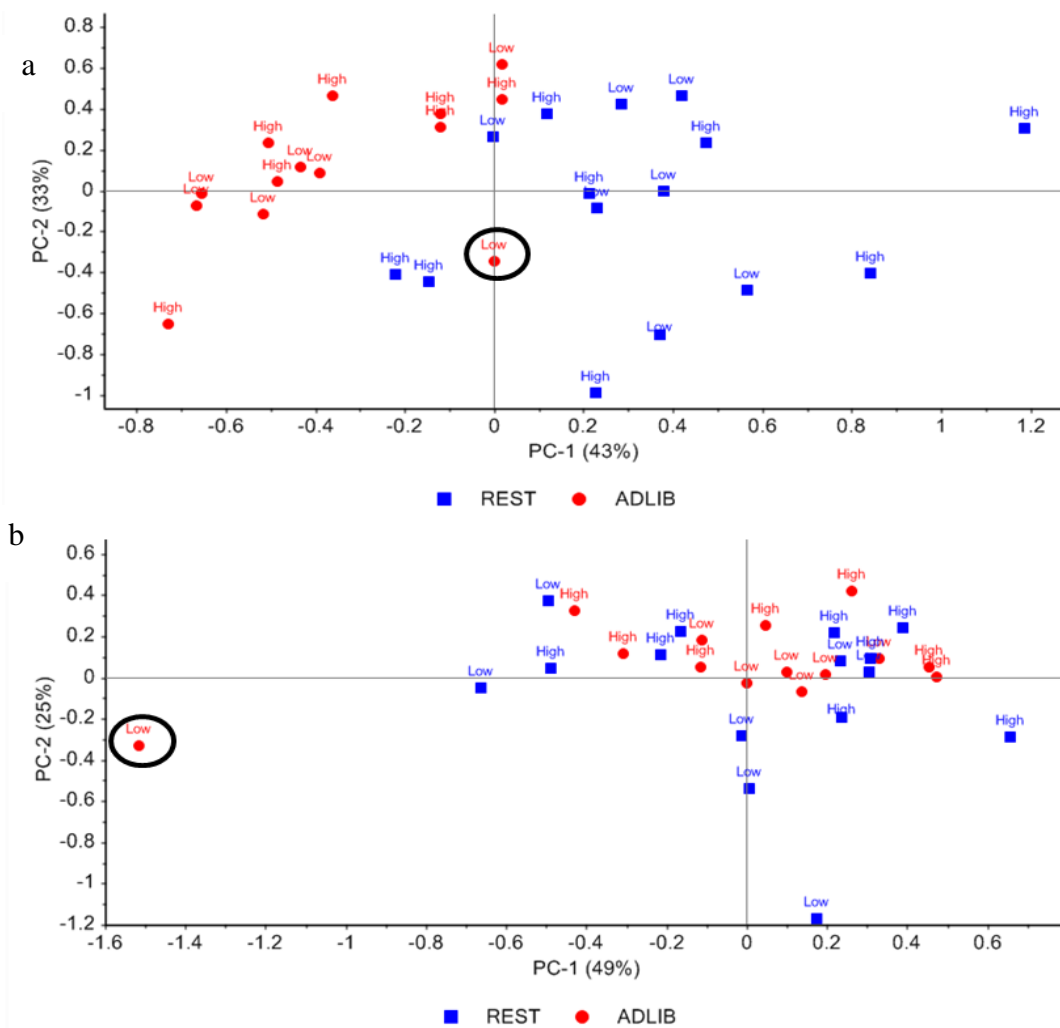


Figure 6.3 – PCA scores plot for NIR spectra (a) during Period 1 and (b) during Period 2. Samples are labelled by FCR level (Low or High) within each intake group. The circled bull had low intake during Period 1 and was seen to be an outlier in Period 2.

6.3.3.2 Differences in FCR level

No grouping was observed for FCR level (Low or High) for either *ad libitum* or restricted diets during Period 1 (Figure 6.3a) or Period 2 (Figure 6.3b). Low or High FCR level was found to have no effect on PC-1 ($P = 0.994$; $P = 0.836$) or PC-2 ($P = 0.769$; $P = 0.219$). However when assessed using regression FCR was found to have a significant effect on PC-1 ($P > 0.001$) during Period 1 and PC-2 ($P = 0.023$) during Period 2. FCR had no effect on PC-2 during Period 1 or PC-1 during Period 2 ($P > 0.05$). As stated above, one bull from the *ad libitum* group had low intake so appeared within the restriction group during Period 1, interestingly this animal was in the low FCR group, as a result of its low DMI.

Faecal samples were analysed using PCA to examine the relationships between samples from bulls on the different feed levels (restricted or *ad libitum*). To investigate the separation between feeding level seen during Period 1, the associated PC loadings were examined. Associated loadings for Period 2 were also assessed to assess spectral regions linked with variation seen between bulls.

The loadings associated with PC-1 and PC-2 from the PC scores plot for Period 1 (Figure 6.3a) are shown in Figure 6.4a. A broad peak was observed for PC-1 between 1240 – 1420 nm with peaks observed at 1332 and 1394 nm. These spectral regions are dominated by C-H absorptions in the 2nd overtone region. A peak was observed at 1732 nm with shoulders at 1660 and 1766 nm. These spectral regions are associated with lipids (1732 and 1766 nm; Flinn *et al.*, 1992; Workman and Weyer, 2012) and absorptions of C-H bonds in the 1st overtone region (1660 nm). A broad peak was observed in spectral regions 1880 – 2030 nm. The spectral region 1900 – 2000 nm is dominated by moisture. Peaks were observed at 2310, 2350 and 2388 nm, these regions are associated with aliphatic C-H bonds, in particular those from lipids. Negative regions were noted at two main spectral regions: (i) a broad trough between 1420 – 1630 nm with minima at 1470 and 1580 nm and (ii) a trough observed at 2100 nm with a shoulder at 2230 nm. Spectral regions 1470 and 1580 nm are associated with protein and cellulose respectively (Murray, 1987; Workman and Weyer, 2012). Spectral regions 2100 and 2230 nm are associated with sugars, starch and cellulose.

For PC-2 positive regions were noted as a broad peak between 1680 – 1900 nm. Peaks were observed in this region at 1750 and 1880 nm, which represent aliphatic C-H bonds (lipids) and C-H bonds. Peaks were observed at 2000 and 2210 nm, these regions represent N-H bonds (Murray, 1987). The main negative region was noted as a trough between 1270 – 1670 nm. Minima was noted at 1450 nm with contributing shoulders at 1350 and 1560 nm, these regions are associated with C-H bonds (1350 nm) and starch and glucose (1560 nm). A second small trough was observed at 1920 nm likely due to differences in moisture.

The loadings associated with PC-1 and PC-2 from the PC scores plot from Period 2 (Figure 6.3b) are shown in Figure 6.4b. During Period 2, for PC-1 peaks were noted at 1730, 1766, 1988, 2210 and 2310 nm in the positive direction. Regions 1730, 1762 and 2310 nm are associated with lipids, spectral region 1988 nm is associated with N-H bands, but could also be affected by moisture (Shenk *et al.*, 1992). Spectral region 2200 nm is associated with carbohydrates. A broad trough was noted in the negative region with minima at 1450 nm, a second trough was noted at 2100 nm. Spectral region 1450 nm is associated with moisture and 2100 nm associated with compounds containing N-H bonds. For PC-2 peaks were noted at 1450, 1934, 2048 nm of which a shoulder was observed at 2170 nm. Spectral regions 1450 and 1934 nm are likely caused by residual moisture. Spectral regions 2048 and 2170 nm are associated with N-H absorptions due to protein. Negative spectral regions were noted as a broad trough between 1270 – 1420 nm, with minima at 1390 nm, and troughs at 1730, 1766, 2310, 2348 and 2390 nm. Spectral regions 1730, 1766, 2310, 2348 and 2390 nm are highly characteristic of lipids.

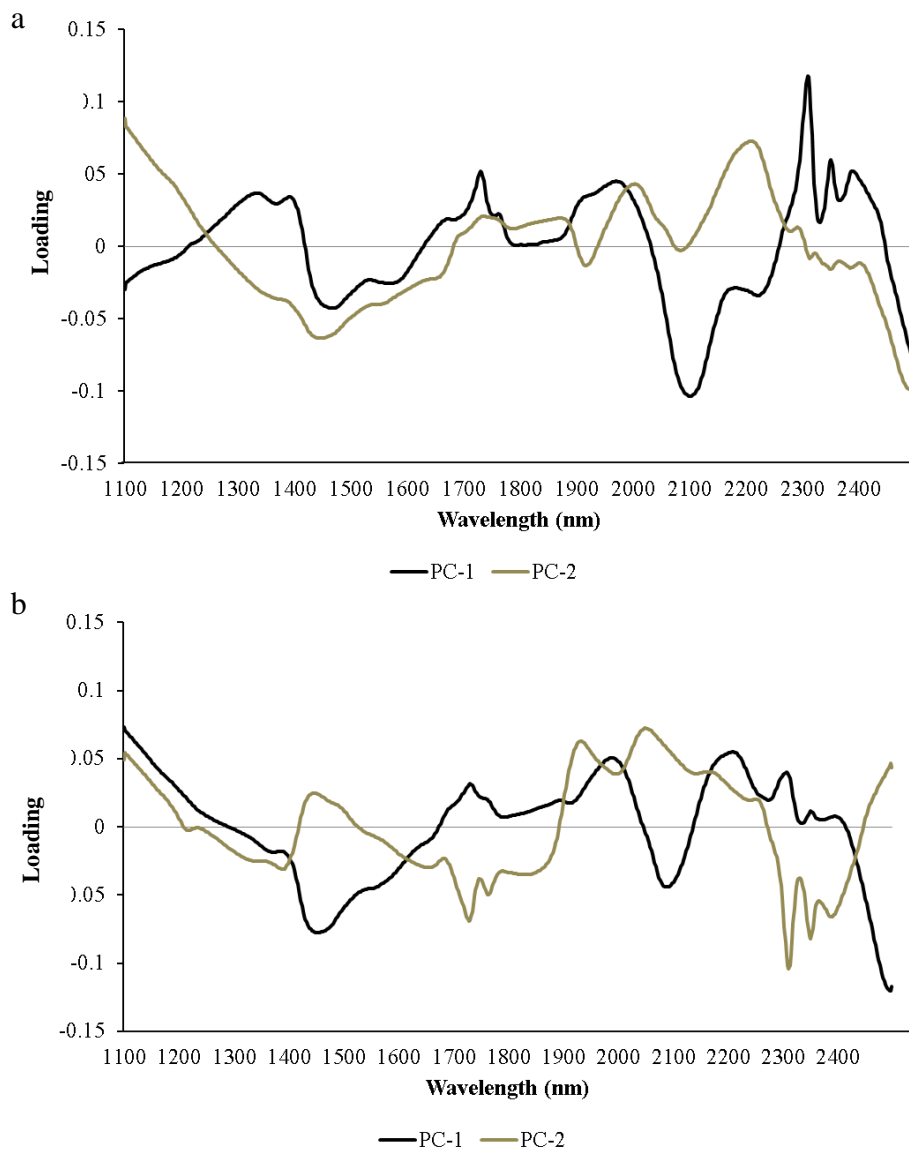


Figure 6.4 - Loadings plots of PC-1 (black) and PC-2 (grey) during (a) Period 1 and (b) Period 2.

Standard deviation (SD) plots were compiled to assess the variation in spectral data at each wavelength (Figure 6.5). Differences between Period 1 and Period 2 can be seen in 3 main spectral areas, of which Period 1 showed more variation in spectral regions 1246 – 1416 nm, with peaks observed at 1326 and 1382 nm showed variation between periods, these regions are largely associated with C-H and O-H bands (Workman and Weyer, 2012). Secondly, spectral regions 2052 – 2230 nm, with peaks at 2092 and 2200 nm showed differences between periods. These broad regions are associated with cellulose and protein respectively (Murray, 1987). Finally, spectral regions 2310 and 2388 nm showed differences in standard deviations between periods, these peaks are characteristic of lipids (Bertrand, 2002; Flinn, *et al.*, 1992).

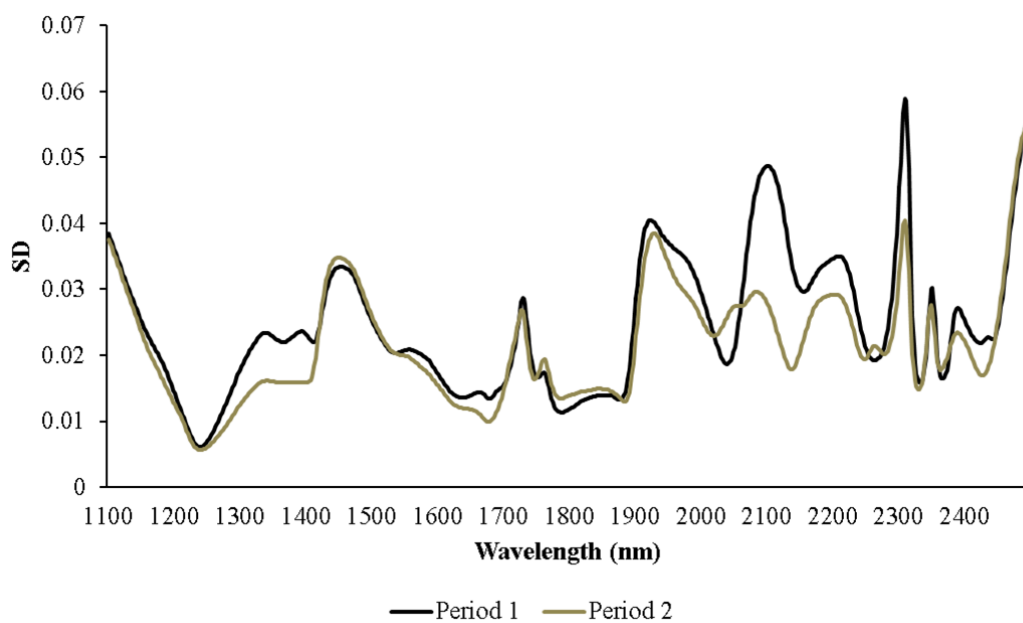


Figure 6.5 – SD plot to show which spectral show greatest variation between Period 1 and Period 2.

6.4 DISCUSSION

The aim of this study was to assess the use of NIRS as a rapid technique to assess potential differences in digestion resulting from feed restriction and subsequent realimentation.

6.4.1 NIR spectral differences

SDT transformed data showed differences between periods and intake levels (Figure 6.1). Differences observed between period and between diets were investigated further using multivariate analysis and difference spectra.

Using PCA scores allows variation between-animals within groups to be visualised, along with differences between groups (restricted vs. *ad libitum*). Chemical differences in the faeces associated with feeding level, either restricted or *ad libitum*, were observed during Period 1, with two clear groups being observed in the PC score plots (Figure 6.3). Reduced intake levels, coupled with increasing efficiency and lower passage rates through the rumen probably account for differences between restricted or *ad libitum* groups. The compensatory index, which describes the success of regrowth, of bulls from this study was found to be 48 % after 55 days of realimentation (Keogh *et al.*, 2015a). However, the lack of differences in the PC scores plot (Figure 6.3) indicates that there was no residual effect of restricted feeding on digestion processes.

Spectral regions associated with lipids, protein and moisture showed highest loadings during Period 1 and thus explained the differences seen between *ad libitum* and restricted groups observed in Figure 6.3. Rust and Owens (1981) reported that low intake resulted in an increase in ruminal ammonia amounts, which was a likely result of increased protein digestion. This study also reported that increasing feed intake resulted in decreased digestibility of starch, fibre and nitrogen using chromic oxide to determine digestibility. In the current study PC loadings of compounds containing C-H bonds, moisture and lipids associated with Period 1 indicated that compounds increased in concentration in restricted bulls relative to *ad libitum* bulls. The higher levels of readily digestible compounds in the faeces of *ad libitum* fed steers indicates

that passage rates were increased allowing for decreased digestion of feed. Spectral regions associated with lipids, protein and moisture also showed highest loadings during Period 2. This suggests that differences observed were due to differences in extremes of intake and not due to differences in FCR values between intake groups. Difference spectra for Period 2 (Figure 6.2b) showed very little variation between *ad libitum* and restricted bulls, indicating that differences seen in the Figure 6.3b were due to natural variation in digestion between-animals. Significant differences between FCR of restricted and *ad libitum* groups were observed for Period 1 and Period 2, and significant differences noted between PC-1 and FCR and PC-2 and FCR during Period 1 and 2 respectively, however separation was only observed in PC scores plot for Period 1.

Greater variation was observed in Period 1 in SD plots. Differences were seen in regions associated with protein, cellulose, moisture and lipids again indicating that differences seen in Figure 6.1 were due to digestion of these components. Spectral regions associated with lipids were observed in the regions of 2310, 2350 and 2388 nm, and repeated in the overtone region at 1730 and 1762 nm. The 2310 nm spectral region has been previously associated with degradability related to methylene stretch (Russell *et al.*, 1989), indigestible fibre (Barton *et al.*, 1986) and carbohydrate, lignin and protein, and waxes (Barton *et al.*, 1992). As discussed in Chapter 5 spectral regions 2310, 2350 and 2388 nm are likely due to lipids found in cuticular waxes (Flinn *et al.*, 1992). Coleman and Murray, (1993) found that these regions were associated with below average apparent digestion in faeces and suggested that these lipids may be of plant or animal (host) origin. Flinn *et al.*, (1992) suggested that these peaks could be due to cuticular waxes and not from animal origin. Lebzien and Paul, (1997) reported that spectral regions 1722 – 1728 nm were characteristic of either lipids or carbohydrates found in duodenal samples of cows. Differences observed in Figure 6.3b showed that lipids were found to be increased within restricted bulls in Period 1 compared to *ad libitum* bulls. It is likely that digestion of grass was increased within the restriction group causing an increase in concentration of cuticular waxes. Longer retention time of feed in the rumen, caused by decreased intake, would potentially allow for increased digestion of feed. Cuticular waxes present in the leaves could also be liberated through increased chewing efficiency

which has been reported in animals offered restricted feeding compared to animals offered *ad libitum* feeding (Dias *et al.*, 2011). Increased rumination time per unit of dry matter has been found in restricted fed lambs compared to *ad libitum* fed lambs (de Souza Rodrigues *et al.*, 2014).

6.4.2 Factors affecting digestibility

Spectral differences between *ad libitum* and restricted groups were observed during Period 1, whilst no apparent differences between groups were seen during the realimentation period during Period 2. Passage time dictates the amount of time feed remains within the rumen. Retention time is an important factor for the control of intake and digestibility (Welch, 1986). Longer retention time within the rumen, or decreased passage rates, allow for further fermentation of digestion of feed. Reduced passage rates allow for further digestion of feed, especially components with slower rates of fermentation, such as fibre. Dias *et al.*, (2011) showed that cattle offered hay *ad libitum* and at three intake levels (1.5, 2.0, 2.5 % body weight) had differing retention times. Lower intake levels led to increased retention time and increased chewing efficiency, which subsequently led to an increase in digestibility ($P < 0.001$). An increase in gut fill has been shown in restricted animals (Sainz *et al.*, 1995), however compensatory growth has been shown to be independent of level of gut fill.

Spectral region 2100 nm has been previously associated with the –OH groups of starch, cellulose and sugars (Murray and Williams, 1987). Coleman and Murray (1993) found that spectral region 2100 – 2200 nm is characteristic of above apparent digestion using difference spectra of faeces and feed of steers fed hay. Spectral region 2145 nm has been found to correspond well with digestible organic matter (DOM) in faecal samples (Lyons and Stuth, 1992). Difference spectra indicate that increased concentration of these compounds were found in the faeces of *ad libitum* fed bulls during Period 1 compare to restricted bulls. This indicates that lesser digestion of these compounds has taken place possibly due to an increase in passage rates. Montgomery *et al.*, (2004) showed that limit-fed steers had a slower passage rates (longer retention time) than *ad libitum* fed steers and thus increases in digestibility, using pulse doses of Ytterbium labelled alfalfa hay. These authors also

found that overall, limit-fed steers had decreased ruminal fill on a DM basis, contrary to results reported by Sainz *et al.*, (1995), however the fill increased at a faster rate for limit-fed steers for the first 4 h after feeding. Previous studies have shown that digestibility increases as feeding level decreases during the restriction period in sheep, due to increased retention time allowing for further digestion of feed (Marais *et al.*, 1991). Feed was offered *ad libitum*, 80%, 65% or 50% of *ad libitum* intake.

6.4.3 Feed efficiency of previously restricted animals

FCR values decreased from 10.18 in Period 1 and 8.82 in Period 2 bulls, which is in agreement with other studies. It has previously been reported that FCR is reduced after a period of feed restriction (Hornick *et al.*, 1998), allowing for greater feed efficiency. The period of restricted feeding typically occurs over winter months when poorer quality feed is available. Subsequently this is followed by an *ad libitum* (realimentation) period when higher quality feed is available, typically this occurs during the spring months. A combination of the lower FCR and increased nutritional value of the silage (Table 6.1) allowed for greater ADG in the realimentation period (Period 2). However, FCR usually increases after around 2 months of *ad libitum* feeding (realimentation period) (Hornick *et al.*, 1998). It has been suggested that the greater efficiency of previously restricted animals is due to the increased deposition of fat and protein and reduced maintenance energy requirement (Keogh *et al.*, 2015a; Ryan *et al.*, 1993). Although useful to describe gross efficiency of animals, FCR does not take into account differences in intake requirements for maintenance energy and growth requirements (Carstens and Tedeschi, 2006).

An increase in FCR was observed (6.89 to 14.21) for *ad libitum* animals between Periods 1 and 2. Although higher quality feed was available during Period 2, the FCR value increased and therefore animals became less efficient, bulls had a final LW of 652 and 557 kg for *ad libitum* and restricted diets respectively. The increase in FCR was likely due to older animals becoming less efficient as they increased fat and reduced protein deposition, during the fattening phase (Wood *et al.*, 2008).

6.4.4 Faecal sample time points

The restriction period for this trial lasted for 125 days, followed by a realimentation period of 55 days. Faecal samples were collected at days 70 and 71 during the restriction period (Period 1), and towards the end of Period 2 on days 43 and 44. Although the length of restriction periods differs between studies, typically the period lasts between 80 (Ryan *et al.*, 1993) and 145 days (Vega *et al.*, 2009), with realimentation typically taking place over 2 to 3 months. Sampling during Period 2 occurred on days 43 and 44 of 55. There was no apparent residual effect on faecal characteristics at this point in time, despite the fact that the animals had not fully compensated. The extent of recovery from compensatory growth has previously been referred to as the compensatory index, which typically ranges from 50 – 100 % (Hornick *et al.*, 2000). In this study the compensatory index was 48 % by the end of the 55 day realimentation period.

6.5 CONCLUSION

Faecal NIRS was able to identify differences in digestion between restricted and *ad libitum* groups during the first (restriction) period of this study, however there were no residual effects 43 and 44 days after the feed restriction ended. Spectral regions due to differences observed during Period 1 were caused by lipids, likely from cuticular waxes, and protein and moisture. The effect of feed restriction on faecal NIR was most likely due to differences in digestion processes linked to reduced rumen passage rates. The wavelengths associated with differences between samples from the *ad libitum* and restriction groups during Period 1 were also responsible for between-animal variation observed when all animals received the same feed during Period 2.

CHAPTER 7

**THE USE OF FAECAL NIRS, FAECAL LASER
DIFFRACTION AND 16S SEQUENCING OF THE
RUMEN MICROBIOME TO ASSESS
DIFFERENCES IN FEED EFFICIENCY OF
STEERS OFFERED FEED ADDITIVES
DESIGNED TO REDUCE METHANE EMISSIONS**

7.1 INTRODUCTION

Livestock systems, especially ruminant systems, are a significant source of greenhouse gas (GHG) emissions worldwide. Enteric methane (CH₄), a by-product of fermentation, accounts for approximately 10 - 12 % of global methane emissions (Hristov *et al.*, 2015; IPCC, 2014). Methane production also represents an energy loss to the animal, therefore reduction of these emissions is of both environmental and economic benefit.

7.1.1 Reducing methane emissions through additives in the diet

The majority of methane production is dependent on the supply of hydrogen, high concentrate diets favour propionate production, whereas forage based diets favour acetate production. The diet has been the basis of effective mitigations of methane emissions through dietary manipulation (McAllister *et al.*, 1996). Hulshof *et al.*, (2012) suggested that methane emissions could be reduced by removing methanogens, reducing hydrogen (H₂) production, or providing an alternative H⁺ sink

The addition of lipid to the diet has reduced methane emissions in a number of previous studies (Grainger and Beauchemin, 2011; Martin *et al.*, 2010). Lipids are typically added as pure fats, oils, or as in this study, the addition of high-fat ingredients such as by-products from distilleries. It has been suggested that lipids may reduce methane emissions in several ways: by (i) directly inhibiting methanogens and protozoa, (ii) decreasing the proportion of fermentable feed (fatty acids are not fermentable), (iii) reducing the digestibility of fibre particles by coating them in the lipid, or (iv) increasing propionate production (Johnson and Johnson, 1995).

Nitrate has previously been assessed as a means of reducing methane emissions from beef cattle (Hulshof *et al.*, 2012; Huyen *et al.*, 2010; Leng *et al.*, 2012) and dairy cows (van Zijderveld *et al.*, 2011). A full review of feeding nitrate to ruminants has been carried out by Lee and Beauchemin (2014). Nitrate is reduced to nitrite, and nitrite reduced to ammonia (Lee and Beauchemin 2014; Ungerfeld and Kohn 2006)

in the rumen. This provides an alternate hydrogen sink for the production of methane. The nitrate reduction pathway proves to be more energetically favourable than CO₂ reduction (Nolan *et al.*, 2010).

Nitrate and the intermediate product, nitrite, are toxic to the animal, especially if animals have not previously been exposed, or adapted. An adaption period is required as nitrate and nitrite can build up in the rumen and be absorbed into the bloodstream. Nitrite binds to haemoglobin and converts it to methaemoglobin. Methaemoglobin has an increased affinity for oxygen, allowing more efficient oxygen binding however, it is less able to release oxygen to the tissues than haemoglobin (Reilly and Winzor, 2000) so has negative consequences for animal performance and health.

7.1.2 Assessment of feed efficiency

Methane emissions result in a significant energetic loss to the animal, accounting for 2 – 12 % of gross energy intake (Johnson and Johnson, 1995). Residual feed intake (RFI), which is the difference between expected feed intake and actual feed intake (Koch *et al.*, 1963), is a useful way of expressing feed efficiency in cattle. Cattle with low RFI values eat less than expected for their weight and growth rate, so are more efficient. Hegarty *et al.*, (2007) reported a significant negative relationship between enteric methane emissions and RFI in beef cattle. They found that beef cattle with lower RFI had lower daily methane production (g/d) using the sulphur hexafluoride (SF₆) technique. Differences in breed also result in differing feed efficiency values. Limousin (LIM) cattle have a larger mature size and faster growing rates than smaller and earlier maturing breeds such as Aberdeen Angus (AA). This leads AA to have higher RFI values (i.e. being less feed efficient) than LIM cattle (Crowley *et al.*, 2010).

7.1.3. Rumen microbial communities

The effects of dietary manipulation on ruminants have typically focused on animal production traits, as traditional culture-based microbiology has been unable to

provide sufficient detail (Lee *et al.*, 2012) to establish links between-animal production traits, such as feed efficiency and the rumen microbiome. Studies using next-generation sequencing (Caporaso *et al.*, 2012; McCabe *et al.*, 2015) have enabled more rapid and detailed descriptions of these microbial communities. Recent studies have shown that even small shifts in the rumen microbial communities can have marked effects on productivity (McCann *et al.*, 2014a; Shabat *et al.*, 2016). Bar-coded next generation sequencing, known as multiplexing, allows for multiple samples to be sequenced in a single run, whilst the amount of sequence generated per run allows for extensive coverage of communities. This multiplexing approach is achieved by adding a short sequence of nucleotides (bar-codes) to the beginning of the sequence, with each short sequence being unique to an individual sample (Caporaso *et al.*, 2011; Caporaso *et al.*, 2012). The majority of studies using this technique have focused on the effects of different diets (Henderson *et al.*, 2015; Poulsen *et al.*, 2012; Zened *et al.*, 2012) but fewer studies have investigated the effect of feed efficiency on the rumen microbiome (Jewell *et al.*, 2015; McCann *et al.*, 2014a; Shabat *et al.*, 2016). However it must be noted that microbial populations differ between-animals (Ross *et al.*, 2012). A recent review by McCann *et al.*, (2014b) discussed the use of high-throughput methods to determine the rumen microbiome and how it was affected by nutrition.

Many rumen methanogenic Archaea produce methane by converting H₂ and CO₂ to methane in a seven step pathway (Attwood and McSweeney, 2008). There has been recent interest in another group of Archaea, Thermoplasmata which utilise methanol and methylamines in the production of methane ('methylotrophic' as opposed to 'hydrogenotrophic' methanogenesis). Poulsen *et al.*, (2013) reported the methanogenic properties of Thermoplasmata due to the presence of methyl-coenzyme M reductase genes. Under their feeding conditions (rapeseed oil supplementation), they found that levels of Thermoplasmata were more important in affecting levels of methane emissions than the previously studied hydrogenotrophs (Methanobacteriales). Thermoplasmata have recently been classified as Methanomassiliicoccales (Seedorf *et al.*, 2014).

7.1.4 Faecal attributes

As shown in previous chapters, analysis of faecal and feed samples using near infrared spectroscopy (NIRS) and laser diffraction (LD) provided information about the chemical and physical breakdown of feed. Determination of spectral regions associated with high and low degradability of grass samples (Chapter 4), and variation between-animals has been assessed (Chapter 5) using NIRS. Thus far, LD has been assessed as a rapid technique to determine differences in faecal particle size without any phenotypic data available to determine differences in digestion processes. The rationale behind the use of this technique is that differences in the size of particles present within and leaving the rumen will affect both microbial activity in the rumen (Bjorndal *et al.*, 1990; Clauss *et al.*, 2015), as well as digestive processes in the intestines. Therefore a higher proportion of smaller particles in faeces may be related to digestive efficiency and, potentially, overall feed efficiency.

7.1.5 Aims

Previous chapters have looked at establishing, or the potential, of 16S sequencing, NIRS and LD as rapid techniques to assess differences in digestion processes, however small numbers of animals have been used. This chapter aims to utilise these techniques on larger number of animals with accompanying phenotypic data. The aims of this chapter are to (i) determine differences in the chemical (NIRS) and physical (LD) breakdown, and rumen microbial populations (16S) associated with dietary differences caused by diet additives; and (ii) explore potential relationships between these measurements and between-animal variation in feed efficiency of animals for each of the dietary treatments.

7.2 MATERIALS AND METHODS

7.2.1 Experimental Design

The animal experiment was carried out between 24th March and 4th November 2014 at Easter Howgate Beef Research Unit, SRUC, Scotland. The study was a two x four factorial design with two breeds; crossbred Aberdeen Angus (AAx) and crossbred Limousin (LIMx), and four experimental diets (section 7.2.2); CONTROL, OIL, NITRATE and NITRATEOIL. The AAx and LIMx steers were bred from a 2-breed reciprocal-crossing programme. In this programme, AAx cows were mated to a LIM sire and LIMx cows were mated to an AA sire. After 3 generations, the proportion of each breed type was 62.5:37.5 within each individual. A total of 80 steers were used for this experiment (40 of each breed) and split into 4 dietary groups with 20 steers (10 of each breed) in each. Due to the risk of nitrate toxicity for cattle fed nitrate as an additive, cattle were subjected to a five week adaption period. Nitrate was included at 25 % of the final concentration during week 1, 50 % during week 2, 75% during week 3 and 100 % during week 4. Animals remained at the 100 % inclusion rate for 2 weeks prior to the start of the performance test.

7.2.2 Experimental diets and performance test

Steers were offered a basal diet consisting of forage to concentrate ratio of 557:443 (g/kg DM). In addition to the basal diet, steers were offered one of four treatments; (i) CONTROL; containing rapeseed meal (RSM) as the main protein source, (ii) NITRATE in the form of calcium nitrate (Calcinit, Yara, Oslo, Norway; 18 g nitrate/kg DM) or (iii) lipid (OIL) in the form of maize dark grains (MDG), a by-product of the distilling industry (acid hydrolysed ether extract (AHEE) increased from 25.0 to 36.7 g AHEE/kg DM in CONTROL and OIL dietary treatments respectively); and (iv) a combination of Nitrate and Lipid diets (NITRATEOIL). Detailed diet formulations and chemical compositions can be found in Tables 7.1 and 7.2 respectively.

Table 7.1 –Ingredient composition for each of the 4 experimental diets (g/kg DM)

Ingredient	CONTROL	OIL	NITRATE	NITRATEOIL
Grass silage	210	209	211	210
Whole-crop barley silage	347	346	347	346
Barley	336	289	388	263
Rapeseed meal	79	0	0	0
Calcinit	0	0	25	25
Maize distillers grains	0	128	0	127
Molasses	19	19	20	19
Minerals and vitamins*	9	9	9	9

*Contained (mg/kg): Iron: 6,036; Manganese: 2,200; Zinc: 2,600; Iodine: 200; Cobalt: 90; Copper: 2,500; Selenium: 30; (µg/kg): Vitamin E: 2,000; Vitamin B12: 1,000; Vitamin A: 151,515; Vitamin D: 2,500

Table 7.2 – Chemical composition of the 4 experimental diets.

Constituent	CONTROL	OIL	NITRATE	NITRATEOIL
DM (g/kg)	533	533	531	533
Ash (g/kg DM)	52	51	48	51
CP (g/kg DM)	135	136	141	162
ADF (g/kg DM)	184	184	166	183
NDF (g/kg DM)	308	317	295	313
Starch (g/kg DM)	281	264	308	295
AHEE (g/kg DM)	25.0	36.7	23.4	35.9
GE (MJ/kg DM)	18.1	18.5	17.6	18.0
ME (MJ/kg DM)	11.7	12.0	11.5	11.7

Steers were subjected to a 56-day performance test after the adaption period was completed. Individual DMI (kg/d) was recorded daily using electronic feeding equipment (HOKO, Insentec, Marknesse, The Netherlands) and body weight (BW) measured weekly before fresh feed was offered. Ultrasonic fat depth was recorded (Aloka 500 machine; BCF Technology Ltd, Scotland, UK) at the 12th and 13th rib at the start and end of the performance test period. RFI and FCR values were calculated

for each steer. FCR was calculated as average daily DMI per day (kg) / average daily gain (ADG) (kg). RFI was calculated as the difference between actual DMI (kg) from predicted DMI (kg) based on linear regression of actual DMI on ADG (kg), mid-MBW (mid-test metabolic body weight; $BW^{0.75}$) and ultrasonic fat depth at the end of the performance test period (Basarab *et al.*, 2003).

7.2.3.1 Rumen sample collection

Rumen samples were collected on days 10 and 11 prior to (PRE-RFI) and on days 56 and 57 of the RFI measurement (POST-RFI) period. Analysis was only carried out on samples collected POST-RFI. Animals were sampled according to dietary grouping with OIL and CONTROL being sampled on day 56 am and pm respectively, and NITRATE and NITRATEOIL being sampled on day 57 am and pm respectively. Steers were restrained in a crush whilst rumen and faecal samples were collected. Rumen samples were collected by inserting a stomach tube (16 x 2700 mm Equivet Stomach Tube, JørgenKruuse A/S, Langeskov, Denmark) nasally and aspirating manually. Samples were taken over a 3 h period, with the first sample being taken approximately 1 h after feed was last offered. Rumen liquor was filtered through 4 layers of cheesecloth. A 5 ml sample of strained rumen fluid was mixed with 10 ml phosphate buffered saline containing glycerol (30% v/v; details in Appendix). After collection, rumen samples were immediately stored on ice and transferred to a -20°C freezer within 3 h of collection.

7.2.3.2 Faecal sample collection

Faecal grab samples were collected on days 10 and 11 prior (PRE-RFI) and day 56 and 57 of the performance test (POST-RFI) after fresh feed was offered. Analysis was only carried out on samples collected POST-RFI. Faecal sampling took place in the same order as described for rumen samples (Section 7.2.3.1). Samples were collected and placed in 150 ml screw top pots, before being stored at -20°C prior to further analysis.

Frozen faecal and rumen samples were transported on dry ice to Teagasc, Grange where they were stored at -20°C.

7.2.4 Faecal Particle Size Using Laser Diffraction

Sufficient faecal sample for LD analysis was available for only 75 steers. Samples were prepared for analysis using LD as described in Section 3.1.1. The wet weight of particles remaining in the tea strainer was recorded to provide an estimate of retained material.

7.2.4.1 Analysis of LD scans

As described in the Section 3.1.2, data was exported to Unscrambler X (Camo Software, Norway). Principal component analysis (PCA) plots were derived and loadings plots examined, firstly using all samples (ALL, n = 75), then subsequently PCA plots for each treatment group (CONTROL, n = 18; NITRATE, n = 19; NITRATEOIL, n = 18; OIL, n = 20). Each sample was coded for the presence or absence of nitrate or oil within the treatment group. The effect of nitrate or oil addition, or interactions, were assessed using REML (Linear Mixed Models) (GenStat V14; VSNI, Hemel Hempstead, UK) using individual principal component (PC) as Y-Variate and nitrate*oil as the fixed model. Steers were ranked by RFI values and each steer was assigned to either a nominally low or high RFI within treatment (CONTROL low n = 9, high n = 9; NITRATE low = 10, high = 9; NITRATEOIL low = 9, high = 9; OIL low = 10, high = 10). Nominal feed efficiency levels were balanced for breed within each diet. The same was carried out so that each steer was nominally ranked by FCR. This was repeated for both NIRS and 16S analysis. Effects of diet, nominal feed efficiency level (RFI/FCR; low/high) and interactions on PC scores were assessed using REML (GenStat). Individual PC's were set as Y-Variate and diet*RFI level or diet*FCR level used as fixed model. Within each diet the effect of breed and nominal feed efficiency level was assessed using REML using PC as Y-Variate and breed*nominal feed efficiency level as the model. The effect of individual feed efficiency values were assessed using General

Linear Regression (GenStat) using breed*feed efficiency value as the fixed model. This was also carried out for NIRS and 16S analysis.

Weights of remaining particles were calculated as a percentage ($[\text{weight in (wet)}/\text{weight out (wet)}]*100$). Differences in retained particles between diets were assessed using REML. Retained particles were set as the Y-Variate and nitrate*oil as the fixed model. The effect of breed and nominal feed efficiency level were assessed using REML in which breed*nominal feed efficiency level was set as the fixed model. Breed and feed efficiency values (FCR or RFI individual values) were assessed within each dietary treatment using general linear regression (GenStat) using breed*feed efficiency value as the model.

Median particle size (MPS) was calculated for each faecal sample, based on cumulative frequency distributions. The cumulative frequency was calculated for each faecal sample separately using the 27 particle size categories (see Table 3.1). Once the cumulative frequency passed 50% the mid-point of that category was used as the MPS. The effect of nitrate or oil addition, or interactions, was assessed using REML (Linear Mixed Models; GenStat) using individual PC's as Y-Variate and nitrate*oil set as the fixed model. The effect of breed and nominal feed efficiency level (FCR/RFI; low/high) on MPS was assessed using REML using MPS as Y-Variate and breed*nominal feed efficiency level as fixed model.

7.2.5 Faecal NIRS

Sufficient faecal sample for NIR analysis was available for only 72 steers. Samples were dried at 40°C for 72 h before being milled through a 0.75 mm sieve, samples were re-dried at 40°C for 48 h and scanned using NIRS as described in Section 3.2.1.

7.2.5.1 Analysis of NIR spectra

As described in the general methods (section 3.2.3), scans were averaged before being transformed using SDT. PCA analysis was carried out using all of the spectral data (ALL; n=72), then for each of the 4 dietary treatment groups individually (CONTROL, n=16; OIL, n=19; NITRATE, n=19; NITRATEOIL, n=16). Graphical

representation of the PC scores was used to observe the grouping within the data sets. Loadings associated with each PC were used to assess spectral regions of interest. Standard deviations were calculated between diets (Excel, 2010). The effect of dietary treatment on PC scores (ALL) was investigated using REML (GenStat) in which nitrate*oil was used as fixed model. As described above (Section 7.2.4.1), steers were ranked on RFI and FCR values and assigned to nominally low or high feed efficiency group (CONTROL low = 9, high = 9; NITRATE low = 10, high = 9; NITRATEOIL low = 8, high = 8; OIL low = 10, high = 9). Effects of diet and nominal feed efficiency level on PC scores were assessed using REML using diet*nominal feed efficiency level as fixed model. The effect of breed and nominal feed efficiency level, and breed and feed efficiency value on PC scores within each diet was assessed. Within each diet and breed, feed efficiency values (FCR and RFI) were ranked in order of lowest (most efficient) to highest (least efficient). SDT spectra for the median feed efficiency value (FCR and RFI) were subtracted from each steer.

7.2.6 16S sequencing

7.2.6.1 Sample preparation

Rumen samples were transferred from the -20°C freezer to a -80°C freezer in preparation for grinding using liquid nitrogen. Samples were processed in sets of 6. Samples were placed in a 1 L dewar containing liquid nitrogen, removed one at a time, loosely wrapped in laboratory tissue and hit with a hammer to break the screw cap tube. The sample was transferred to a cooled pestle and mortar (using clean liquid nitrogen), taking care not to include any shards of plastic from the screw top tube, and crushed to a fine powder. Clean liquid nitrogen was added as necessary to prevent the sample defrosting. The crushed sample was transferred to a sterile 15 ml screw top tube using a spatula and placed in the -80°C freezer. All equipment was cooled with liquid nitrogen before use and thoroughly cleaned between samples.

7.2.3 Next Generation Sequencing

7.2.3.1 DNA extractions

In brief, 1 ml lysis buffer was added to the thawed crushed rumen sample (600 mg) in a screw cap tube containing 0.3 g of 0.1 mm and 0.1 g of 0.5 mm zirconia beads. DNA extractions were carried out as described in Section 3.3.1. Extracted DNA samples were assessed for quality on a 1% agarose gel and for quantity using the NanoDrop 1000.

7.2.3.2 Library preparation and sequencing

Library preparation and sequencing of samples were carried out as described in the General Methods Section (3.3.2).

Due to limitations of Illumina sequencing kits, 40 samples were sequenced per run. Samples from NITRATE and NITRATEOIL dietary treatments were sequenced in run 1 and samples from OIL and CONTROL dietary treatments were sequenced in run 2. Further information and details of run contents can be found in the Appendix.

7.2.3.3 Data analysis

7.2.3.3.1 Data clean-up

Sequence data was analysed as described in Section 3.3.3. Unassigned taxonomies (unassigned at any level) and taxonomies containing cyanobacteria were removed and relative abundances of taxa were reported from kingdom to phyla level.

Samples were rarefied to the lowest read number across all samples (~22,000) ten times, with Shannon indexes calculated at each iteration. The average of each iteration was used as a Shannon index value and used in rarefaction plots.

7.2.3.3.2 Statistical analysis

PCA (using the singular value decomposition (SVD) algorithm; Unscrambler X) was carried out on all samples and subsequently for each of the treatment groups using relative abundances (at genus level), with unassigned taxonomies removed.

The effect of dietary treatment on PC scores (ALL) was investigated using REML (GenStat) in which nitrate*oil was used as Treatment Structure. Each sample was coded for the presence or absence of nitrate or oil within the treatment group. As described above, steers were assigned to nominal low or high RFI and FCR levels (CONTROL, NITRATE and OIL; low = 10, high n = 10; NITRATEOIL low = 9, high = 10). Effects of diet and nominal feed efficiency level on PC scores were assessed using REML using diet*nominal feed efficiency level as fixed model. As described in Section 7.2.4.1 the effect of breed and nominal feed efficiency level, and breed and feed efficiency value on PC scores within each diet was assessed.

Taxonomies that averaged 0 % relative abundances across any individual diet (at genus level) were removed to give a core microbiome across all diets.

Firmicutes to Bacteroidetes ratio (F:B) was assessed from relative abundances (at the phylum level). Correlations (Pearson) were examined between nominal FCR and RFI values and F:B within each treatment group and also within breed for each treatment group.

Differences in genera across all diets were calculated (STAMP; Parks *et al.*, 2015) using ANOVA with a Benjamini-Hochberg false discovery rate (FDR) applied. Each of the treatment groups (NITRATE; NITRATEOIL; OIL) were subsequently compared to CONTROL diet using ANOVA and Benjamini-Hochberg FDR. Values with $P < 0.05$ were classed as significant, with particular interest being taken in taxonomies associated with methane emissions. Nominal low and high nominal feed efficiency levels and breed were assessed using ANOVA and Benjamini-Hochberg FDR for individual dietary treatment groups.

Differences in Shannon diversity indexes within each diet were assessed using REML (GenStat) using breed*nominal feed efficiency level as fixed model and using general linear regression using breed*feed efficiency value were set as fixed model.

Relative abundances that averaged < 1 % across each of the diets were removed at both phylum and family level as described by McCann *et al.*, (2014a). The effect of taxonomic group (phylum and family level) on feed efficiency, whether expressed as nominal levels or individual values, was assessed using General Linear Regression for each treatment group separately.

7.3 RESULTS

7.3.1 Performance and methane data

One steer, from NITRATEOIL dietary treatment, was removed from the trial for health reasons not related to the treatment, therefore feed efficiency data was only available for 79 cattle. Full details of performance data can be found in NUTRIBEEF Final Report (Roehe *et al.*, 2015). In brief, inclusion of nitrate or oil in the diet did not affect DMI. The NITRATE diet was found to have an affect ($P < 0.05$) on FCR, but not on RFI. The OIL diet did not ($P > 0.05$) affect feed efficiency (FCR or RFI). AAx steers had higher ADG ($P < 0.01$) and higher DMI ($P < 0.001$) compared to LIMx steers and were therefore less efficient (RFI; $P < 0.01$). No interaction ($P > 0.05$) between breed and dietary treatment was found. NITRATE had a significant effect on reducing methane emissions, expressed as both g/day and g/kg DMI ($P < 0.001$ and $P < 0.001$, respectively). OIL led to a numerical reduction in methane emissions but this was found to be not significant. NITRATE x OIL interactions were also found to be non-significant ($P > 0.05$). This data is summarised in Table 7.3 and 7.4.

Table 7.3 - Performance results within breed for each of the 4 dietary treatments (results from Roehle *et al.*, 2015).

Breed	AAx				LIMx				Significance ²			
	Treatment ¹	C	O	N	NO	C	O	N	NO	Breed	Nitrate	Oil
ADG (kg/d)		1.78	1.78	1.66	1.77	1.69	1.66	1.41	1.49	**	**	NS
FCR		6.79	6.92	7.53	6.82	6.91	6.87	7.50	7.54	NS	*	NS
RFI		0.04	0.44	0.51	0.06	-0.20	-0.36	-0.39	0.01	**	NS	NS

¹ Treatment – C = CONTROL, O = OIL, N = NITRATE, NO = NITRATEOIL

² Significance - NS, not significant; *P<0.05; **P<0.01; ***P<0.001.

Table 7.4 - Methane production results from steers within each of the 4 dietary treatments (results from Roehle *et al.*, 2015).

	Treatment ¹				Significance ²		
	C	O	N	NO	Nitrate	Oil	Nitrate x Oil
DMI (kg/d)	10.35	10.23	9.82	10.21	NS	NS	NS
CH ₄ (g/d)	245.5	238.2	218.6	209.9	***	NS	NS
CH ₄ (g/kg DMI)	23.98	23.38	22.09	20.89	***	NS	NS

¹ Treatment – C = CONTROL, O = OIL, N = NITRATE, NO = NITRATEOIL

² Significance - NS, not significant; *P<0.05; **P<0.01; ***P<0.001.

7.3.2 Laser Diffraction

The average faecal dry matter was 14.4 % (s.d. 3.2) across all diets. Median faecal particle size was 213.89 μm , 170.39 μm , 163.89 μm and 181.88 μm for CONTROL, NITRATE, NITRATEOIL and OIL diets respectively. Average particle size distribution for each dietary treatment can be seen in Figure 7.1. The addition of nitrate to the diet was found to have a significant effect ($P = 0.004$) on MPS, whilst the addition of oil or interactions were found to be non-significant ($P > 0.05$). There was no significant relationship between RFI value, FCR value, Breed or interactions with MPS within each dietary treatment group ($P > 0.05$).

An average estimation of 17.9 %, 21.9 %, 21.7 % and 15.6 % of the original sample remained in the tea strainer after the pre-sieving stage for the CONTROL, NITRATE, NITRATEOIL, OIL diets, respectively. The majority of the retained particles were undigested grains and husks that passed through the digestive tract. An interaction between nitrate*oil ($P = 0.01$) and MPS was observed, nitrate or oil were found to have no effect ($P > 0.05$). When assessed within each dietary treatment, FCR (value) was found to have a significant effect on particles >1.8 mm within the NITRATEOIL diet ($P = 0.031$). No significance was noted between FCR value, RFI value, breed or interactions.

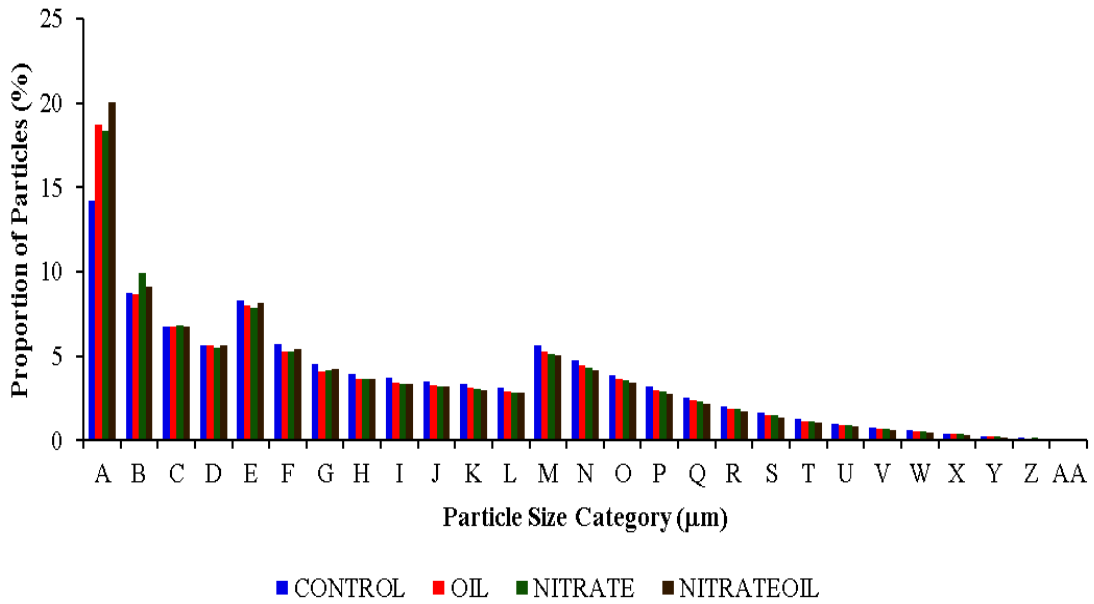


Figure 7.1 – particle size distribution for each size category for each of the 4 dietary treatments. See Table 3.1 for full particle size category details.

7.3.2.1 Differences between diets

PC scores plots were compiled to assess differences between particle sizes (<1.8 mm) from the different dietary treatments. PC-1 accounted for 89 %, PC-2 accounted for 8% and PC-3 accounted for 3% of the variation (Figure 7.2d) Separation of CONTROL diet was observed when comparing PC-1 vs PC-2 (Figure 7.2a). Three groupings were observed when comparing PC-1 vs PC-3 and PC-2 vs PC-3 (Figure 7.2b, c), however some overlap does occurs; (i) CONTROL, (ii) NITRATE and (iii) OIL and NITRATEOIL.

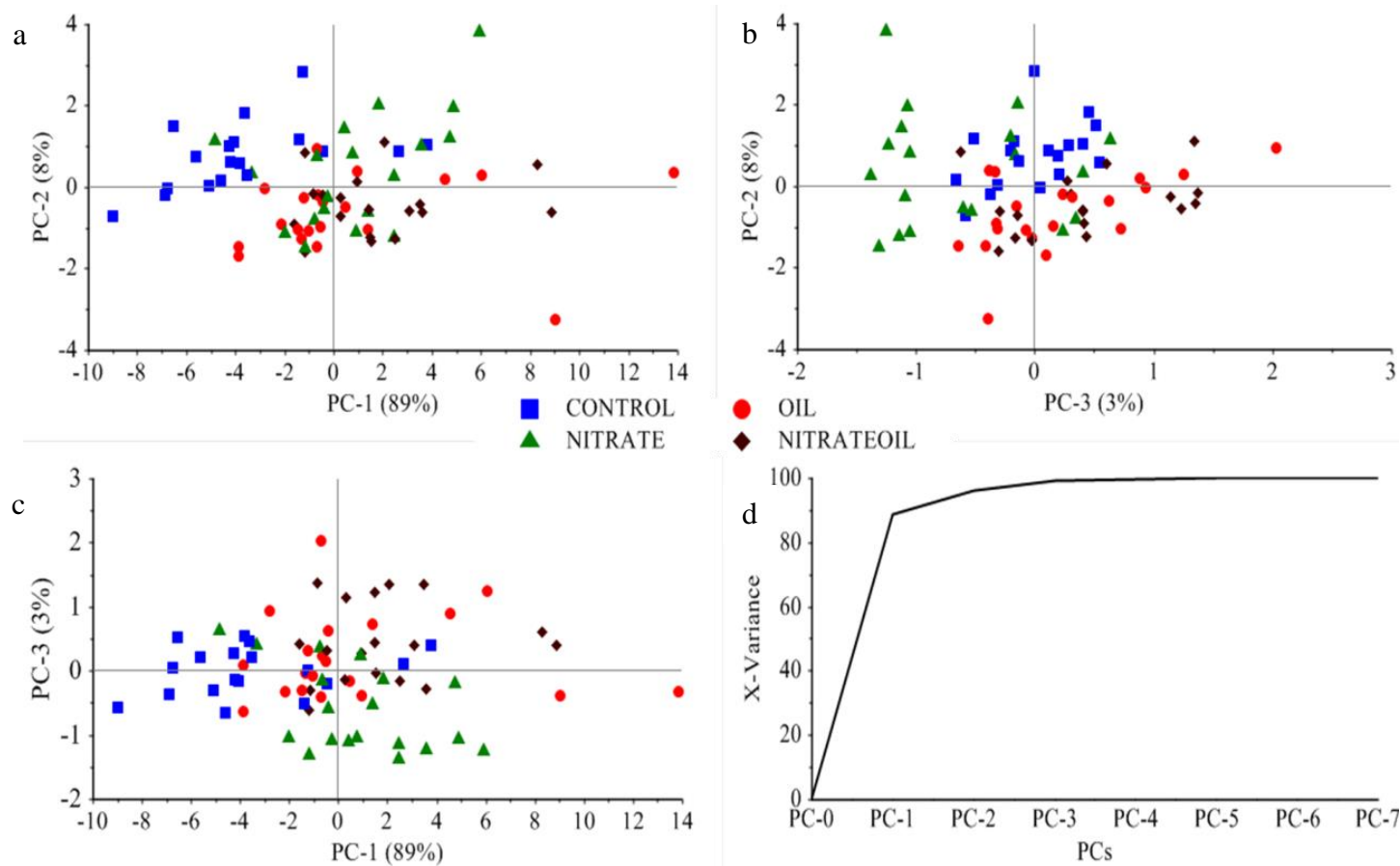


Figure 7.2 – PCA overview of LD analysis of all 4 dietary treatments (a) scores plot containing PC-1 vs. PC-2 (b) scores plot containing PC-3 vs. PC-2 (c) scores plot containing PC-1 vs. PC-3 (d) explained variance plot.

The addition of nitrate ($P = 0.002$), oil ($P = 0.001$) and nitrate*oil ($P = 0.037$) were found to be significant for PC-1. The addition of oil was found to be significant for PC-2 ($P < 0.001$), nitrate or nitrate*oil had no effect ($P > 0.05$). The addition of oil ($P < 0.001$) and nitrate*oil ($P = 0.004$) had a significant effect on PC-3. These results are summarised in Table 7.5. REML was used to assess the effect of diet, nominal feed efficiency level (FCR/RFI; low/high) and interactions. Diet was found to have a significant effect ($P < 0.001$) on PC-1, PC-2 and PC-3, however nominal feed efficiency level or interactions had no effect ($P > 0.05$), full details can be found in Appendix.

Table 7.5 – The effect of nitrate and oil addition on LD results using PC-1, PC-2 and PC-3.

Dietary Treatment	P Value		
	PC-1	PC-2	PC-3
nitrate	0.002	0.935	0.132
oil	0.001	<0.001	<0.001
nitrate*oil	0.037	0.203	0.004

7.3.2.2 Differences in feed efficiency

PC scores plots were compiled for each dietary treatment to assess between-animal variation with particular interest taken in feed efficiency. PC-1, PC-2 and PC-3 accounted for 99 % of the variation for all of the dietary treatments. No clustering or grouping was observed when assessing nominal feed efficiency levels within each diet. Figure 7.3 shows PCA overview of LD results within the NITRATE diet, samples were coded by FCR level (low or high). PC score plots and explained variance for the CONTROL, OIL and NITRATEOIL diets can be found in the appendix.

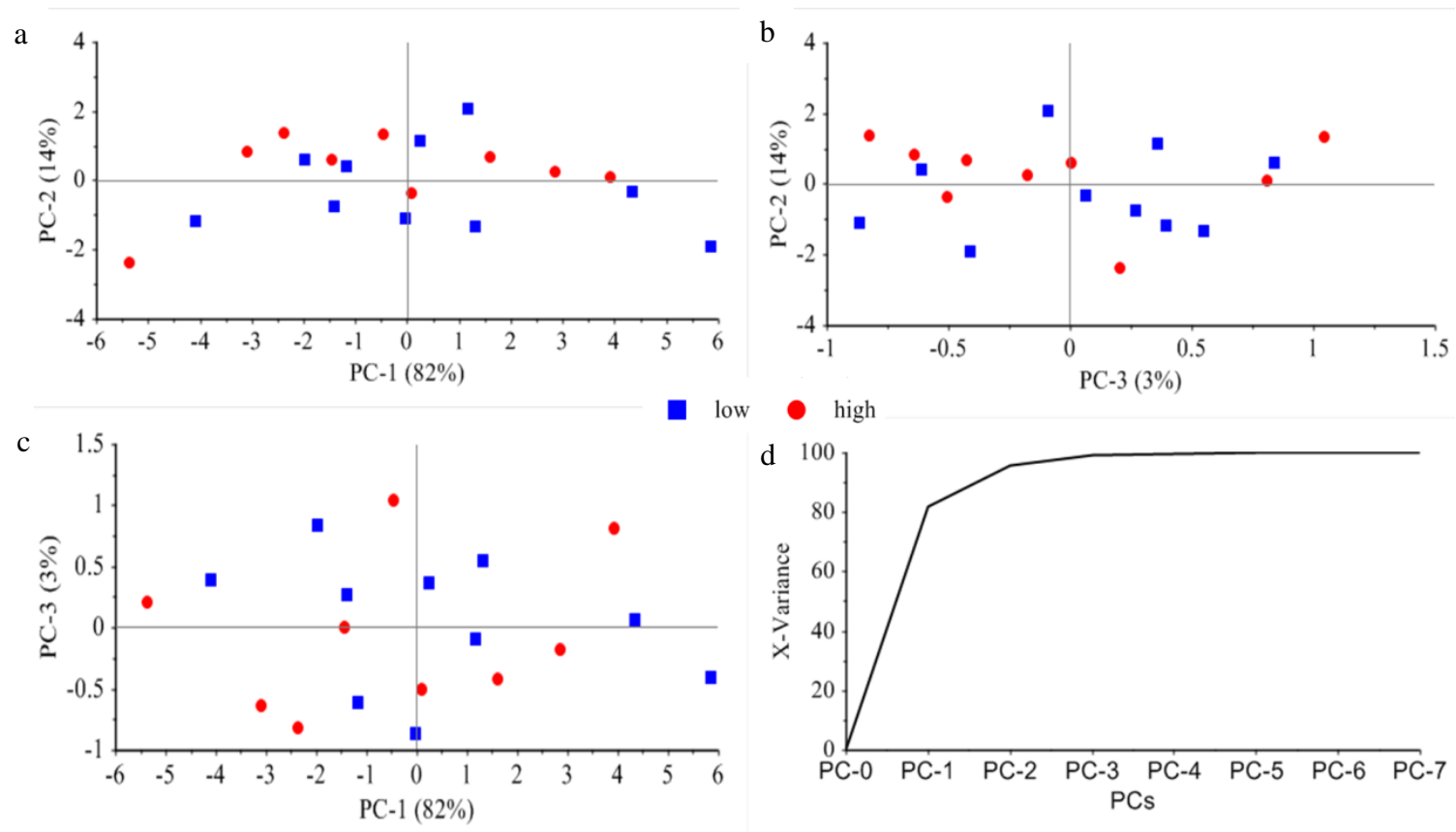


Figure 7.3 - PCA overview of LD analysis of NITRATE dietary treatment, samples labelled according to FCR level (low/high). (a) scores plot for PC-1 vs. PC-2 (b) scores plot for PC-3 vs. PC-2 (c) scores plot for PC-1 vs. PC-3 (d) explained variance plot.

The effect of breed and nominal feed efficiency level (FCR/RFI; low/high) was assessed within each diet using REML. Within the CONTROL and OIL diets, breed, nominal feed efficiency level or interactions were non-significant ($P > 0.05$) for PC-1, PC-2 or PC-3. Within the NITRATE diet, breed*FCR was found to have a significant effect on PC-2 ($P = 0.011$). Within the NITRATEOIL diet, FCR was found to have a significant effect on PC-2 ($P = 0.041$), no significance was noted for PC-1 or PC-3. Full results can be found in the Appendix.

The effect of breed and nominal feed efficiency value (FCR/RFI) was assessed within each diet using General Linear Regression. Within the CONTROL, OIL and NITRATEOIL dietary treatments, breed, feed efficiency value, or interactions were non-significant ($P > 0.05$) for PC-1, PC-2 or PC-3. Within the NITRATE diet breed ($P = 0.032$) and Breed*FCR ($P = 0.034$) for PC-2, no significance was noted for PC-1 or PC-3.

7.3.3 Faecal NIRS

Spectra of the 4 dietary treatments are shown in Figure 7.4 as (a) Log (1/R) spectra and (b) SDT transformed spectra. Differences between diets using SDT were observed between diets at spectral regions 1260 – 1480 nm, with minima at 1310 nm, 1430 – 1480, 1714, 1860, 1930, 2056 – 2120, 2220, and 2310 nm with contributing shoulders at 2270 and 2330 nm. Spectral regions 1430 – 1480 and 1930 nm are due to moisture (Shenk *et al.*, 1992).

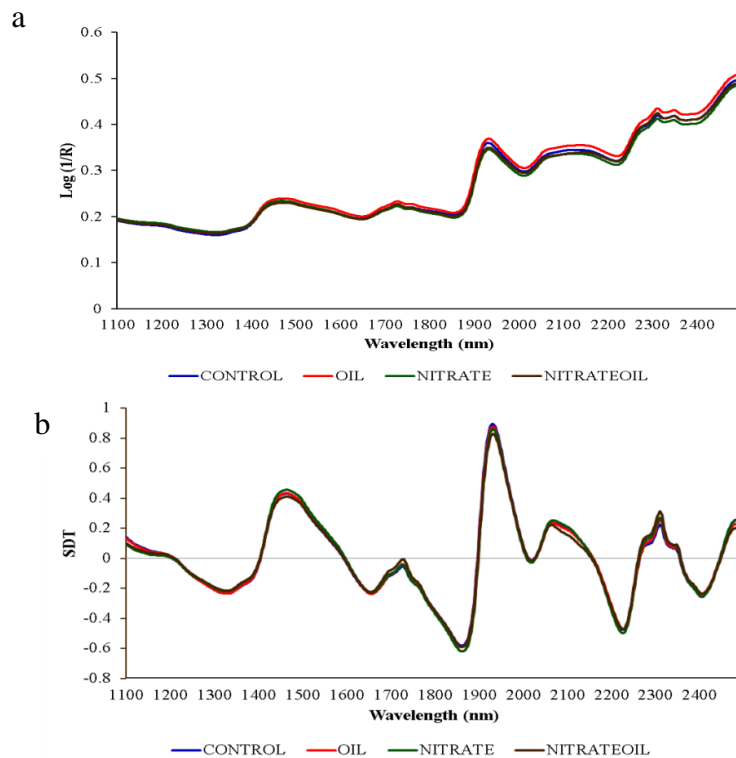


Figure 7.4 – Spectra of faecal samples from all 4 dietary treatments as (a) Log (1/R) and (b) transformed spectra using SDT.

7.3.3.1 Differences between diets

PC scores were derived to assess differences between diets. PC-1 accounted for 45 % of the variation, PC-2 accounted for 39 % of the variation and PC-3 accounted for 7 % of the variation (Figure 7.5d). No separation of diet was observed when comparing PC-1 vs. PC-2 (Figure 7.5a). Two groupings were observed when comparing PC-3 vs. PC-2 (i) CONTROL and OIL and (ii) NITRATE and NITRATEOIL (Figure 7.5b). Three groupings were observed when comparing PC-1 vs PC-3 (i) CONTROL and OIL, (ii) NITRATE and (iii) NITRATEOIL (Figure 7.5c), however some overlap does occur.

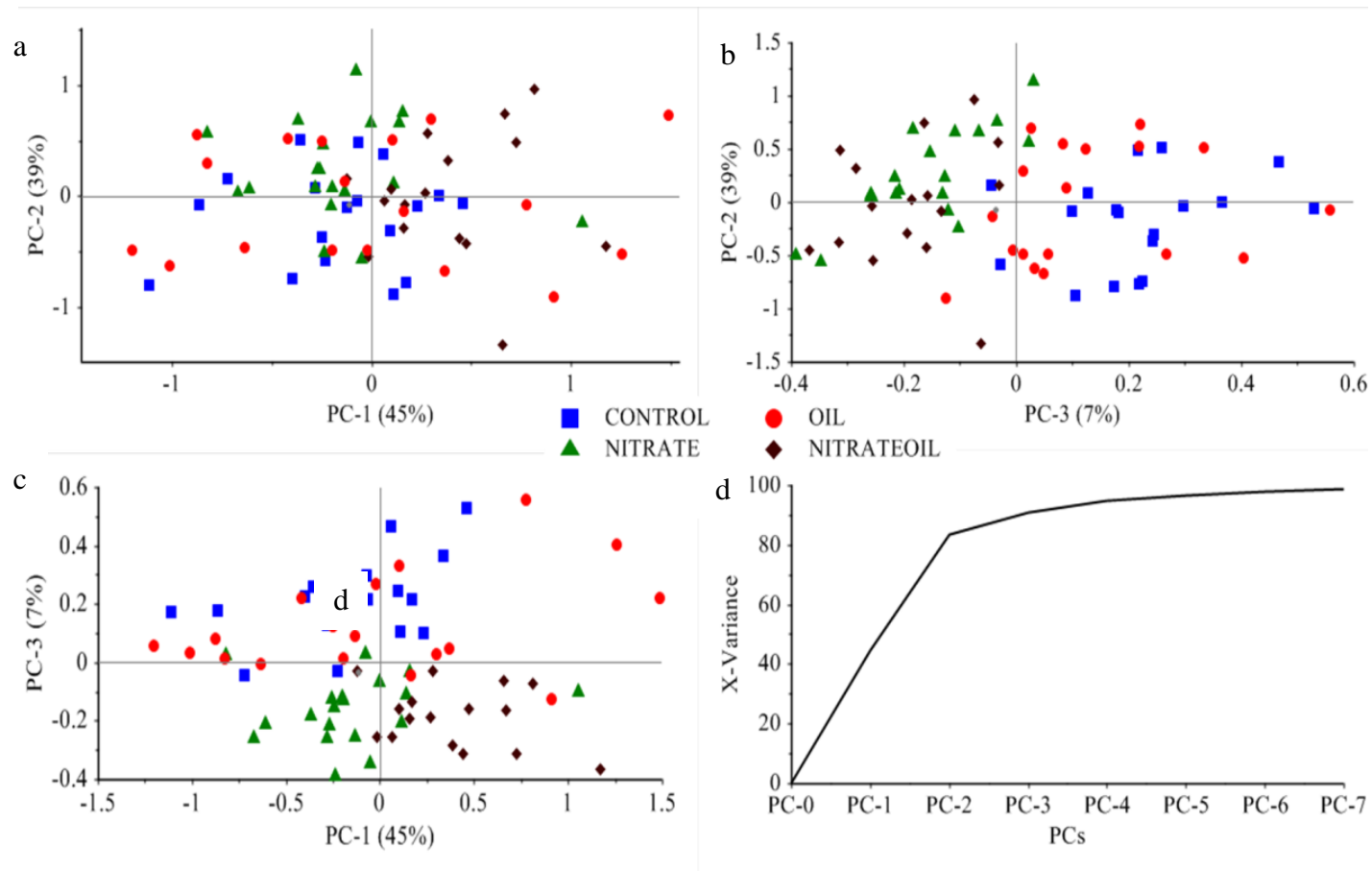


Figure 7.5 – PCA overview of NIRS analysis of all 4 dietary treatments (a) scores plot for PC-1 vs. PC-2 (b) scores plot for PC-3 vs. PC-2 (c) scores plot for PC-1 vs. PC-3 (d) explained variance plot.

The addition of oil to the diet had a significant effect on PC-1 ($P = 0.006$). The addition of nitrate to the diet had a significant effect on PC-2 ($P = 0.05$) and PC-3 ($P < 0.001$). These results are summarised in Table 7.6. REML was used to assess the effect of diet, nominal feed efficiency level (FCR/RFI; low/high) and interactions. Diet was found to have a significant effect on PC-1 ($P = 0.07$ / $P = 0.08$) and PC-3 ($P < 0.001$), nominal feed efficiency level or interactions had no significant effect on PC-1, PC-2, or PC-3. Full details can be found in Appendix.

Table 7.6 – The effect of nitrate and oil addition on NIRS results using PC-1, PC-2 and PC-3.

Dietary Treatment	P Value		
	PC-1	PC-2	PC-3
nitrate	0.131	0.050	<0.001
oil	0.006	0.647	0.073
nitrate*oil	0.104	0.116	0.265

Loadings associated with PC scores plots are shown in Figure 7.6. For PC-1 positive peaks were noted at 1726 nm, with shoulders observed at 1686 and 1746 nm, 2306, 2350 and 2372 nm. A broad peak was also noted at 2194 nm. Spectral regions 1726, 1746, 2306, 2350 and 2372 nm are strongly associated with lipids (Murray, 1987), in particular cuticular waxes as described in Chapter 4. The spectral region 2200 nm is strongly associated with carbohydrates (Workman and Weyer, 2012) and spectral region 1686 nm is dominated by C-H absorptions. For PC-1 negative regions were observed as a broad trough at 1440 nm, with a slight shoulder at 1540 nm, 1920 and 2070 nm. Spectral region 1540 nm is associated with 1st overtone stretch of N-H bonds (Murray, 1987) and 2070 nm associated with N-H stretch associated with amide III (protein) (Murray, 1987). Spectral regions 1440 and 1920 nm are both associated with moisture from 1st and 2nd overtone regions respectively. For PC-2 positive peaks were observed between 1400 – 1650 nm, with peaks at 1450 and 1550 nm, 2100, 2274 and 2316 nm. Spectral region 1450 nm is associated with moisture and 1550 nm due to N-H bonds in the 1st overtone. Spectral regions 2100, 2274 and 2318 nm are due to cellulose and lipids. For PC-2 negative regions were associated at 1940 and 2190 nm, these regions are associated with moisture and amide III (protein). For PC-3 positive regions were observed at 1870 nm, with a contributing

shoulder at 1780 nm, 2110 and 2210 nm. Spectral regions 1780, 2110 and 2210 nm are associated with cellulose, carbohydrates, N-H bonds respectively (Workman and Weyer, 2012). It is unclear what spectral region 1870 nm is, however the region is associated with C-H and O-H bonds. For PC-3 negative regions were observed in as a broad trough 1230 – 1574 nm. Spectral region 1510 nm is associated with protein and 1720, 2346 and 2370 nm associated with lipids. It is unclear what compound causes absorption at 1330 nm. Spectral region 1958 is likely caused by moisture. Spectral region 1684 nm is dominated by C-H bonds and 2036 nm associated with N-H bonds (Murray, 1987).

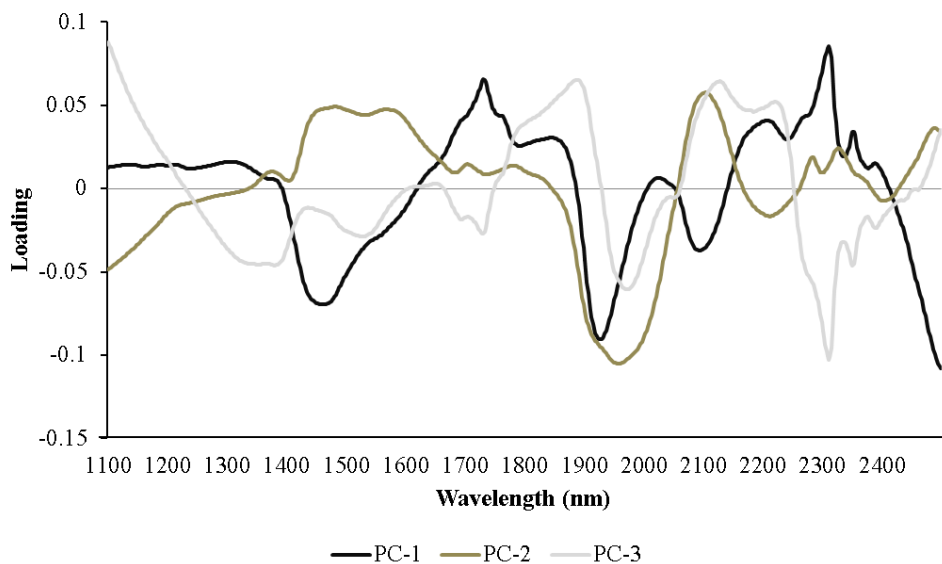


Figure 7.6 – loadings plot for PCA scores plot containing all 4 dietary treatments.

SD plots were compiled and are able to show differences observed in Figure 7.7 but in a more condensed format. Spectral variation was observed in the same areas across all dietary treatments. The main regions of variation were observed at 1450 and around 1920 nm, and 1728, 1756, 2308, 2348 and 2384 nm, which are associated with moisture (Shenk *et al.*, 1992) and lipids (Flinn *et al.*, 1992; Murray, 1987) respectively. Spectral regions 1208, 1300, 1570, 1840, 2100 and 2184 nm showed variation, although at a lower variation than seen with moisture and lipids. Regions 1208 and 1300 nm are likely due to C-H bonds (Workman and Weyer, 2012).

Spectral regions 1570 and 2184 nm are associated with protein (Murray, 1987) and 1840 and 2100 nm associated with cellulose (Workman and Weyer, 2012). A small peak was observed at 2254 nm, this was most prominent in samples from the NITRATE dietary treatment, this is associated with lignin (Givens *et al.*, 1992). A small peak was also observed at 1366 nm within the NITRATEOIL dietary treatment, this is associated with C-H absorptions (Workman and Weyer, 2012). With the exception of spectral region 2100 nm, the OIL dietary treatment showed highest variation. NITRATEOIL showed higher spectral variations compared to CONTROL and NITRATE diets in regions associated with lipids.

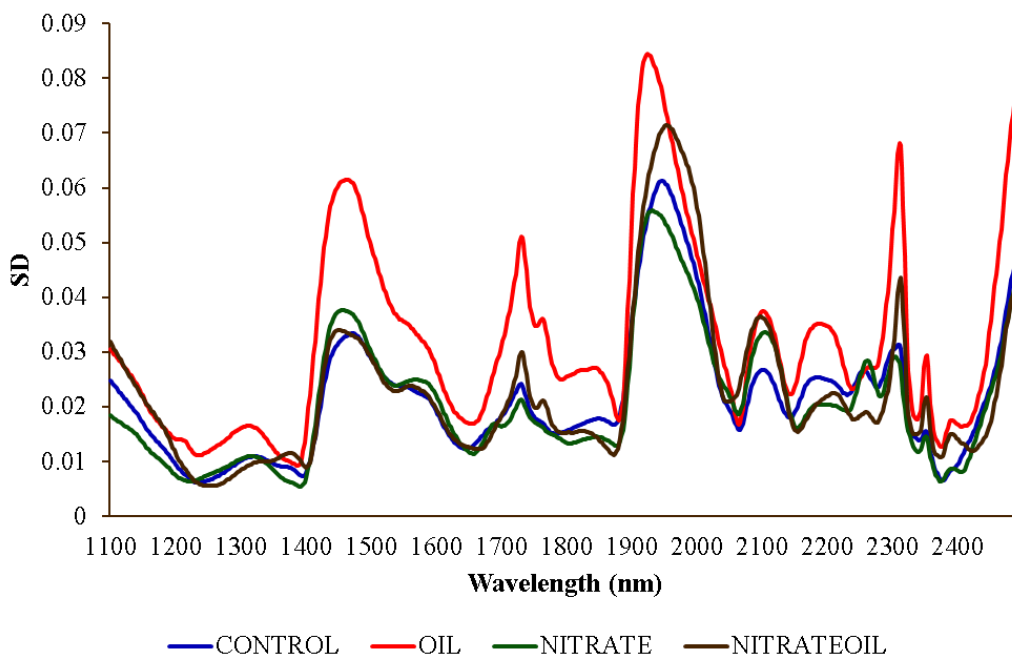


Figure 7.7 – SD plot for all NIRS data, grouped by dietary treatment

7.3.3.2 Differences in feed efficiency

Difference spectra were calculated within each breed and within each diet to assess regions of the spectra related to increased or decreased feed efficiency relative to the median value. The difference spectra of LIMx and AAX steers relative to median FCR and RFI values within the NITRATEOIL diet can be seen in Figure 7.8 expressed as more efficient (ME) or less efficient (LE) relative to the median.

Difference spectra for LIMx and AAX steers from CONTROL, OIL and NITRATE dietary treatments can be found in the Appendix. Greatest variation was observed at regions associated with moisture (1450 and 1940 nm) across both breeds and efficiency measures. Differences were also noted at spectral regions associated with lipids (1724, 1752, 2306, 2350 and 2386 nm), protein (1552 and 2190 nm) and cellulose (2090 nm). Similar regions were seen within CONTROL, OIL and NITRATE dietary treatments. Difference spectra presented show variation between-animals however differences in concentrations were are not related to differences in feed efficiencies. Greater magnitude of peaks and troughs were observed in AAX steers relative to LIMx, however this was not found to be consistent throughout other dietary treatments.

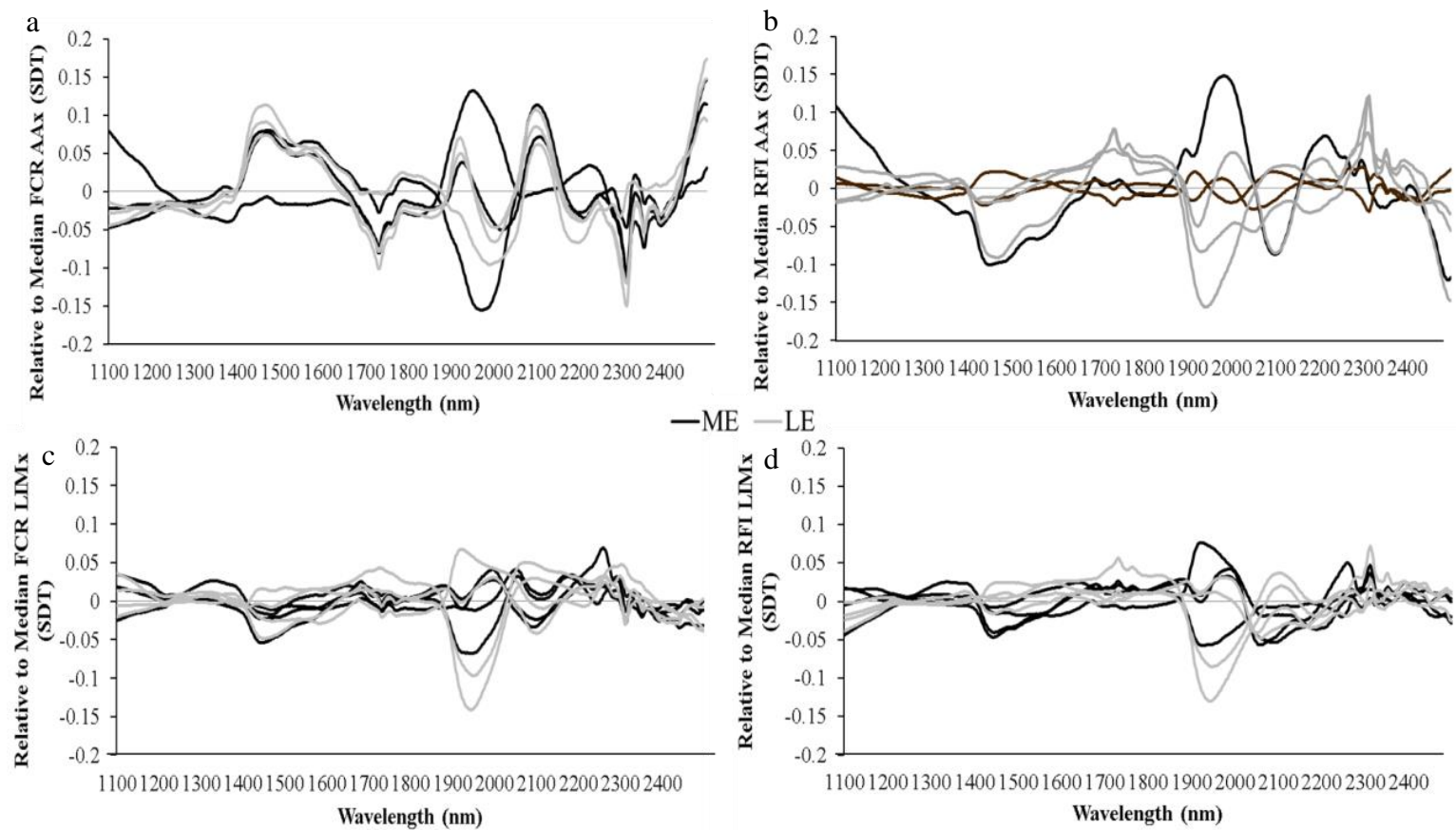


Figure 7.8 – Difference spectra (SDT transformed) of NITRATEOIL of feed efficiency value for individual steers (FE - more efficient; LE – less efficient) relative to median value of (a) FCR within AAX, (b) RFI within AAX, (c) FCR within LIMx and (d) RFI within LIMx

PC scores plots were compiled for each dietary treatment to assess effect of feed efficiency. The first 3 PCs accounted for 93 %, 96 %, 95 % and 97 % of the variation for CONTROL, OIL, NITRATE and NITRATEOIL diets respectively. Figure 7.9d shows explained variance of samples from NITRATEOIL dietary treatment. No clustering or grouping was observed when assessing nominal feed efficiency level within each dietary treatment. Figure 7.9 shows PCA overview of NITRATEOIL diet, samples labelled by nominal low or high RFI level. PCA overviews for CONTROL, OIL and NITRATE diets can be found in the Appendix.

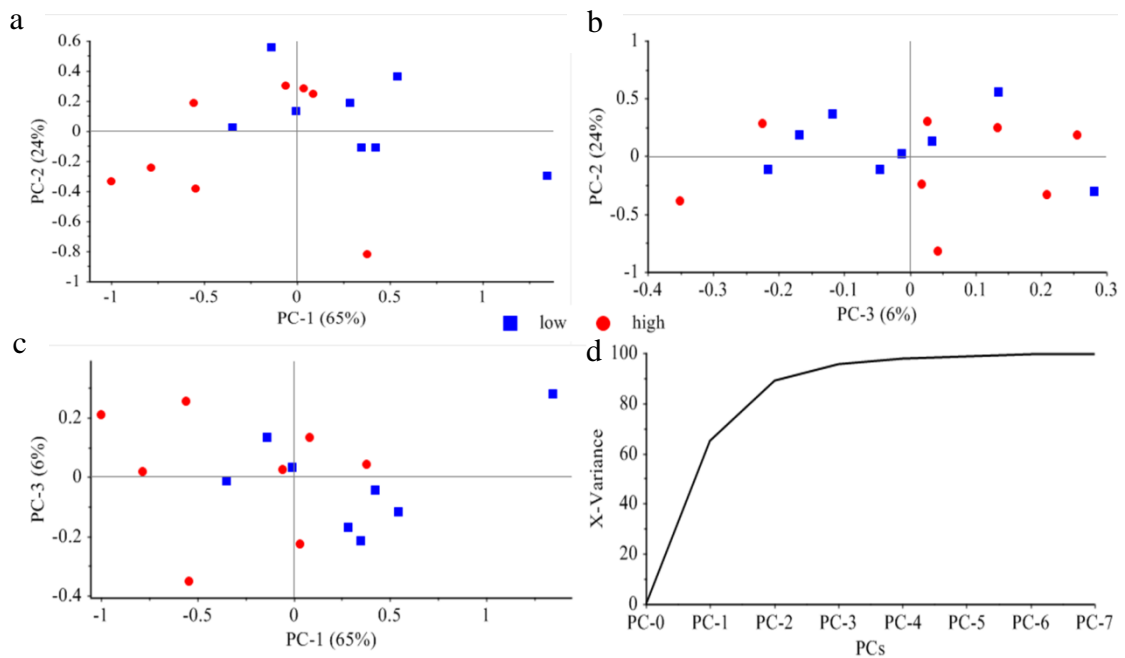


Figure 7.9 - PCA overview of NIR analysis of NITRATEOIL dietary treatment, samples labelled according to RFI level (low/high). (a) scores plot for PC-1 vs. PC-2 (b) scores plot for PC-3 vs. PC-2 (c) scores plot for PC-1 vs. PC-3 (d) explained variance plot

The effect of breed and nominal feed efficiency level was assessed within each diet using REML. Within the NITRATE and OIL dietary treatments, breed, nominal feed efficiency level or interactions were found to be non-significant ($P > 0.05$). Within

the CONTROL diet RFI had a significant effect on PC-2 ($P = 0.041$). RFI level had a significant effect on PC-1 ($P = 0.04$) of the NITRATEOIL dietary treatment.

The effect of breed and feed efficiency value were assessed using General Linear Regression. Within the CONTROL and OIL dietary treatments, breed, feed efficiency value or interactions were non-significant ($P > 0.05$). RFI and FCR were found to have a significant effect on PC-1 within the NITRATEOIL diet ($P = 0.02$; $P = 0.011$). FCR was found to have a significant effect on PC-3 within the NITRATE diet ($P = 0.012$). Full details of these statistical tests can be found in the Appendix.

To investigate the differences in PC scores plots for individual diets, the associated loadings were examined. Particular interest was taken on loadings associated with the NITRATEOIL dietary treatment due to associations between PCs and feed efficiency described above. Loadings plots associated with each diet can be found in Figure 7.10, the main (highest) loadings observed are described within each diet below.

Loadings plots associated with PC-1, PC-2 and PC-3 within the CONTROL dietary treatment can be found in Figure 7.10a. For PC-1, a trough was observed at 1932 nm, this region is associated with moisture. For PC-2 regions with highest loadings were noted at 1454, 1550 and 2090 nm in the positive region. These wavelengths are associated with moisture, N-H bonds and cellulose respectively. Highest loadings were noted in spectral regions 1724, 2000, 2176 and 2292 nm in the negative region. These spectral regions are associated with lipids, N-H bonds, amide II (proteins) and proteins respectively (Murray, 1987; Workman and Weyer, 2012). For PC-3 wavelength 1872 nm showed highest loading in the negative direction, this is associated with C-H and O-H bonds. Highest loadings were noted at 2046 and 2290 nm for negative regions, these regions are associated with N-H absorptions, in particular proteins (Murray, 1987).

Loadings plots associated with PC-1, PC-2 and PC-3 for the OIL dietary treatment can be found in Figure 7.10b. Similar spectral regions were observed as described for CONTROL dietary treatment. For PC-1 highest loadings were observed at 1440 and

1906 nm in positive regions, these regions are associated with moisture and C=O stretch (such as carboxylic acids) (Workman and Weyer, 2012). Highest loadings were observed at 1728 and 2304 nm in the negative region, both are characteristic of lipids. For PC-2 spectral region 1940 nm showed highest loading in the positive regions, this is associated with moisture. Spectral regions 1440, 1550 and 2090 nm showed highest loadings in the negative direction. These regions are associated with moisture, N-H bonds and cellulose respectively. For PC-3 highest loadings were observed at 2040, 2156 and 2240 nm in the positive direction, these regions are associated with CHO bonds, likely from lignin (workman and Weyer, 2012). Highest loadings were observed at 1728, 1952, 2310 and 2350 nm. These are associated with lipids, except spectral region 1952 nm which is likely caused by moisture (Shenk *et al.*, 1992).

Loadings plots associated with PC-1, PC-2 and PC-3 for the NITRATE dietary treatment can be found in Figure 7.10c. For PC-1 spectral regions 1450, 1550 and 2090 nm showed highest loadings in the positive region, these are associated with moisture, N-H bonds and cellulose respectively. Spectral region 1960 showed highest loading in the negative region, this likely caused by moisture. For PC-2 spectral regions 1440 and 1920 nm showed highest loadings in the negative direction, these are both highly characteristic of moisture. Spectral regions 2200, 2256 and 2290 nm showed highest loadings in the positive direction, a small peak was also observed at 1712 nm. Spectral regions 1712, 2200, 2256 and 2290 nm are associated with lipids, carbohydrates and lignin respectively. For PC-3 spectral regions 1430, 2040 and 2160 nm showed highest loadings in the positive direction. These regions are associated with moisture, amide II (protein) and amide III (protein) respectively (Murray, 1987). Spectral region 2254 nm showed highest loadings in the negative direction, this is associated with lignin.

Loadings plots associated with PC-1, PC-2 and PC-3 for the NITRATEOIL dietary treatment can be found in Figure 7.10d. Positive loadings were dominated by a peak at 1940 nm for PC-1, this is associated with moisture. Spectral region 2086 nm showed highest loading in the negative direction, this is associated with amide II (protein). For PC-2 spectral regions 1442, 1920 and 2088 nm showed highest loadings in the positive direction. These regions are associated with moisture (1442

and 1920 nm) and Amide III (protein) respectively. Negative regions were dominated by regions associated with lipids (1724 and 2310 nm). For PC-3 spectral regions 2046 and 2160 nm showed highest loadings in the positive direction, these are associated with proteins. Spectral region 2310 nm showed highest loading in the negative direction, this is characteristic of lipids.

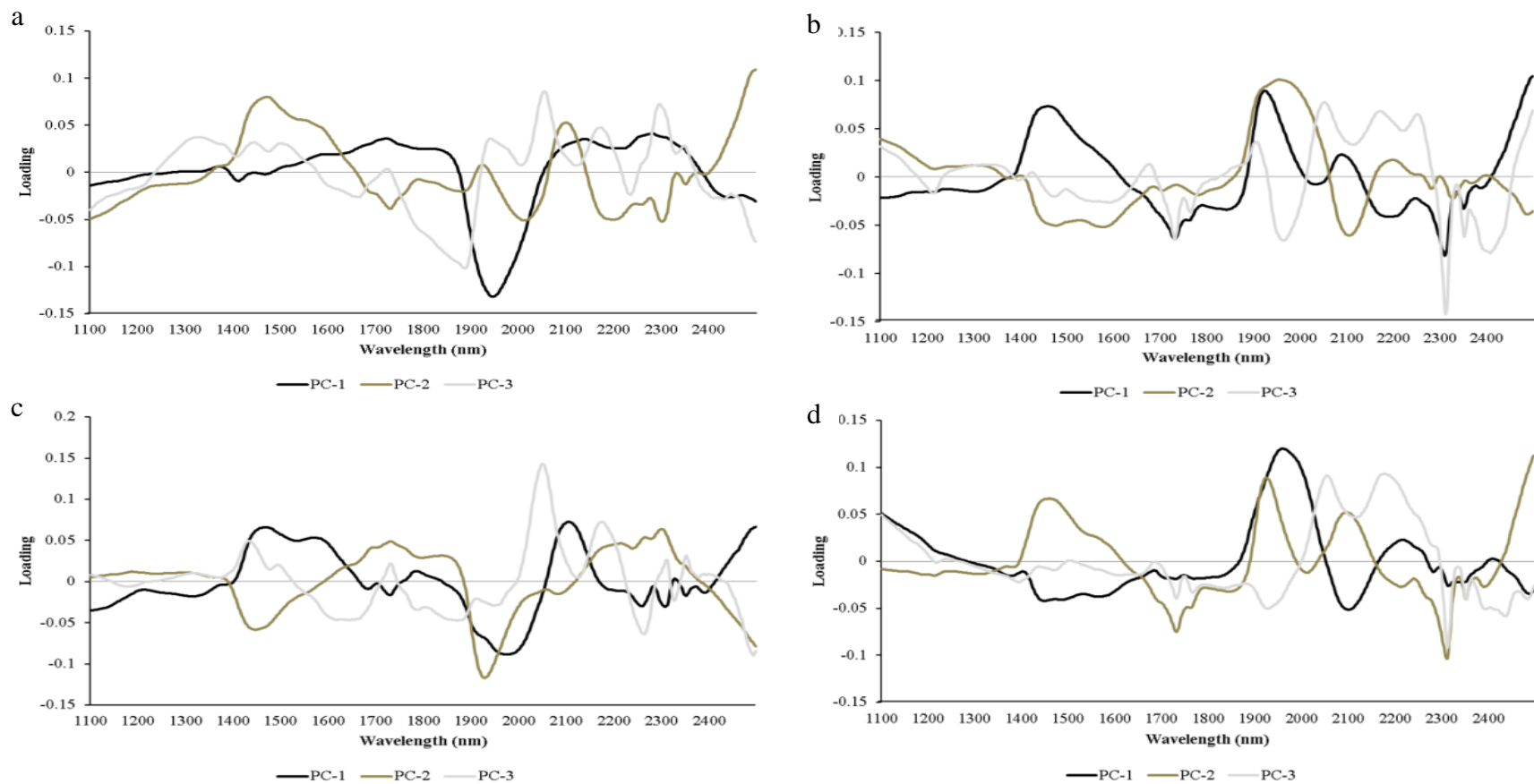


Figure 7.10 – Loadings plots associated with PC-1, PC-2 and PC-3 for (a) CONTROL, (b) OIL, (c) NITRATE and (d) NITRATEOIL

7.3.4 Rumen communities

7.3.4.1 16S sequencing data

Overall, 6,667,019 reads were generated, giving an average of 83,337 reads \pm 36,460 per sample. The reads were reduced to 6,562,696 after quality filtering. The average number of counts per sample that were assigned to an OTU (post filtering) was 82,033 \pm 36,021. Rarefaction plots for observed species can be found in Figure 7.11.

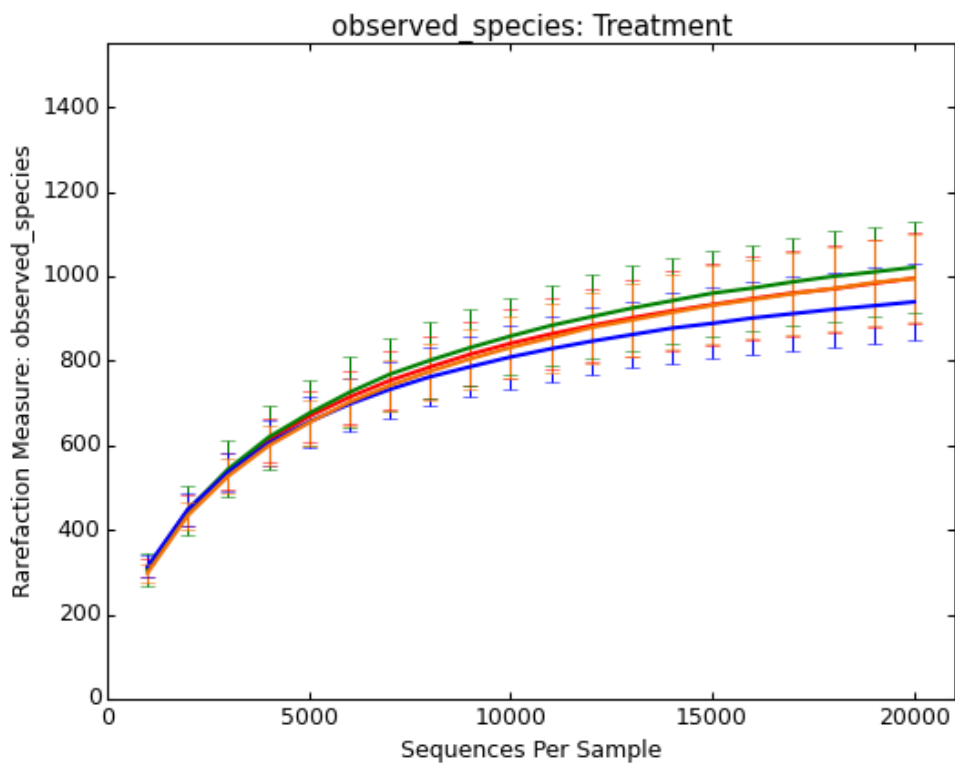


Figure 7.11 – Rarefaction plot showing observed species for dietary treatments; CONTROL (red), OIL (green), NITRATE (blue) and NITRATEOIL (orange).

7.3.4.2 Microbial community structure

Once unclassified taxonomies were removed, 19 were recorded at the phylum level. Details of the taxonomies with highest abundances at the phylum level can be found in Table 7.7. Firmicutes, Bacteroidetes, Proteobacteria, Euryarchaeota,

Verrucomicrobia, Spirochaetes, Tenericutes, Fibrobacteres and Actinobacteria were present at relative abundances > 1 %. Other phyla were present at < 1 % relative abundances. Figure 7.12 represents relative abundances at phylum level for CONTROL, OIL, NITRATE and NITRATEOIL dietary treatments. One hundred and eighty six taxonomies were recorded at the genus level. Once unclassified reads were moved (unclassified at any level), 183 genera remained. *Prevotella* accounted for the largest taxonomic group at the genus level, present as 25.0 %, 25.4 %, 32.4 % and 24.1 % of relative abundances within CONTROL, OIL, NITRATE and NITRATEOIL diets respectively. Other taxonomies present at relative abundances > 5 % at the genus level include *Ruminococcus*, *Methanobrevibacter*, families *Succinivibrionaceae*, *Methanobacteriaceae* and *Ruminococcaceae* and the order Clostridiales. Full details of relative abundances at the phylum and genus level can be found in the Appendix.

Table 7.7 – Mean relative abundance (%) of phyla within each of the 4 dietary treatments (CONTROL, OIL, NITRATE, and NITRATEOIL).

Taxonomy (Phylum)	CONTROL Mean Rel Ab. (%)	NITRATE Mean Rel Ab. (%)	NITRATEOIL Mean Rel Ab. (%)	OIL Mean Rel Ab. (%)
Firmicutes	36.7 (8.8)	31.5 (7.6)	35.4 (9.3)	38.4 (8.7)
Bacteroidetes	34.0 (7.4)	42.0 (10.6)	33.8 (10.7)	33.7 (10.5)
Proteobacteria	12.0 (7.2)	10.7 (6.3)	13.8 (7.4)	4.4 (3.2)
Euryarchaeota	7.3 (3.6)	4.3 (2.2)	5.6 (2.3)	10.5 (6.0)
Verrucomicrobia	2.4 (1.1)	2.4 (0.9)	3.4 (2.1)	2.7 (1.4)
Spirochaetes	1.9 (0.7)	2.8 (1.5)	1.6 (0.6)	2.0 (1.1)
Tenericutes	1.9 (0.7)	2.3 (0.7)	2.2 (0.8)	2.3 (1.2)
Fibrobacteres	1.1 (0.5)	1.5 (0.6)	1.1 (0.5)	2.6 (2.0)
Actinobacteria	1.1 (0.54)	1.4 (1.1)	1.8 (0.8)	1.3 (0.6)

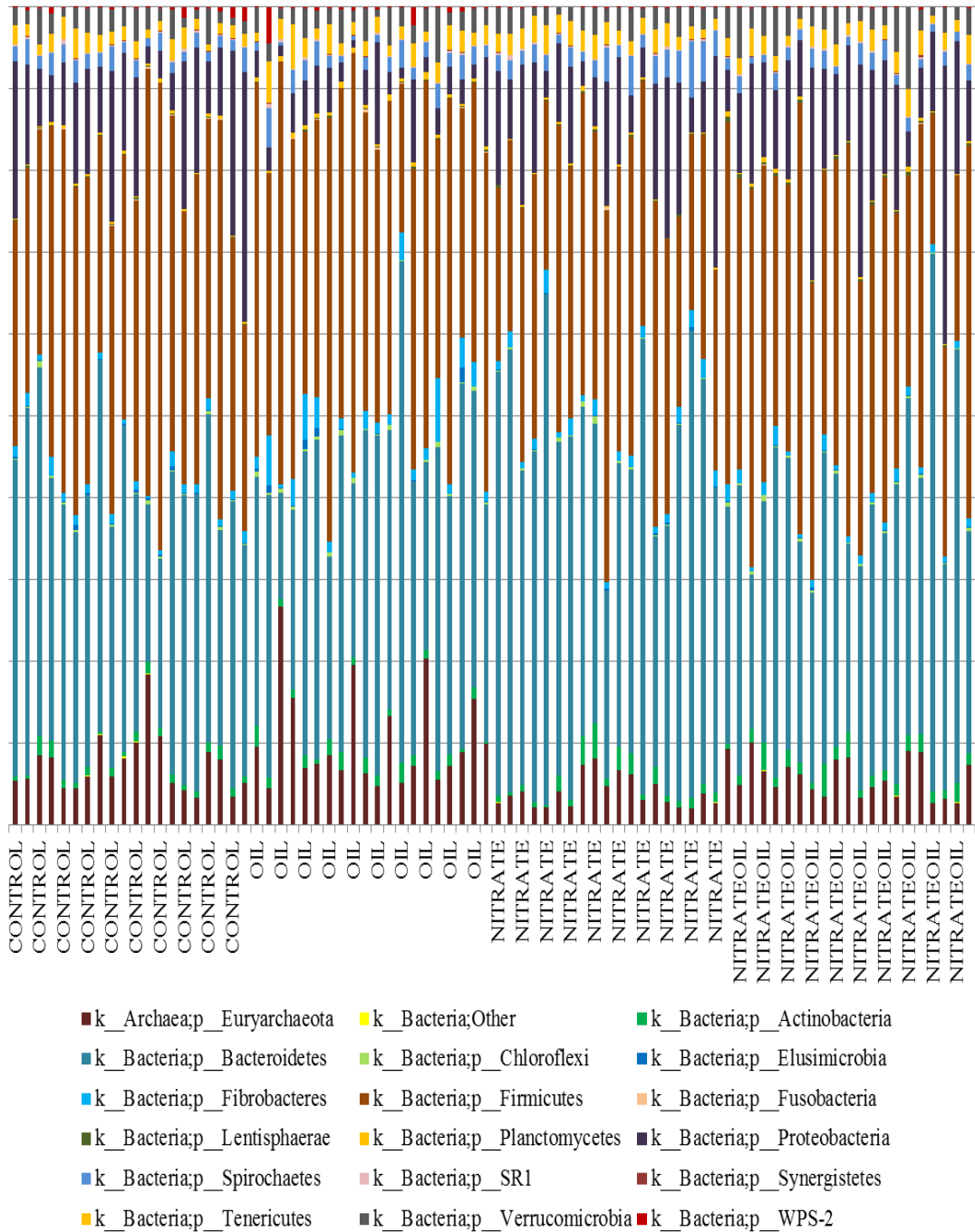


Figure 7.12 – Taxa summary plot of relative abundances of CONTROL, OIL, NITRATE and NITRATEOIL diets for individual rumen samples at the phylum level.

7.3.4.3 Dietary effects on rumen microbial communities

PC scores plots were compiled to assess overall differences in microbial communities (at the genus level) from each of the dietary treatments. PC-1 accounted for 59 % of the variation, PC-2 accounted for 24 % of the variation and PC-3 accounted for 6 % of the variation (Figure 7.13d). A slight grouping of OIL was observed when comparing PC-1 vs. PC-2 (Figure 7.13a), no separation or grouping was observed within PC-1 vs. PC-3 (Figure 7.13c). Two groupings were observed when comparing PC-3 vs. PC-2 (Figure 7.13b), however some overlap occurs (i) CONTROL, NITRATE and NITRATEOIL and (i) OIL.

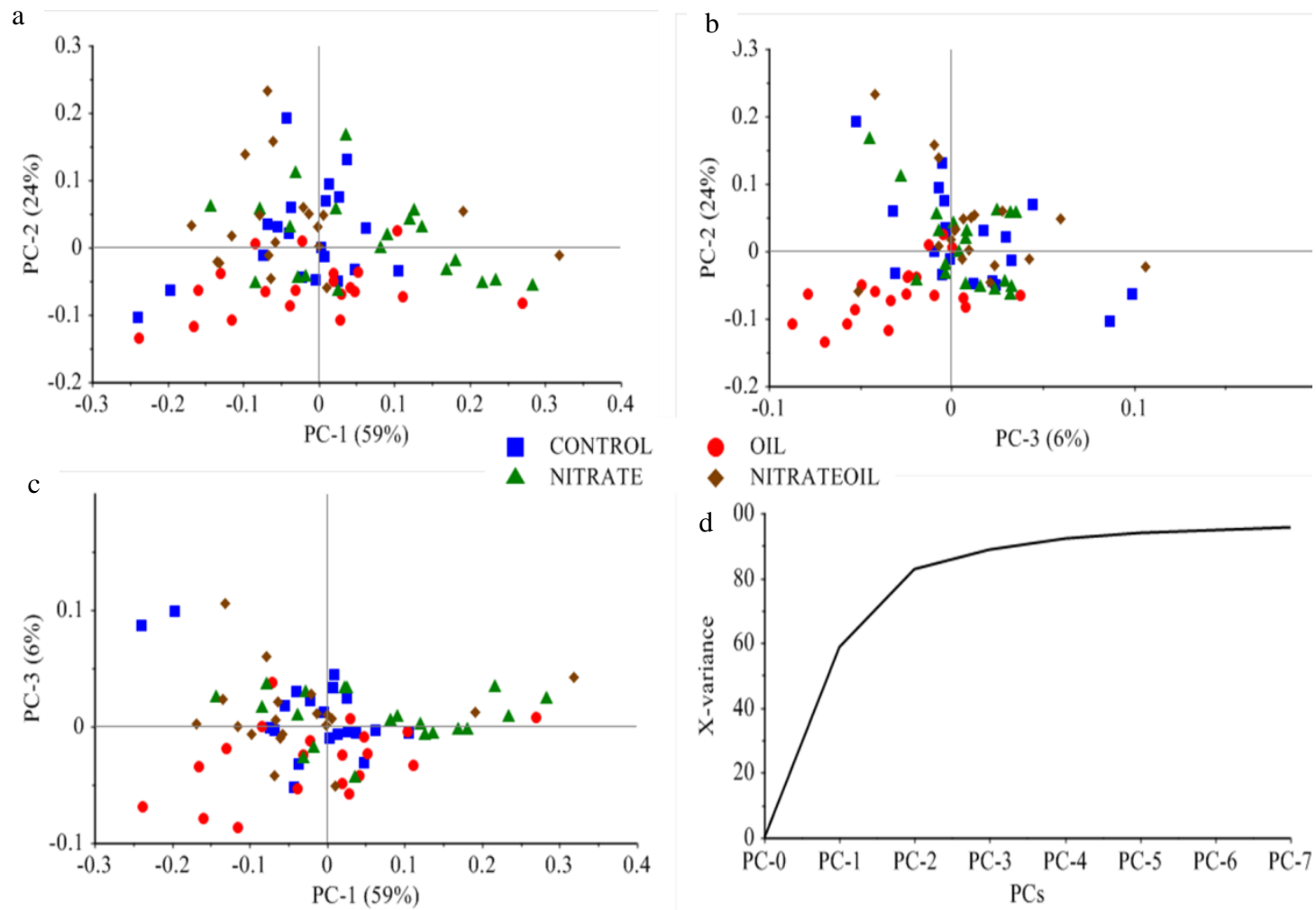


Figure 7.13 – PCA overview of 16S analysis of all 4 dietary treatments (a) scores plot for PC-1 vs. PC-2 (b) scores plot for PC-3 vs. PC-2 (c) scores plot for PC-1 vs. PC-3 (d) explained variance plot.

An interaction between nitrate*oil addition was observed for PC-1 ($P = 0.045$). The addition of nitrate or oil was found to be non-significant ($P > 0.05$) for PC-1. The addition of nitrate was found to be significantly different for PC-2 ($P = 0.002$) and for interactions between nitrate*oil ($P < 0.001$). The addition of oil was found to be non-significant ($P > 0.05$). The addition of nitrate ($P = 0.013$), oil ($P = 0.014$) and interaction between nitrate*oil ($P = 0.003$) were significant for PC-3. These results are summarised in Table 7.8. REML was used to assess the effect of diet, nominal feed efficiency level (FCR/RFI; low/high) and interactions. Diet ($P = 0.02$) and diet*FCR level ($P = 0.05$) interactions were significantly different for PC-1. Diet had a significant effect on PC-2 ($P < 0.001$). Diet ($P < 0.001$) and diet*RFI level ($P = 0.048$) for PC-3. Full details can be found in Appendix.

Table 7.8 – The effect of nitrate and oil addition on NIRS results using PC-1, PC-2 and PC-3.

Dietary Treatment	P Value		
	PC-1	PC-2	PC-3
nitrate	0.119	0.002	0.013
oil	0.076	0.077	0.014
nitrate*oil	0.045	<0.001	0.003

Ninety-one genus level taxonomies were found to be significantly different ($P < 0.05$) between diets when assessed with ANOVA with FDR using STAMP statistical software (full details in Appendix). When comparing NITRATE to CONTROL diet, 80 genus level taxonomies were significantly different ($P < 0.05$), 53 were found to be significantly different for the NITRATEOIL diet and 16 for the OIL diet.

Changes in relative abundances of bacteria and archaea associated with methane emissions were assessed between diets, due to the potential link with feed efficiency. The relative abundance of *VadinCA11* was greater in the CONTROL diet relative to other dietary treatments. *Methanosphaera* and *Methanobrevibacter* were reduced by the NITRATE (P < 0.001; P = 0.007) and NITRATEOIL (P > 0.05; P > 0.05) diets, but increased by the OIL (P = 0.005; P > 0.03) diet, significance levels of these were different between dietary treatment groups (Table 7.9). Relative abundances of *Campylobacter* were significantly increased for both the NITRATE (P < 0.001) and NITRATEOIL (P < 0.001) treatment groups.

Table 7.9 – Mean relative abundance (Mean rel. ab.) (s.d.) for each additive treatment (NITRATE, OIL and NITRATEOIL) and significance of genera associated with methane emissions of each diet additive relative to the CONTROL diet. P Value corrected using Benjamini-Hochberg FDR.

Taxonomy	CONTROL Mean rel. ab. (%)	NITRATE Mean rel. ab. (%)	NITRATEOIL Mean rel. ab. (%)	OIL Mean rel. ab. (%)
<i>VadinCA11</i>	0.6 (0.2)	0.3 (0.1)	0.4 (0.2)	0.3 (0.1)
<i>Methanosphaera</i>	0.09 (0.06)	0.03 (0.03)	0.07 (0.05)	0.2 (0.1)
<i>Methanobrevibacter</i>	6.6 (3.4)	3.9 (2.2)	5.2 (2.3)	10.1 (6.1)
<i>Succinivibrio</i>	0.1 (0.1)	0.3 (0.2)	0.3 (0.2)	0.1 (0.1)
<i>Succinivibrionaceae*</i>	10.0 (6.8)	8.6 (6.0)	11.7 (7.1)	3.2 (3.2)
<i>VadinCA11</i>		P < 0.001	P = 0.001	P < 0.001
<i>Methanosphaera</i>		P < 0.001	0.351	0.005
<i>Methanobrevibacter</i>		0.08	0.164	0.03
<i>Succinivibrio</i>		P < 0.001	0.006	0.347
<i>Succinivibrionaceae*</i>		0.518	0.442	P < 0.001

*Family *Succinivibrionaceae*, unidentifiable at the genus level

At the genus level, 181 taxonomies were identified. Of the 181, 164 were found to be present in all diets so were identified as the core microbiome. The remaining 17 taxonomies were found to be unique to at least one of the dietary treatments, no taxonomy was completely unique to a diet. Breed was found to have no effect on taxonomies when assessed within the CONTROL, NITRATE or NITRATEOIL dietary treatments. Relative abundances of *SHD-231* from the family *Anaerolinaceae* were significantly increased in LIMx steers offered OIL diet ($P = 0.049$). Full details can be found in Appendix.

7.3.4.4 Differences in microbial communities due to feed efficiency (comparisons within dietary treatment groups).

PC scores plots were compiled for each dietary treatment to assess relationship with nominal feed efficiency level. The effect of breed and nominal feed efficiency level (FCR/RFI; low/high) was assessed within each diet using REML. The first 3 PCs accounted for 87 %, 90 %, 93 % and 95 % of the variation within CONTROL, OIL, NITRATE and NITRATEOIL dietary treatments. Figure 7.14 shows PCA overview of 16S results within the OIL diet, samples labelled by FCR level (low or high). The remainder of the PC scores plots and explained variance can be found in the Appendix.

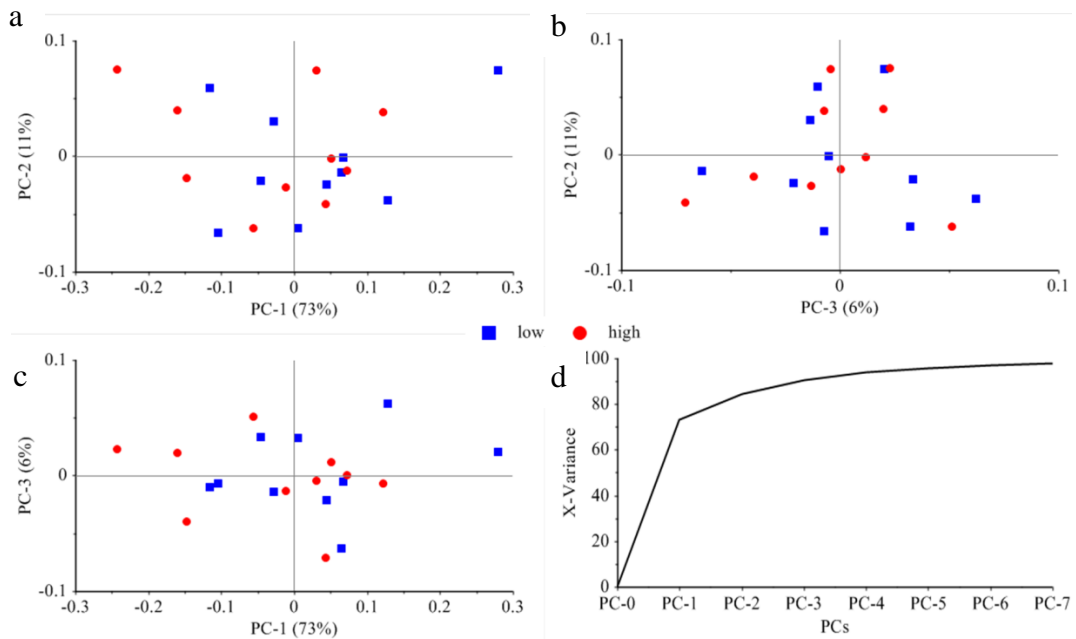


Figure 7.14 – PCA overview of 16S analysis of OIL dietary treatment, samples labelled according to nominal FCR level (low/high). (a) scores plot for PC-1 vs. PC-2 (b) scores plot for PC-3 vs. PC-2 (c) scores plot for PC-1 vs. PC-3 (d) explained variance plot.

The effect of breed and nominal feed efficiency level (FCR/FCR; low/high) was assessed within each diet using REML. Within the OIL diet a breed*FCR level interaction was observed for PC-1 ($P = 0.003$). No significance was noted within CONTROL, NITRATE or NITRATEOIL dietary treatments for FCR, RFI, breed or interactions.

The effect of breed and feed efficiency value was assessed using general linear regression. Within the OIL dietary treatment, breed ($P = 0.001$), FCR value ($P = 0.005$) and breed*FCR value ($P = 0.002$) had a significant effect on PC-3. No significance was noted within CONTROL, NITRATE or NITRATEOIL dietary treatments.

Firmicutes to Bacteroidetes ratios were 36.7 : 34.0, 38.4 : 33.6, 31.6 : 42.0 and 35.6 : 33.4 for CONTROL, OIL, NITRATE and NITRATEOIL diets respectively. Relationship between RFI, FCR and breed were assessed within each diet using Pearson's correlation. Within the CONTROL dietary treatment strong correlations

were observed for RFI within LIMx steers ($r^2 = 0.66$), within the OIL diet strong correlations were observed between RFI and AAx steers ($r^2 = 0.76$). Strong negative correlations between RFI and LIMx steers were observed within the NITRATE diet ($r^2 = -0.65$). All other correlations were weak ($r^2 < 0.05$), full details can be found in Appendix.

No differences were observed between taxonomies and feed efficiency was discovered when comparing genera using STAMP ($P > 0.05$). Nominal RFI and FCR levels (low vs. high) and Shannon indexes within each diet were compiled into a rarefaction plot (Figure 7.15), however there were no clear differences between nominal low and high groups. There were no significant differences ($P > 0.05$) in Shannon indexes between nominal feed efficiency level, Breed, or interactions when assessed within each treatment group. When assessed using feed efficiency value, breed ($P = 0.008$) and breed*RFI value interactions ($P = 0.049$) were observed within the NITRATE diet. AAx steers had higher microbial diversity (7.6) compared to LIMx steers (7.2) within the NITRATE diet. Full details can be found in the Appendix.

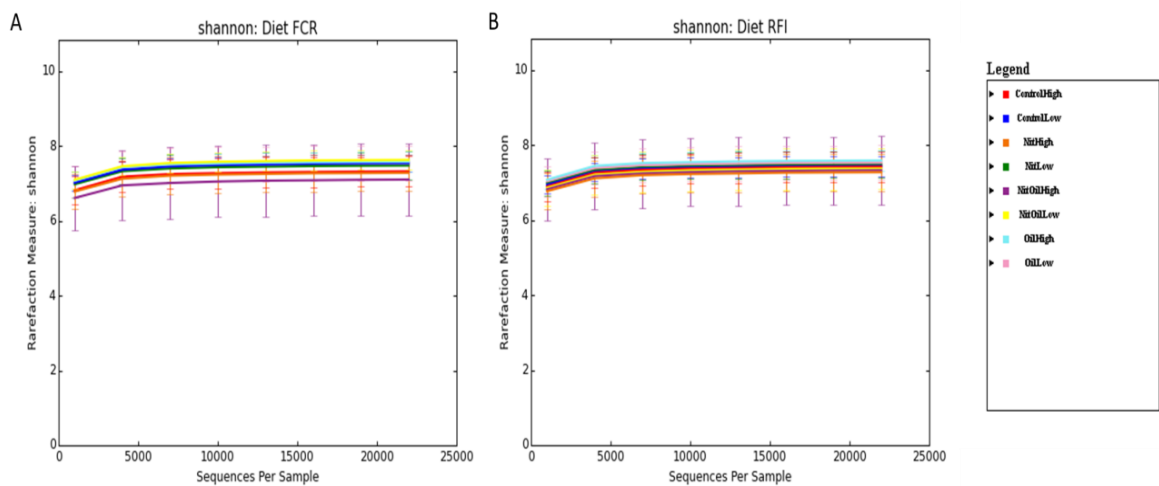


Figure 7.15 – Rarefaction plots containing Shannon diversities for nominal (a) FCR and (b) RFI groups (Low and High; as described in Section 7.2.3.3.2), shown separately for diet treatment groups (CONTROL, OIL, NITRATE, NITRATEOIL).

Taxonomies present at < 1 % relative abundance were discarded and the remaining assessed for links to feed efficiency using regression. At the phylum level a breed*FCR level was observed (P = 0.024) for Spirochaetes, a breed*RFI value interaction (P = 0.016), a breed*FCR value (P = 0.043) and a breed*RFI value (P = 0.005) interaction was observed for Verrucomicrobia within the CONTROL diet. Verrucomicrobia also has a significant effect on breed (P = 0.031) within the CONTROL diet. For the OIL diet, Actinobacteria influenced by FCR level (P = 0.028), Bacteroidetes were influenced by RFI value (P = 0.025), Breed (P = 0.049) and RFI value (P = 0.014). Bacteroidetes were influenced by RFI value (P = 0.010), breed (0.030) and and interaction between breed*RFI value was observed (P = 0.014). An interaction between breed*RFI value (P = 0.041) was observed for Firmicutes, and interactions between breed*RFI value (P = 0.019) observed for Proteobacteria. Proteobacteria was also influenced by breed (P = 0.010) within the OIL dietary treatment. No significant differences were detected for the NITRATE diet. RFI level*breed interactions (P = 0.012) affected the relative abundance of Tenericutes and FCR level*breed (P = 0.033) had a significant effect on Spirochaetes in samples from steers offered the NITRATEOIL diet.

At the family level, a breed*FCR level (P = 0.021) interaction was observed for *Spirochaetaceae*, breed* FCR level (P = 0.041) interactions and breed (P = 0.021) for *RFPI2* within the CONTROL dietary treatment. A breed * RFI value (P = 0.005) and level (P = 0.014) interaction was observed within *RFPI2*. Breed (P = 0.047) had a significant effect on *Erysipelotrichaceae*. For the OIL dietary treatment FCR value (P = 0.034) had an effect on *Veillonellaceae*. RFI level (P = 0.025), RFI value (P = 0.009), breed (P = 0.017) and breed*RFI value (P = 0.009) interactions were observed for *Prevotellaceae*. RFI value (P = 0.014) and breed*RFI value (P = 0.049) interactions were noted within *Methanobacteriaceae*. A breed*RFI value interaction was observed within *Ruminococcaceae*. *Lachnospiraceae* was influenced by RFI value (P = 0.037), breed (P = 0.008) and breed*RFI value interaction observed (P = 0.044). *Succinivibrionaceae* was influenced by RFI value (P = 0.026) and breed (P = 0.015) within the OIL dietary treatment. Within the NITRATEOIL dietary treatment breed (P = 0.003) and breed*FCR value (P = 0.002) had significant effects on *RFI2*.

Within the NIRATEOIL dietary treatment a breed*FCR level ($P = 0.033$) interaction was observed within *Spirochaetaceae*. *Veillonellaceae* was influenced by FCR value ($P = 0.017$) and a breed*RFI value interaction was noted for *RF39*. *Fibrobacteraceae* was influenced by RFI value ($P = 0.019$), breed ($P = 0.047$) and breed*RFI value interactions ($P = 0.017$). Full details of the regression analysis of relationships between feed efficiency measures and 16S communities at the phylum and family levels can be found in the Appendix.

7.4 DISCUSSION

The objectives of this study were (i) to determine differences in the chemical (NIRS) and physical (LD) breakdown, and the rumen populations (16S) associated with diet manipulation using additives and (ii) to explore any potential links with feed efficiency measures.

7.4.1 Laser Diffraction

Although LD shows promise as a rapid technique to assess faecal particle size a large amount of faecal sample (approximately 19 % on a wet basis) remained after the pre-sieving stage, the majority of which was comprised of grain husks. It is important to note that the amount of faecal matter remaining in the sieve was only recorded on a wet weight basis, so it is possible that the proportion may be different on a DM basis. The pre-sieving stage acted to remove particles larger than 1.8 mm because in theory these cannot be measured by the LD instrument (maximum particle size 2 mm). However, in the current study, these results show that particles between 1.8 and 2.0 mm were recorded suggesting that this upper limit is not absolute. Schadt *et al.*, (2012) reported that particles of a longer length are able to escape if the width is below the required size. LD results are based on an equivalent sphere of the particle size (Malvern Instruments, 2012), which overcomes issues associated with describing long, thin particles using a single numerical value.

LD results are expressed as a percentage volume across all size categories (Eshel *et al.*, 2004). It is therefore not possible to focus on individual size categories to determine effects of the diet or relationships with feed efficiency. In this study, MPS was calculated by taking the mid-point of the size category at which the cumulative frequency passed 50 %. Although this is a useful indicator for the median size, it is worth noting that comparisons to other studies may be difficult due to differences in technique used and the method used to determine median particle size. The low number of studies using LD as a technique to measure faecal particle size means that comparisons with studies that have used other techniques to assess faecal particle size distribution need to be made.

7.4.1.1 Differences between diets

Particle size was affected by diet and addition of additives to the diet. Three groupings were seen in the PCA scores plots for PC-1 vs. PC-3 and PC-2 vs. PC-3.

An increase in MPS of particles <2 mm was seen for the CONTROL diet. RSM was the only dietary constituent that was present in the CONTROL diet and not NITRATE, NITRATEOIL or OIL diets. During the preparation of RSM, seeds are crushed and oil removed, leaving seed husks which are high in protein (see chemical composition of diets; Table 7.1 and Table 7.2). These husks are largely undigested within the rumen (Kendall *et al.*, 1991), which may explain the increased MPS in faeces from the CONTROL diet.

Calcium nitrate, added to the diet as a salt, exerts its effects on faecal particle size in the absence of changes in feed particle distribution. Allen *et al.*, (2000) suggested that reduction in numbers of cellulolytic bacteria, which causes reductions in cellulolytic activity, could increase retention time in the rumen and decrease DMI. However, there was no reduction in DMI for steers offered the NITRATE diet in this study (Roehle *et al.*, 2016). Garwacki and Redel (1989) carried out a series of experiments that assessed the effect of nitrite (intermediate step in conversion of nitrate to ammonia) at various doses via intravenous infusion on rumen motility in sheep. These studies showed that rumen contractions decreased, even at low levels of nitrite. Rumen motility and contractions are an important mechanism that enables separation of digesta within the rumen, allowing particles to leave the rumen whilst retaining other particles for further digestion. Kaske *et al.*, (1997) showed the

importance of reticular contractions for passage of digesta from the rumen of sheep. They recorded an increase in the proportion of large particles in faeces when weights were added to the reticulum to decrease reticular contractions. In the current study, the increase in large particles retained after the pre-sieving stage is likely due to nitrite, or potentially nitrate, inhibiting rumen contractions and thus increasing faecal particle size.

7.4.1.2 Between-animal variation

Although variation between-animals was observed in the PCA scores plot, this variation was not related to differences in feed efficiency, with the exception of FCR having an effect on PC-2 for the NITRATEOIL diet. This coupled with RFI or FCR value, or breed, having no effect on MPS indicates that although LD is able to determine variation in faecal particle size between groups of animals, this variation is unrelated to variation in feed efficiency.

7.4.2 NIR

Spectral regions were dominated by regions associated by 2310, 2350 and 2388 nm, which have been previously associated with lipids (Bertrand, 2002; Decruyenaere *et al.*, 2009; Wetzel, 1983), in particular cuticular waxes (Flinn *et al.*, 1992). Moisture was dominating spectral regions 1450 and 1940 nm (Shenk *et al.*, 1992).

7.4.2.1 Differences between diets

Differences in the PC-2 and PC-3 direction were driven by the presence or absence of nitrate in the diet, with wavelengths associated with protein, cellulose, lipids and moisture showing highest loadings. Differences observed were not due to initial protein levels in the diet as the OIL dietary treatment contained higher amounts of CP than the CONTROL dietary treatment (Table 7.2). As the addition of nitrate may reduce rumen contractions (Garwacki and Redel, 1989) it may also lead to reduced

digestion (Kaske and Midasch, 1997). In the experiment of Kaske and Midasch, (1997), reduced reticulorumen contractions were coupled with a decreased rate of flow of digesta out of the reticulo-omasal orifice to the abomasum. The abomasum was reported to have been enlarged with digesta as a result of the change in consistency of the digesta, with potentially, a slower rate of passage. However this phenomenon disappeared 2 days after the ruminal weight was observed. Nevertheless, Nolan *et al.*, (2010) reported no difference in digesta outflow between nitrate and urea supplementation treatments. The abomasum is the primary site of protein digestion due to the presence of pepsins (proteolytic enzymes) (Knowles, 1970). It is possible that an increase in retention time in the abomasum would allow for increased protein digestion, explaining high loadings for wavelengths associated with protein and the separation observed. However it must be remembered that high loadings were also observed for protein in the PC-1 direction, which was not affected by the addition of nitrate. A second possible reason for the spectral differences observed in regions associated with protein is due to the fact that nitrate is able to replace non protein nitrogen as a source of N for microbial synthesis in the rumen (Latham *et al.*, 2016). Nolan *et al.*, (2010) also reported increased microbial protein outflow in response to nitrate supplementation (4 % potassium nitrate), however the increase was not found to be significant.

The addition of oil to the diet had a significant effect on PC-1. Wavelengths associated with lipids, moisture and cellulose showing highest loadings. The addition of oil to the diet has been previously found to reduce fermentability of fibre within the rumen (Tager and Krause, 2011). However the addition of nitrate causes a reduction in ruminal contractions and therefore potentially increases retention time. This could explain why separation was seen between NITRATE and NITRATEOIL diets but not OIL and CONTROL dietary treatments observed when assessing PC-2 vs PC-3. The OIL diet showed greatest spectral variation, indicating greatest variation in digestion between groups.

7.4.2.2 Between-animal variation

When assessed within each diet, feed efficiency or breed was found to have no effect on PC scores, with the exception of PC-2 for the CONTROL treatment groups and PC-1 for the NITRATEOIL treatment group. Wavelengths associated with moisture

and protein, and cellulose showed highest loadings for PC-2 of the CONTROL treatment groups. However it must be noted that wavelengths associated with moisture, protein, cellulose and lipids showed highest loadings regardless of dietary treatments. In agreement with data presented here, Lebziem and Paul (1997) also reported the presence of peaks associated with lipids (2310 – 2348 nm) in duodenal samples from dairy cows. However spectral region 210 nm has also been associated with indigestible fibre (Barton *et al.*, 1986), carbohydrates, lignin and protein, and waxes (Barton *et al.*, 1992) and methylene stretch (Russel *et al.*, 1989) Spectral region 2260 nm has been reported as being characteristic of digestible fiber, however Barton *et al.*, (1986) reported that this region was associated with indigestible fiber. Wavelengths associated with protein were also observed for PC-1 within the NITRATEOIL diet. Moisture was also observed in spectral regions 1900 – 2000 nm (centred around 1940 nm) was observed in this study. Even though these spectral regions described above were associated with differences in feed efficiency, these spectral regions were common among PCs for each dietary treatment. The use of difference spectra showed no conclusive evidence that NIRS was able to predict LE or ME steers relative to the median value. It is therefore not likely that these wavelengths or faeces alone contribute to differences in feed efficiency.

7.4.3 16S Sequencing

Two separate sequencing runs were carried out to provide sufficient depth of coverage – this was confirmed by achieving an average of over 80,000 reads per sample. A lower number of OTUs was observed in samples from the OIL diet when assessed using rarefaction plots however, when analysed using Shannon diversity no difference was seen. This is in agreement with Veneman *et al.*, (2015), who found no significant differences in Shannon diversity, in either bacteria or archaea, when comparing nitrate, linseed oil and control diet treatments. These authors also found that nitrate supplementation significantly decreased methane (g/kg DMI) as opposed to linseed oil supplementation which did not affect methane yield. Although no differences in Shannon diversities were observed in the current study, the lack of balancing for diet across sequencing runs could have led to sequencing bias (Schirmer *et al.*, 2015). However, Kennedy *et al.*, (2014) found that sample

sequences were consistent when samples were run on separate lanes on different days, with PCR and library preparation variations likely causing more of an affect.

7.4.3.1 Differences between diets

Fibrobacter, Butyrivibrio, Pseudobutyrvibrio and Ruminococcus are cellulolytic bacteria (Nyonyo *et al.*, 2014). In the current study, relative abundances in Fibrobacter, Ruminococcus and Pseudobutyrvibrio were reported for the NITRATE diet relative to the CONTROL, which agrees with Veneman *et al.*, 2015 who found increased Fibrobacter abundance with nitrate supplementation. Even though statistically significant increases in Fibrobacter and Pseudobutyrvibrio were observed in the study described in this chapter, these were numerically small differences - less than 1%, and so unlikely to be a factor contributing to variation in feed efficiency. Marais *et al.*, (1988) reported that whilst increasing the abundance of bacteria associated with nitrate reduction, the addition of nitrate reduces cellulolytic activity which disagrees with the findings of the current study. Latham *et al.*, (2016) reported that bacterial denitrifiers increase in number in the rumen with the addition of nitrate; in particular there are increases in Pseudomonas, Propionibacterium and Nitrosomonas. In the current study, only Pseudomonas was identified and, as expected, levels were increased ($P < 0.05$) for the NITRATE diet relative to the CONTROL. Asanuma *et al.*, (2002) and Iwamoto *et al.*, (2002) reported increases in Veillonella, which has been reported as a denitrifier, when nitrate was added to the diet (Iwamoto *et al.*, 2002; Lin *et al.*, 2013). Results of the current study show that Veillonella was significantly different when comparing NITRATE and NITRATEOIL to CONTROL diets; however, Veillonella was higher in samples from the CONTROL diet in comparison with both nitrate-supplemented diets, which contradict the findings of Asanuma *et al.*, (2002) and Iwamoto *et al.*, (2002). Both these studies used culturing techniques whilst the study described in this chapter used relative abundances and not absolute counts. It is possible that effects of other microbial populations are having an effect on relative abundances of Veillonella.

Latham *et al.*, (2016) suggested that an undesirable effect of nitrate addition to the diet is increased number of microbes associated with food borne pathogens. In the present study, relative abundances of Campylobacter, a known food borne pathogen

(Silva *et al.*, 2011), increased for both the NITRATE and NITRATEOIL diets relative to the CONTROL (both $P < 0.05$) which agrees with the findings of Lin *et al.*, (2013) who also found that *Campylobacter* increased with the addition of potassium nitrate to the diet. No significant differences ($P < 0.05$) were observed for the Order YS2 in the NITRATEOIL or OIL relative to CONTROL diets. Zhao *et al.*, (2015) proposed the nitrate reducing function of *Campylobacter*. However, it must be remembered that these are relative abundances and fluctuations in abundances may be due to decreases in other taxonomies as much as increases in a group of interest. Poulsen *et al.*, (2013) showed that rapeseed oil added to the diet significantly reduced methane emissions. Additionally, Poulsen *et al.*, (2013) also observed reductions in Thermoplasmata, along with increases in Methanosphaera and Methanobrevibacter. This indicated that under the dietary conditions of that study, Thermoplasmata levels were more important for methane emissions than the previously studied Methanobacteriales (Methanosphaera and Methanobrevibacter). Numbers of Methanobacteriales (indicated by levels of 16S rRNA rumen cluster C (RCC)) reported by Poulsen *et al.*, (2013) were relatively low, approximately 0.8 % for control diet and 0.2 % for fat supplementation diet (using rapeseed oil). Data from the present study showed an average of 6% Methanobacteriales were present in samples from steers offered the CONTROL diet, which increased on average to 10.2% for the OIL diet. This is likely counteracting the effect of the Thermoplasmata (VadinCA11), which decreased from 0.6 % to 0.2 %, for the CONTROL relative to OIL diet. The increase in Thermoplasmata (VadinCA11) in the CONTROL diet in the current study may be partially explained by the presence of rapeseed meal in the CONTROL diet. Rapeseed meal, which was present only in the CONTROL diet, contains precursors (Glucosinolates and sinapine) for trimethylamine (TMA) (Kretzschmar *et al.*, 2013) which is a substrate utilised by Thermoplasmata (VadinCA11). This is one possible explanation why methane emissions were higher for the CONTROL diet and why Thermoplasmata were reduced across all additive diets in the current study.

It has been proposed that *Succinivibrionaceae* is able to compete with the methanogens (Archaea) for hydrogen, with succinate and propionate being produced in the place of methane (McCabe *et al.*, 2015). Veneman *et al.*, (2015) noted that no

clustering was observed between linseed oil or nitrate supplementation in relation to a control diet, when comparing bacteria or archaea. However, non-metric dimensional scaling (NMDS) was performed in the Veneman study, this technique uses rank order correlations.

7.4.3.2 *Between-animal variation*

Diet has been previously reported to affect rumen microbial communities (Henderson *et al.*, 2015; Singh *et al.*, 2014; Veneman *et al.*, 2015). Breed has also been reported to affect rumen microbial communities, Lee *et al.*, (2012) found that growing Holstein-Friesian dairy cows and Hanwoo steers had different microbial communities when assessed using pyrosequencing, however the effect of gender must not be ignored. A recent study found that differences in rumen microbial communities between Holstein and Jersey cows occurred when cows were under same dietary conditions (Paz *et al.*, 2016). In the current study, FCR, RFI, Breed and interactions were found to affect taxonomies at both the phylum and family levels. There were no consistent taxonomies that were related to feed efficiencies across the different diets. This is not surprising given the well documented ability of diet to modify microbial populations which may have countered any variation in taxonomies caused by differences in feed efficiency (Lee *et al.*, 2012; Carberry *et al.*, 2012). Breed and breed*RFI level interactions was found to have an effect on an relative abundance of an unidentified family in the order RF39 within samples from the OIL and NITRATEOIL diets; this group has previously been reported to have a positive, although weak, association with RFI (Jami *et al.*, 2014). However, Jami *et al.*, (2014) noted very little research has been carried out on this order suggesting that further work is warranted in ascertaining the relationship between feed efficiency and microbial populations.

The present study did not select for low and high feed efficiencies, as all samples were sequenced. In contrast Rius *et al.*, (2012) found that PCA scores based on relative abundances of rumen microbial populations were not able to determine differences between low and high RFI dairy cows. Similarly, Myer *et al.* (2015; 2016) also found that UniFrac PCoA plots of microbial communities from low and high ADFI and ADG steers were not able to detect clustering. However, Rius *et al.*,

(2012) did report that abundances of *Fibrobacteraceae* and *Prevotellaceae* were higher in less efficient cattle, and *Lachnospiraceae* were more abundant in more efficient animals. Jewell *et al.*, (2015) also found that inefficient dairy cows had increased levels of *Anaerovibrio*, Clostridiales, *Prevotella* and *Ruminococcaceae*.

Fibrobacteres to Bacteroidetes ratio has been previously associated with feed efficiency in cattle (Jami *et al.*, 2014; Ramirez Ramirez *et al.*, 2012; Turnbaugh, 2006), with many studies also focusing on this relationship within humans and mice. Firmicutes to Bacteroidetes ratio was found to be highest for samples from the NITRATE diet compared to other diets. However low R^2 values were reported for correlations between F:B and feed efficiency measures. Jami *et al.*, (2014) reported strong correlations between F:B ratio and milk-fat yield ($R^2=0.72$), with strong correlations also being observed in mice (Turnbaugh *et al.*, 2006). Ramirez Ramirez *et al.*, (2012) found that dried distillers grains with solubles (lipid based additive to the diet) significantly reduced the ratio. However, the findings of Ramirez Ramirez *et al.*, (2012) are not in agreement with the current study where there were no significant differences in F:B ratio for OIL relative to CONTROL diets.

In the present study, steers fed NITRATE were found to have higher FCR values compare to steers offered the CONTROL diet ($P < 0.05$), although no significant difference was observed in RFI between the diets. Ley *et al.*, (2006) showed that obese mice had fewer Bacteroidetes compared to lean mice, which the authors suggested to be the result of more effective release of calories from digestion. In production terms this would make the animal more efficient. A greater relative abundance of Bacteroidetes in samples from the NITRATE diet could explain the lower efficiency of these animals compared to CONTROL, OIL or NITRATEOIL diets (Roehe *et al.*, 2015).

A recent study by Shabat *et al.*, (2016) found that more efficient animals have a less diverse rumen microbial community. Shabat *et al.*, (2016) suggest that this decreased diversity allows for more relevant metabolites to be produced and thus, more substrates for the host animal to use. In this present study significant differences in Shannon diversities were seen between LIMx and AAX steers associated with RFI within the NITRATE diet, with lower diversity observed in LIMx steers. LIMx have been shown to be more efficient breeds than AAX (Crowley *et al.*, 2010), which

supports the findings of Shabat *et al.*, (2016). However there were no differences between feed efficiencies (either RFI or FCR) and rumen microbial diversity using the Shannon index in CONTROL, OIL or NITRATEOIL diets. Myer *et al.*, (2015) reported no differences in Shannon diversities between efficient and inefficient animals. In the study of Myer *et al.*, (2015), rumen samples from high and low average daily feed intake (ADFI) and ADG steers were sequenced using Illumina MiSeq platform. A further study by Myer *et al.*, (2016) reported no difference in Shannon diversities between efficient and inefficient animals using samples of jejunal digesta taken from the same group of steers.

7.4.3.3 Differences over time

Huws *et al.*, (2016) found that the order Clostridiales and family *Lachnospiraceae* increased significantly between 1-2 and 4-8 h after feeding using 16S rRNA (cDNA) pyrosequencing. The authors also found that families *Prevotellaceae* and *Fibrobacteriaceae* did not change significantly over time. There were no significant differences in the relative abundances over a 3-h sampling period (per treatment group) in the present study. This lack of significant change could be due to several factors. Firstly variation between rumen microbiomes has been documented (Ross *et al.*, 2012). It is likely that any differences in microbial populations that may have arisen over time are counteracted by the overall differences in the microbial communities between-animals. A second possible reason for the absence of an effect of time on microbial populations is that this study used DNA to perform sequencing whereas Huws *et al.*, (2016) performed sequencing using RNA. Finally, the present study essentially comprised of liquid associated bacteria, as samples were strained through cheesecloth before being stored prior to analysis. Huws *et al.*, (2016) study likely involved a greater proportion of solid associated bacteria as samples were washed to remove loosely attached microbes. As seen in Chapter 4, sample site within the rumen has a marked effect on microbial populations. Li *et al.*, (2009) found that sampling rumen contents -3, +3 and +9 h relative to feeding had no effect on DGGE bands or qRT-PCR. qRT-PCR was carried out using primers which were selected to target *Fibrobacter*, *Ruminococcus*, *Butyviribrio*, *Eubacterium*, *Prevotella*,

Streptococcus and Selenomonas. Due to limitations of DGGE only a limited number of bacterial groups were analysed.

7.5 CONCLUSION

NIRS, LD and 16S based techniques were able to differentiate between different dietary treatments. It is interesting to note that nitrate addition to the diet likely reduced rumen contractions and this may explain increases in particles >2 mm. the addition of nitrate also reduced relative abundances of cellulolytic bacteria. Breed appeared to have an effect on microbial communities within the NITRATE dietary treatment, however no consistent links were observed between feed efficiencies and proxies across all dietary treatments.

CHAPTER 8

GENERAL DISCUSSION

8.1 SUMMARY OF MAIN FINDINGS

Feed represents up to 75 % of variable costs associated with beef production (Ahola and Hill, 2012; Hersom, 2009), so that small increases in feed efficiency are likely to have large effects on profitability. Variation between-animals in cattle has long been known (Koch *et al.*, 1968) and there have been previous attempts to describe the components of variation in feed efficiency, including differences in digestion, metabolism and animal activity (Herd *et al.* 2004). At present there are fundamental gaps in knowledge about the relationship between feed efficiency and between-animal variation in digestion. Current techniques to assess digestion processes are time consuming, laborious, sometimes invasive, and generally not practical for characterising large numbers of animals. The overall aim of this thesis was to develop and apply rapid techniques to address these gaps.

Next generation sequencing using the 16S rRNA gene is a rapid technique to assess rumen communities, which has been adopted widely over the last few years. However, variability associated with sampling, DNA extraction, library preparation and sequencing techniques has resulted in conflicting results. Primers used (Caporaso *et al.*, 2012) in the studies described here have allowed bacteria and archaea to be detected. The technique identified differences in the rumen microbial community associated with different diets (perennial ryegrass vs. white clover; Chapter 4), and also large differences between communities associated with the solid- and liquid-phase of the rumen. Rumen samples from fistulated cows were used in this initial study, but in subsequent work (Chapter 7) samples were taken via a naso-gastric tube as this is the only viable approach when working with large numbers of animals. The ability of the 16S community profiling technique developed in Chapter 4 to distinguish dietary treatments was confirmed in the later study (Chapter 7), which showed that NITRATE and OIL dietary treatments had a large effect on bacterial and archaeal communities. However, no consistent relationship between feed efficiency measures and the rumen microbiome was observed across dietary treatment groups. For the NITRATE dietary treatment, a significant reduction in diversity (measured using the Shannon index) with increasing feed efficiency was present for LIMx steers. The lack of consistent relationship across diets and breeds suggest that there is

no strong basis for relationships between feed efficiency and the microbial (at least the bacterial and archaeal) community.

No previous study, to our knowledge, has explored the use of NIRS to assess between-animal variation in digestion processes without the use of calibrations. Although faecal NIRS showed differences between restricted and *ad libitum* animals (Chapter 6), no differences were seen when both groups were fed *ad libitum* during the subsequent realimentation period. Since there were differences in feed efficiency during both the feed restriction and realimentation periods (Table 6.2), these results suggest that there is little or no involvement of differences in digestive processes in explaining compensatory growth. Faecal NIRS was also able to discriminate samples from different diets (Chapter 7), however there was no relationship between digestive processes (faecal NIRS) and variation in feed efficiency within treatment groups, whether expressed as FCR or RFI.

Interestingly spectral regions associated with lipids (1428, 2310, 2350 and 2380 nm), were noted in Chapters 5, 6 and 7. These spectral regions have previously been associated with cuticular waxes (Flinn *et al.*, 1992) and correspond well with the C₃₂ alkane and oleic acid. Differences in these spectral regions were found between nominal leaf and stem fractions (Chapter 5) and were present at higher levels in faeces from restricted animals relative to *ad libitum* animals (Chapter 6). This shows potential for further investigation using these spectral regions as proxies to assess intake/digestibility of grass using naturally occurring alkanes (C₃₂). Key regions of difference spectra from the time course of rumen digestion (Chapter 5) were similar to those when comparing faeces from bulls on *ad libitum* or restricted feeding (Chapter 6). Differences in retention time were simulated using different incubation times in Chapter 4. It seems likely that these observations are connected by a common mechanism with differences due to either the retention time of feed in the rumen or the intensity of rumen fermentation. During the realimentation period (Period 2; Chapter 6), there was little residual difference between faecal NIR spectra from animals that had previously been fed at *ad libitum* or restricted levels. NIR difference spectra were also used to further analyse differences between low and high feed efficiency steers relative to the median (Chapter 7), but no significant relationships were found.

Previous work on faecal particle size has assessed the effects of type of feed (Maulfair *et al.*, 2011). To our knowledge, direct links between feed efficiency and faecal particle sizes have not been investigated. The LD technique was developed over a number of studies described in Chapter 4 and subsequently tested with data from feed efficiency measures and faecal particle size of steers (Chapter 7). Faecal particle size was affected by diet, but no clear relationships were found between feed efficiency measures and faecal particle size of the steers. Addition of nitrate to the diet increased the percentage of particles retained (>1.8 mm) during the preparatory step of the procedure. The addition of nitrate to diets reduces rumen contractions (Garwacki and Redel, 1989), and this may result in reduced mixing of digesta, so that faecal particle size is increased.

Overall, there were no consistent relationships between faecal NIRS, LD or rumen 16S community analysis and feed efficiency. All three techniques showed effects of diets, as well as between-animal variation. In contrast to other recent studies that have explored relationships with feed efficiency (McCann *et al.*, 2014a; Shabat *et al.*, 2016), this study used samples taken from a range of animals rather than only from extreme high- and low-feed efficiency animals. Although studies with samples from extreme animals are useful for identifying potential mechanisms, it is the variation between all animals in the population that is more relevant to the commercial setting, or for implementation in animal breeding.

The fact that techniques used in these studies did not prove effective for determining differences in feed efficiency measures, confirms the suggestion that only 10 – 14 % of the variation observed in RFI between-animals can be accounted for by digestion processes (Arthur and Herd, 2008; Herd *et al.*, 2004). These authors suggested that the remaining variation could be explained by differences in heat increment of feeding, energy retention, activity and host processes such as protein turnover. It is evident from the work carried out in this thesis that differences in digestion processes, whether from rumen or faecal samples, only play a small part in feed efficiency differences.

8.2 PRACTICAL CONSIDERATIONS OF METHODOLOGY

Faeces contain a mix of undigested food, sloughed intestinal cells, microbes and other endogenous secretions. The amount of feed digested, not taking endogenous losses into account, is described as ‘true’ digestion (van Soest, 1994). One of the major questions raised when using faecal NIRS and LD as techniques to assess between-animal variation in digestion is whether they are picking up true digestion or endogenous losses. These and other limitations of the sampling and analytical techniques will be discussed further in the following sections.

8.2.1 LD

Despite considerable effort to refine the LD technique for analysis of faeces (Chapter 4), there remained variation between replicate runs of the same sample (Section 4.2.3, 4.2.3 and 4.2.5), so an average of three scans was used in subsequent work (Section 4.2 and Chapter 7). This variation must be borne in mind when considering how far this technique can be used to distinguish treatment groups or between-animal variation. The instrument could measure a maximum particle size of 2.0 mm, therefore the pre-sieving stage was introduced to remove particles larger than 1.8 mm. However particles were recorded between 1.8 and 2.0 mm during each of the runs and this probably reflects the way in which particle size is defined by the software. Particle sizes are described on an equivalent sphere basis (Malvern Instruments, 2012). In reality faecal samples contain particles in a range of shapes, including long thin fibrous particles derived from forage fibre. Whilst this approach results in some anomalies (particles that are apparently larger than the sieve size), it does provide a common basis for comparisons. However, this approach was limited by the range in particle sizes measured by the instrument used and the pre-sieving step used to removed particles that could potentially contribute to feed efficiency. Newer LD instruments are able to assess particles up to 3.5 mm, which corresponds to the size of faecal particles of up to 3.6 mm observed in cattle (Cardoza, 1985).

During all LD studies in this thesis the smallest particle size fraction reported by the instrument was 0.01 – 25 μm . It seems likely that the finest particles are heavily influenced by sloughed cells and other endogenous processes such as hindgut

fermentation. It is conceivable that differences in feed efficiency related to digestive processes could be associated with either differences in large particles (i.e. physical breakdown of large particles) or small particles (e.g. endogenous components of faeces), but in this work there was little evidence of relationships between any of the particle size fractions and feed efficiency.

8.2.2 NIRS

NIRS is a rapid and low-cost technique for determining chemical composition of samples. Moisture, even present in small amounts, can have a large effect on NIR spectra, so it is essential that samples are completely dried before scanning. Samples were re-dried before scanning in each study, but variation in the moisture peak was noted in all studies, with particularly large effects in Section 3.3. Residual moisture is seen as peaks around 1940 and 1440 nm and small variation in residual moisture of silages affected spectral regions 1900 – 2000 nm (Baker *et al.*, 1994). It has been suggested that water is able to interact with protein and starch (Coleman and Murray, 1993).

Time of sampling relative to feeding affects NIRS spectra as described by Lister *et al.* (1997) for duodenal digesta collected from silage-fed steers at various points throughout the day. Differences in the NIR spectra of duodenal digesta were associated with fibre fractions and fractions containing nitrogen, particularly evident for steers offered low DM silage. A single faecal sample was collected for each steer for the study conducted in Chapter 7, faecal samples were collected over approximately a three h period. The study by Lister *et al.* showed that spectral variation was present, even for samples collected 3 h post feeding. Samples collected for use in Chapter 7 were carried out approximately 1 h after last feeding, and sampling lasted for approximately 3 h. Collecting several faecal samples and averaging NIRS spectra may overcome this issue in future studies.

8.2.3 Rumen microbiome

Next generation sequencing has allowed identification of previously unculturable rumen microbes, however error is introduced at each step of the process from rumen sampling to sequencing.

The study reported in Section 4.3 demonstrated substantial differences in microbial communities based on sampling solid and liquid rumen digesta. *Prevotella* was found at higher relative abundances in the liquid phase of the rumen, and this was associated with lower diversity. This study also demonstrated clear diet effects on the rumen microbiome. Thus, in order to explore potential relationships between the microbiome and feed efficiency, it is essential to take account of diet and sample type effects. Sample storage has also been shown to affect microbiome measurements; McKain *et al.*, (2013) reported a loss of Bacteroidetes in unprotected samples, compared to samples preserved with glycerol as a cryoprotectant. This led to an apparent higher proportion of Firmicutes but archaea were not affected by the addition of the cryoprotectant.

Library preparation and sequencing are other areas that can result in variation. One limitation of next generation sequencing is that only portions of the 16S rRNA gene (in this case the hypervariable V4 region) can be sequenced. It has recently been suggested that hypervariable regions V4 – V6 are the most reliable for determining bacterial species, whilst V2 and V8 hypervariable regions are the least reliable (Yang *et al.*, 2016). Differences in hypervariable regions used can influence relative abundances, the choice of pyrosequencing platform can also have an effect on the apparent bacterial community (Salipante *et al.*, 2014), however agreement between MiSeq and HiSeq platforms has been noted (Caporaso *et al.*, 2012).

8.3 FUTURE WORK

Techniques used in this thesis provided rapid proxies to determine differences in the composition of faeces, the rumen microbiome or both, which were associated with dietary manipulation (feed restriction or different diets). However, the techniques were not sensitive enough to identify differences associated with between-animal variation in feed efficiency in Chapter 7. It is possible that the lack of association

between feed efficiency and the microbiome may be related to lack of association between the microbial community (estimated from analysis of 16S genes in the current work) and microbial genes. Other workers (Roehe *et al.*, 2016) identified relationships between feed efficiency and a small group of rumen genes and it may be possible to improve relationships using software that predicts rumen genes based on 16S communities (PICRUSt; Langille *et al.*, 2013). This approach would be less expensive than sequencing the whole rumen metagenome, which is currently prohibitively expensive.

It would be interesting to evaluate the relationship between the rumen microbiome and breeds in greater depth, due to the suggestion (Chapter 7) of a relationship for the NITRATE dietary treatment group.

The study reported in Chapter 7 looked at relationships between feed efficiency and the microbiome using primers that were able to detect bacteria and archaea (Caporaso *et al.*, 2012). Whilst these primers were designed to detect bacteria and archaea, the presence of protozoa and fungi should not be ignored. Protozoa make up a large proportion of the rumen biomass due to their size, however their role is largely unknown (Williams and Coleman, 1992). It has been suggested that eliminating ciliated protozoa could potentially reduce methane production by up to 11 % (Newbold *et al.*, 2015) because around one-third of methanogens are associated with protozoa. Methane represents an energetic loss to the animal therefore determining the role of protozoa in feed efficiency would be a necessary next step. Fungi, although present in lower numbers than bacteria, archaea or protozoa, play an important role in digestion. This is particularly true as fungi appear to preferentially adhere to lignified tissues (Ushida *et al.*, 1997). Digestion is carried out in a different way to that seen by bacteria as hyphae are able to penetrate into the plant tissue and it has been suggested that they play a large role in the digestion of cellulose (Bauchop, 1981). Future studies should investigate the rumen community as a whole and not just focus on bacteria and archaea. However, at present molecular techniques are better developed for bacteria and archaea than fungi and protozoa.

As suggested above, it is thought that the addition of nitrate to the diet is able to increase faecal particle size by reducing rumen contractions. An increase in large particle (>1.8 mm) passed into faeces may have contributed to feed efficiency

differences, but the current techniques were not able to provide a definitive answer. The next logical step would be to carry out a study where nitrate is added to the diet, faecal particle size distribution characterised more fully and passage rates measured using marker techniques.

8.4 CONCLUSIONS

Whilst able to determine between-animal variation, no significant relationships were observed between feed efficiency measures (using FCR or RFI), faeces composition (using LD and NIRS) or the rumen microbial community (using 16S based techniques). Whilst these techniques could identify between-animal variation in rumen and digestive processes, it seems that these make only a small contribution to variation in feed efficiency; it is likely that differences in animal metabolism and activity explain a much larger proportion of variation.

CHAPTER 9

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CHAPTER 10

PUBLICATION LIST

Bowen, J. M., Lister, S. J., Sanderson, R. Dewhurst, R. J. 2016. The use of faecal NIRS to assess diet effects on digestive processes in steers. Proceedings of the European Federation of Animal Science (EAAP) annual meeting, Belfast, Ireland, September 2016, pp 563

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