Harper Adams University

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Effect of dietary factors on rumen metabolism and the microbiome in high yielding dairy cows

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Declaration

I declare that this thesis is my original work and it has been written by the author and none of this work been accepted in any previous application for a degree. I have acknowledged all the sources of information which have been used in this thesis by means of references.

Catherine Anne Johnson

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On to the next adventure!

Catherine Anne Johnson

Publications

Johnson, C., Snelling, T., Huntington, J., Taylor-Pickard, J., Warren, H. and Sinclair, L. 2020. The effect of pattern of concentrate allocation and Yea-Sacc® supplementation on the performance and rumen pH in high yielding dairy cows. Advances in Animal Biosciences, 11, p 107. (Abstract)

Johnson, C., Snelling, T., Huntington, J., Taylor-Pickard, J., Warren, H. and Sinclair, L. 2021. Short-term feed restriction and re-feeding alters rumen metabolism and performance of high yielding dairy cows fed different concentrate patterns and either with or without a live yeast. Animal Science Proceedings, 12, p.99. (Abstract)

Johnson, C., Snelling, T., Huntington, J., Taylor-Pickard, J., Warren, H. and Sinclair, L. 2022. Response of the rumen microbial community to a short-term restriction and re-feeding period in high yielding dairy cows. Animal Science Proceedings. (Abstract)

Abstract

Previous work has established that poor diet mixing, diet selection and short-term feed restriction (FR) are common on many UK dairy farms. When cattle sort through a total mixed ration they can alter both their level and pattern of concentrate intake which can increase the risk of subacute rumen acidosis (SARA), although the effects on rumen metabolism and the microbiome are unclear. Periods of short-term FR can occur due to a shortage of the ration, machinery malfunction, or poor management, and when cattle re-feed they can overeat concentrates increasing the risk of SARA, defined as periods of pH depression lasting more than 5 to 6 h/d with rumen pH <5.8. Supplementing the diet with a live yeast is common practice in ruminant nutrition to improve rumen conditions as yeasts can increase rumen pH by promoting lactate utilising bacteria growth and scavenging oxygen from the rumen which can compromise fibrolytic bacteria activity. There is a lack of understanding of the precise effects of live yeasts on rumen metabolism and the microbiome in dairy cows. There is interest in increasing the nitrogen efficiency of dairy cows along with reducing ammonia emissions from dairy farms. Feeding *Yucca schidigera* (De-Odorase®) is more common in monogastric animals to reduce volatile ammonia emissions from slurry into the environment, however, its effects on rumen metabolism, the microbiome and nitrogen efficiency in dairy cows is unclear. The objective of the thesis was to determine the effect of pattern of concentrate allocation, short-term FR and supplementation with live yeast or *Y. schidigera* on rumen metabolism, the microbiome, and performance of high yielding dairy cows. In the first study changing the pattern of concentrates fed (even/uneven) had little effect on performance or rumen metabolism, while yeast supplementation tended to increase rumen pH, rumen ammonia concentration and the relative abundance of operational taxonomic units (OTU) related to Clostridiales, associated with fibre degradation. In the second study, a FR period followed by re-feeding decreased dry matter intake (DMI) by 5.14 kg/cow/d during FR and increased by 4.96 kg/cow/d on recovery day 1 (rec d1) compared to the baseline, whilst milk yield decreased in the recovery period, returning to baseline levels after four days. Rumen pH and ammonia concentration also decreased during the recovery period. Following FR the relative abundance of *Methanobrevibacter* and lactate producing bacteria *Bifidobacterium longum* increased, and with yeast supplementation *Treponema bryantii* abundance increased during the recovery period. In the final study supplementing the diet with *Y. schidigera* had a greater effect on the microbiome than live yeast. Supplementation of *Y. schidigera* along with a live yeast decreased DMI but had no effect on milk yield, whole tract digestibility, plasma metabolites or rumen metabolism. Supplementation of *Yucca schidigera* tended to decrease slurry pH compared to the Control after 6 h which may reduce volatile ammonia loss, although ammonia losses from slurry were similar between treatments. In conclusion, common issues on farm such as diet selection, short term FR

and feeding diets high in RDP can have negatives effects on performance, rumen metabolism and the rumen microbiome, and therefore measures should be taken to avoid occurrence on farm. When supplementing the diet with a live yeast in conjunction with these situations, the effects of yeast on performance and the rumen microbiome were inconclusive.

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Abbreviations

16S rRNA	16S small subunit ribosomal RNA
18S rRNA	18S small subunit ribosomal RNA
ADY	Active dry yeast
AMOVA	Analysis of molecular variance
A:P	Acetate to propionate ratio
BCS	Body condition score
BHB	Beta hydroxybutyrate
BW	Bodyweight
CH_4	Methane
CHO	Carbohydrate
CO_2	Carbon dioxide
CP	Crude protein
ddNTP	Dideonucleotide
DM	Dry matter
DMI	Dry matter intake
DUP	Digestible undegradable protein
ETDA	Ethylenediaminetetraacetic acid
E	Even (allocation of concentrates)
ECM	Energy corrected milk
F:C	Forage to concentrate ratio
FPS	Forage particle size
FR	Feed restriction
GHG	Greenhouse gas
GS	Grass silage
HPO_3	Metaphosphoric acid
LDA	Linear discriminant analysis
LPD	Liquid phase digesta
LEfSe	Linear discriminant analysis (LDA) effect size
MP	Metabolisable protein
MPS	Microbial protein synthesis
MS	Maize silage
N	Nitrogen
NAN	Non ammonia nitrogen
NDF	Neutral detergent fibre
NGS	Next Generation Sequencing
NH_3	Ammonia

NH_3-N	Ammonia nitrogen
NH_4^+	Ammonium
NO_3	Nitrate
NPN	Non-protein nitrogen
OM	Organic matter
OTU	Operational taxonomic unit(s)
PCR	Polymerase chain reaction
peNDF	Physically effective fibre
PMR	Partial mixed ration
RDP	Rumen degradable protein
Rec d1	Recovery day 1
Rec d2	Recovery day 2
Rec d3	Recovery day 3
Rec d4	Recovery day 4
SARA	Subacute ruminal acidosis
SBS	Sequencing by synthesis
SPD	Solid phase digesta
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TMR	Total mixed ration
U	Uneven (allocation of concentrates)
UDP	Undegradable dietary protein
VFA	Volatile fatty acid(s)
WSC	Water soluble carbohydrates

Chapter One: Literature Review

1.1 Introduction

The main priorities when managing dairy cattle are health, production and fertility, and a significant factor in controlling these is by diet (Bowen *et al*., 2018). It is common for dairy cattle to be housed throughout the year and receive nutrients in the form of a total mixed ration (TMR) to better monitor and control balance of nutrients, ensuring maximum production while maintaining optimal health (March *et al*., 2014). However, studies have shown that cattle are able to sort through the TMR and tend to favour shorter particles such as concentrates over longer forage particles, a problem that can be exacerbated by poor mixing by the mixer wagon (Tayyab *et al*., 2018). Furthermore, due to errors such as insufficient supply of feed or pasture, or feed equipment failure, cattle may experience periods of short-term feed restriction, and enter a period of overeating when feed is reintroduced, which can increase the risk of cattle developing subacute rumen acidosis (Thomson *et al*., 2018). Often cows receive diets which contain high levels of rumen degradable protein (RDP) which can lead to increased levels of N being excreted (Chowdhury, 2022) which can have negative environmental effects. With these situations (diet selection, short-term feed restriction, diets high in RDP) commonly occurring on UK dairy farms, central to improving dairy cow performance, health and nutrient use efficiency is understanding their effects on rumen metabolism and the rumen microbiome.

1.2 The rumen and the microbiome

Ruminants such as cattle, sheep and goats differ from monogastric animals (pigs and poultry) as they have four stomachs (reticulum, rumen, omasum and abomasum) as part of their digestive tract (Dijkstra *et al*., 2005). This allows ruminants to digest fibre, which can make up more than 30% of the diet (Sova *et al*., 2013). In cattle the rumen has a 100-150 L capacity and is filled with fluid, acting as a fermentation chamber which provides a suitable environment for microbiota (Czerkawski, 1986). These microorganisms provide ruminants with the ability to digest fibrous substances such as cellulose, which like monogastrics which lack the endogenous enzymes to do so (Russell *et al*., 1992; de Mulder *et al*., 2017). Additionally, monogastrics are unable to break down urea, a source of non-protein nitrogen (NPN) and it is excreted as a waste product, while in ruminants, bacteria convert urea to ammonia which they utilise as a nitrogen (N) source (Stewart and Smith, 2005).

The anatomy of the reticulo-rumen is described in Figure 1.1, and the rumen contents can be split into three main phases: liquid, solid, and small particles in a liquid suspension (Zebeli *et al*., 2012). The liquid phase is situated in the ventral part of reticulorumen, while

the rumen mat, which consists of solid digesta with mainly large, newly ingested, buoyant feed particles, extends from the dorsal to the central region (Zebeli *et al*., 2012). Rumination is the cyclical process of regurgitation, remastication and reswallowing partially digested feed (Beauchemin, 2018). Reducing the particle size by repeated chewing enables the feed to pass through the rumen to the omasum for further digestion (Beauchemin, 1991). Rumination also increases the proportion of saliva in the rumen which plays a crucial role in digestive function, as saliva contains bicarbonate (125 mEq/L) and phosphate (65 mEq/L), therefore has a high buffering capacity (Beauchemin, 2018). Bicarbonate is also supplied across the rumen wall by absorption, as part of bicarbonate dependant volatile fatty acids (VFA) uptake (Dijkstra *et al*., 2012). In the rumen, bicarbonate acts as a buffer against the reduction in pH caused by VFA production, and provides nutrients to the rumen microbiota (Beauchemin, 1991).

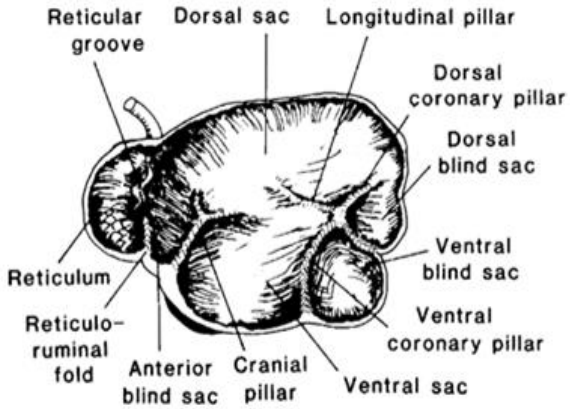


Figure 1.1: Diagram of the reticulo-rumen (Czerkawski, 1986)

In ruminant nutrition there is a lot of focus on the effect of diet on voluntary intake, digestibility, rumen metabolism, N balance and animal performance. According to McCann *et al*. (2014) each of these measures are inseparably linked with the rumen microbial community. Marchesi and Ravel (2015) defined the microbiome as the entire habitat, including the microbiota (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes (i.e. genes), and the surrounding environmental conditions. The proportions of the microbial community in the rumen are described in Table 1.1. The rumen microbiome is a diverse environment containing microorganisms which live synergistically within the rumen. The rumen provides a nutrient rich environment for the microbiota to inhabit, while the products of microbial protein synthesis (MPS) provide the host with sources of additional N and energy (McCann *et al*., 2014).

Table 1.1: Rumen microbiota (Adapted from Nagaraja, 2016 and Kumar *et al*., 2015)

Type	Domain	Number (per ml of rumen contents)	Percentage of total mass (%)
Bacteria	Eubacteria	10^9-10^{11}	40-90
Methanogens	Archaea	10^5-10^8	2-4
Protozoa (Ciliates)	Eukarya	10^4-10^6	0-60
Fungi	Eukarya	10^6	10

In the rumen there are nine predominant phyla of bacteria: Bacteroidetes, Firmicutes, Proteobacteria, Spirochetes, Actinobacteria, Fusobacteria, TM7, Tenericutes and Deinococcus-Thermus (Khafipour *et al.*, 2009). However, as shown in Figure 1.2, more than 98% of rumen bacteria can be derived from Bacteroidetes, Firmicutes and Proteobacteria (Jami and Mizrahi, 2012). Prior to molecular advancements rumen bacteria were categorised by morphology, gram stain, or function (Table 1.2; Dehority, 2003). Rumen bacteria can be distributed freely within the fluid (approximately 30%), attached to feed particles (approximately 65%; loosely or tightly) or attached the rumen wall (epimural bacteria; <5%), although epimural bacteria do not significantly contribute to rumen digestion (Nagaraja, 2016; Dehority, 2003). There are distinct differences between the microbiota associated with the different digesta phases, with bacteria in the liquid phase being diverse in function often with both amylolytic and cellulolytic properties such as *Prevotella* (de Mulder *et al*., 2017). Cellulolytic bacteria from families such as *Ruminococcaceae* and *Christensenellaceae* are often more abundant in the solid phase as they adhere to the feed particles as part of digestion (Bowen *et al*., 2018). Epimural bacteria are facultative anaerobes as the rumen wall is highly oxygenated and it is suggested that epimural bacteria maintain anaerobiosis by scavenging any oxygen that may otherwise diffuse into the rumen (Nagaraja, 2016). de Mulder *et al*. (2017) reported that while bacteria in the solid and liquid phases of rumen fluid were from similar taxonomic groups, though in different levels of abundance, the bacteria found in the epimural fraction were from different taxonomic groups due to their specialised function.

Table 1.2: Summary of common rumen bacteria (Adapted from Stewart *et al*., 1997, Dehority, 2003)

	Metabolism		Gram stain	Carbohydrate energy source			Fermentation by-product					
Bacteria	Anaerobe	Facultative anaerobe		Hemicellulose	Cellulose	Starch	Acetate	Lactate	Succinate	Formate	Butyrate	Ethanol
Ruminococcus flavefaceins	*		+	*	*		*	*		*		
Ruminococcus albus	*		+	*	*		*			*		
Fibrobacter succinogenes	*		-	*	*	*	*		*	*		
Prevotella species	*		-	*		*	*		*	*		
Eubacterium species	*		+	*	*		*	*		*	*	
Streptococcus bovis		*	+			*		*				
Ruminobacter amylophilus	*		-			*	*		*	*		*
Succinimonas amylolytica	*		-			*			*			

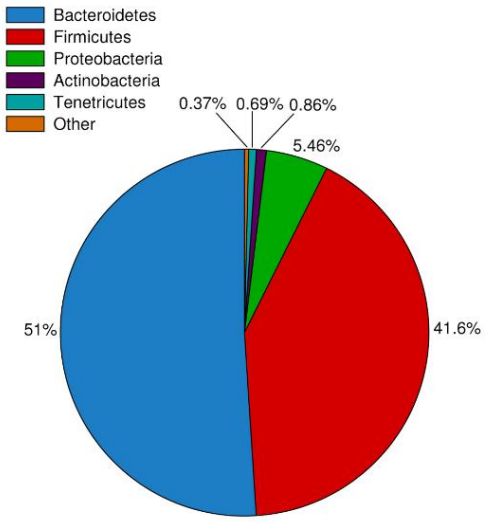


Figure 1.2: Composition of rumen bacterial taxa (Jami and Mizrahi, 2012)

Ciliate protozoa have a lower prevalence than bacteria, although due to their large size (5-250 µm) and they can comprise up to 60% of the total microbial biomass in the rumen (Newbold *et al*., 2015; Williams and Coleman, 1997). Protozoa have been found to predate rumen fungi and bacteria, and therefore have the capacity to regulate the microbiota populations within the rumen (Williams *et al*., 2020). Ciliates are the only type of protozoa found in the rumen, and are split into two main families: entodiniomorphs and holotrichs (Figure 1.3; Williams and Coleman, 1997). Differences between protozoa are described in Table 1.3. Entodiniomorphs are important in rumen digestion as they hydrolyse structural polysaccharides and utilise bacteria as their primary protein source (Nagaraja, 2016). In contrast, holotrichs have minimal predatory activity and contribute little to fibrolytic activity, however, their ability to engulf starch and soluble carbohydrates may have positive effects on rumen conditions (Newbold *et al*., 2015). Holotrichs are more closely associated with methanogenesis, and in the presence of excess carbohydrates rapidly synthesise glycogen which generates hydrogen (Denton *et al*., 2015) and can contribute to methane production.

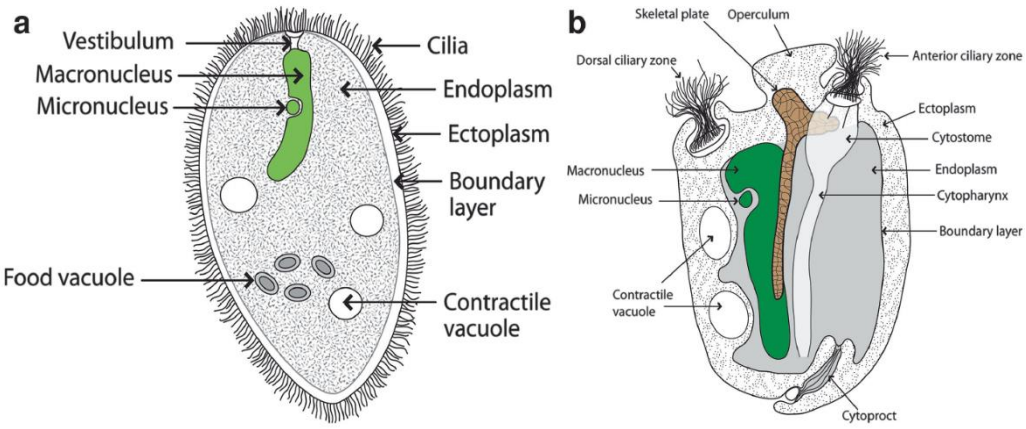


Figure 1.3: Diagram of protozoa, a) holotrich b) entodiniomorph (Nagaraja, 2016)

Table 1.3: Comparison of holotrich and entodiniomorph protozoa (Adapted from Nagaraja, 2016 and Denton *et al*., 2015)

	Holotrich	Entodiniomorph
Ciliary arrangement	Distributed on entire surface of cell	Restricted ciliary zones
Proportion in rumen	10-25%	75-90%
Diurnal variation	Two to fourfold increase in first 1-2 h after feeding	Numbers generally decrease after feeding
Function	Hydrolyse soluble polysaccharides	Hydrolyse structural polysaccharides
Substrates fermented	Starch, pectin, soluble sugars, proteins	Cellulose, hemicellulose, starch, pectin, soluble sugars, proteins
Common genera	*Isotricha, Dasytricha, Charonina*	*Entodinium, Diplodinium, Metadinium, Epidinium*

1.2.1 Analysing the rumen microbiota

Bacteria and other rumen microbiota were traditionally cultured and characterised based on their morphology, gram stain and nutrient metabolism before the development of DNA sequencing and metagenomics allowed researchers to compare microbiota on a molecular level (Gruninger *et al*., 2019; Yu and Morrison, 2004). In recent years there have been great advances in the understanding of the rumen microbiome with the development of the Hungate1000 collection and other projects which identified 336 organisms in the metagenomic dataset, and estimated that the data accounted for

approximately 75% of the genus level bacterial and archaeal taxa in the rumen (Seshadri *et al*., 2018; Stewart *et al*., 2018).

There are many methods available to sequence DNA including pyrosequencing, amplicon, shotgun and next generation sequencing (Gruninger *et al*., 2019). The sanger sequencing process, also known as chain termination method, was developed in 1977 (Sanger *et al*., 1977) to determine the nucleotide base sequence of DNA less than 1000 bp in length, and has since been referred to as the gold standard for sequencing (Figure 1.4; Fakruddin and Chowdhury, 2012). The process involves denaturation of the double stranded DNA, addition of primers complimentary to the template strand, four polymerase solutions, each with one type of dideonucleotide (ddNTP; A, T, C, G) added, and the synthesis reaction which initiates extending the chain until the termination nucleotide is incorporated at random. The four different reaction products (A, T, C, G) and sample are then loaded onto gel and subjected to electrophoresis and the sequence determined based on the migration (Gauthier, 2008). However, there many limitations as the method expensive and highly labour intensive, additionally only few samples can be analysed in parallel and there can be sequencing errors (Fakruddin and Chowdhury, 2012; Tsiatis *et al*, 2010).

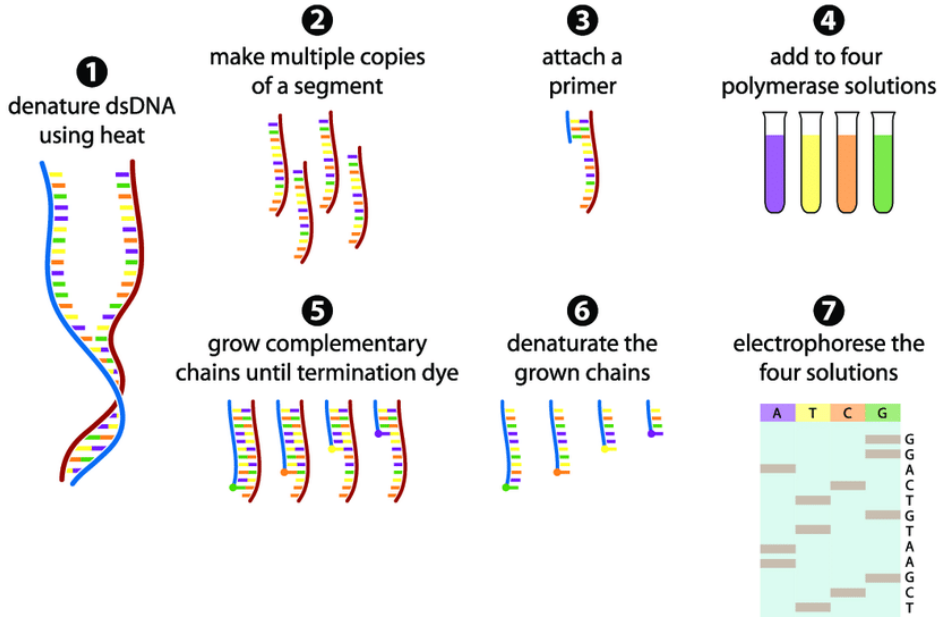


Figure 1.4: Diagram of Sanger sequencing method (Gauthier, 2008)

Amplicon sequencing involves amplifying short sequences of DNA using the 16S small subunit ribosomal RNA (16S rRNA) gene as a marker, as it is the most conserved length of RNA found within all bacterial species (Rajendhran and Gunasekaran, 2011; Woese, 1987). Similarly eukaryotes (protozoa and fungi) are analysed by sequencing the 18S small subunit ribosomal RNA (18S rRNA) genes (Newbold *et al*., 2015; Wallace *et al*., 2014). The methods are shown in Figure 1.5, and the main applications for amplicon

sequencing are identification and classification of isolated pure cultures, and estimation of bacterial diversity while using non metagenomic approaches (Rajendhran and Gunasekaran, 2011). However, amplicon sequencing does not provide information regarding the function of the sequenced microbes, and as with all DNA based methods there is no direct measure of activity, as it is unable to distinguish between the DNA of live, dead, inactive or lysed cells (Gruninger *et al*., 2019; Li *et al*., 2016). Despite improvements made to methodology results are still often inaccurate. Throughout the analysis process errors and inconsistencies can occur such as sample collection and storage, DNA extraction, library preparation and sequencing pipeline (Pollock *et al*., 2018). Therefore, it is important to ensure when conducting the analysis to follow 'best practice' by identifying where errors can occur and minimising risk where possible (Pollock *et al*., 2018).

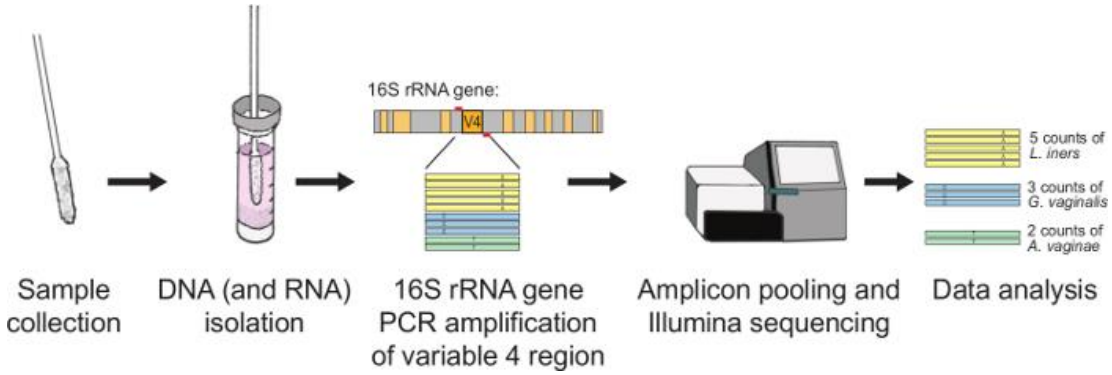


Figure 1.5: 16S rRNA amplicon sequencing method (Bowman and Kwon, 2016)

Next generation sequencing (NGS) is a method based on sequencing by synthesis (SBS) and allows for large scale throughput of sequences (Gruninger *et al*., 2019). There are multiple sequencing technologies which have been developed and are currently used including Illumina Miseq, PacBio HiFi and Oxford Nanopore MinION sequencing, which vary in cost, portability and throughput (Hu *et al*., 2018). Due to the complexity of the microbial community in the rumen using a single approach often leads to an incomplete picture, and the approach of combining multi-omics (metagenomics, metatranscriptomics, metaproteomics and metabolomics) is becoming more common (Gruninger *et al*., 2019). The metagenome is defined as the collection of genomes and genes from members of the microbiota (Marchesi and Ravel, 2015). Shotgun metagenomics and NGS are both able to provide both the taxonomic composition and metabolic activity of the rumen microbiota (Gruninger *et al*., 2019). For example, metagenomics have become a key tool in enhancing the understanding of methane emissions including biomarkers to predict rumen methanogenesis (Huws *et al*., 2018). Metatranscriptomics provides more information

regarding the function of the microbiota, by assessing the quantity and quality of RNA in the genome. The RNA profiles express genes and form the substrate for protein synthesis, which at secondary and tertiary level have functional roles in the microbiome (Bunnik and Roch, 2013). Metatranscriptomics are closely linked to epigenomics, the study of changes in organisms derived from modifications of gene expression rather than the alteration of the actual genetic code, as the regulation of gene expression is fundamental for cell development and differentiation (Hasin *et al*., 2017). Comparing the RNA transcript between organisms and conditions can aid in identifying genes involved in cell differentiation, although the correlations between mRNA and protein levels have been reported to be weak and variable potentially due to post-transcriptional modifications (Huws *et al*, 2018). Metaproteomics measures peptide abundance of proteins involved in intracellular signalling, enzyme activity, protein transport, and cell maintenance. The protein content of cells can be performed by two-dimensional gel electrophoresis, separating the proteins first by size and then by charge (Mesuere *et al*., 2018). Proving more accurate than RNA at identifying individual proteins, metaproteomics also allows proteins to be identified by their amino acid sequence and therefore their function can be linked to taxa using sequencing alignment tools e.g. UniPept (Mesuere *et al*., 2018). Still in relatively early stages of development, metabolomics comprehensively studies the products of cellular metabolic function such as amino acids, VFA, carbohydrates, although there can be challenges due to the chemical complexity and heterogeneity of the metabolites (Huws *et al*., 2018). There are two approaches for metabolic investigation, targeted which focusses on known metabolites and untargeted which covers a large number of compounds but may aid in determining novel compounds and pathways (Patti *et al*., 2012). Metabolomics more directly reflect metabolic function yet the metabolites cannot be linked to the function of the individual members of the microbial community (Hasin *et al*., 2017).

1.3 Carbohydrate metabolism in the rumen

There are three main groups of carbohydrates, complex structural polysaccharides such as cellulose and hemicellulose, storage reserve carbohydrates such as starch and sucrose which are active in plant intermediary metabolism, and soluble carbohydrates such as glucose and fructose (Bannink and Tamminga, 2005; Van Soest, 1994). In ruminants, 70% of host energy supply is derived from VFA (mainly acetate, propionate and butyrate) which are the by-products of microbial fermentation (France and Dikjstra, 2005; Russell *et al*., 1992). Other by-products include lactate, methane (CH_4) and carbon dioxide (CO_2; Hobson, 1997). Carbon dioxide and hydrogen are utilised by methanogens (Morgavi *et al*., 2010). Despite VFA being the main energy source in ruminants, acetate

and butyrate do not contribute to glucose supply, a key component of milk production, and propionate is the most significant contributor to gluconeogenesis (Russell *et al*., 1992). Longer chain VFA such as valerate can also be used to synthesise glucose but in smaller quantities (Bergman, 1990).

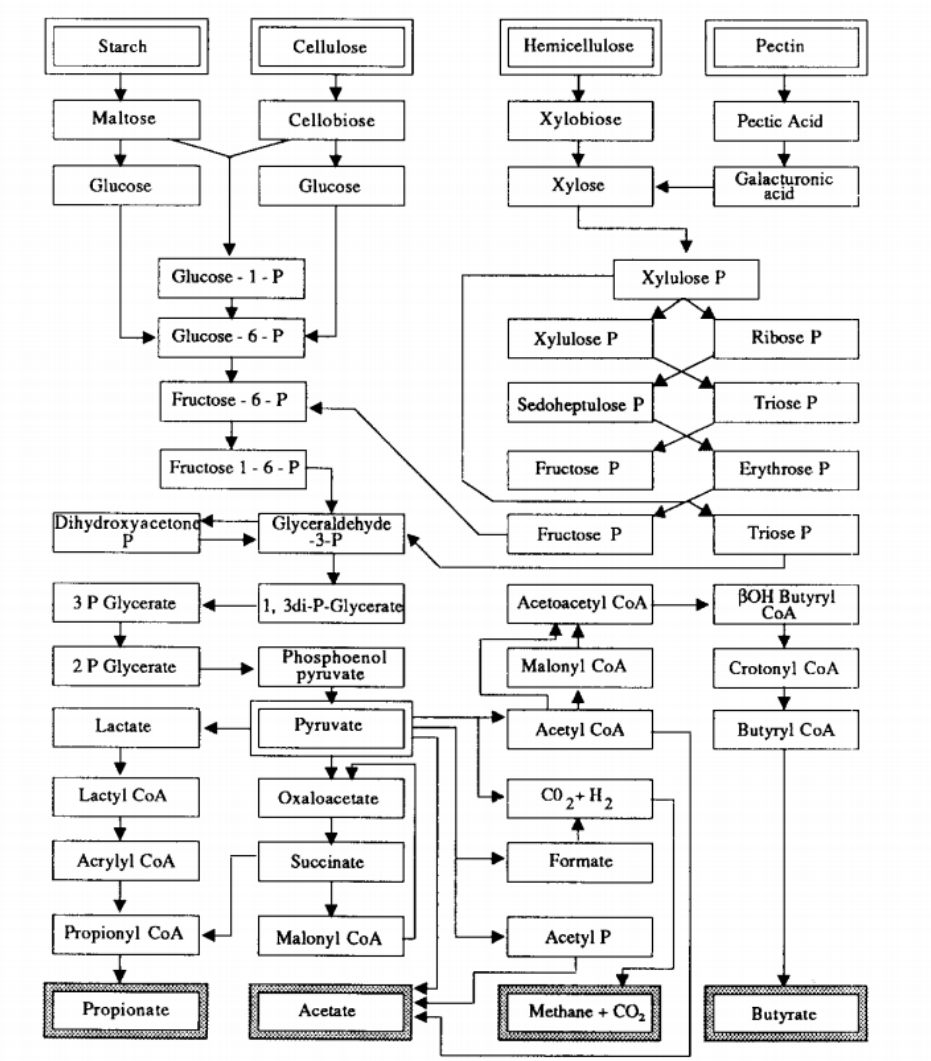


Figure 1.6: Carbohydrate fermentation pathways via pyruvate to the main volatile fatty acids (Van Houtert, 1993)

The process of fermentation of carbohydrates (Figure 1.6) is carried out by the rumen microbiota, and their presence and activities determine the quality and extent of fermentation of feedstuffs (Russell *et al*., 1992). Figure 1.7 describes the pathways of structural and non-structural carbohydrates during digestion in the rumen with the aid of buffering agents from saliva. The majority of VFA are absorbed in a free form directly across the rumen wall into the bloodstream (Bergman, 1990), and only 35% pass through the abomasum to the small intestine for absorption (Dikjstra *et al*., 1993). Volatile fatty acid absorption rate can also be influenced by VFA type, osmolality of the fluid and

effective surface area of the rumen epithelium (Bergman, 1990; Dijkstra *et al*., 1993). Absorption rate is concentration dependant and therefore, for the main three VFA, acetate has the highest absorption rate followed by propionate, and butyrate has the lowest (France and Dijkstra, 2005). Volatile fatty acids, particularly butyrate, can be metabolised by the rumen wall, maintaining the concentration gradient between the rumen and blood across the epithelium and therefore sustaining rapid absorption (Van Soest, 1994). Degeneration of the rumen epithelium can be caused by feeding low energy diets in the dry period, as epithelial growth is stimulated by high concentrations of VFA, particularly butyrate and propionate (Ingvartsen *et al*., 2001; Kauffold *et al*., 1977).

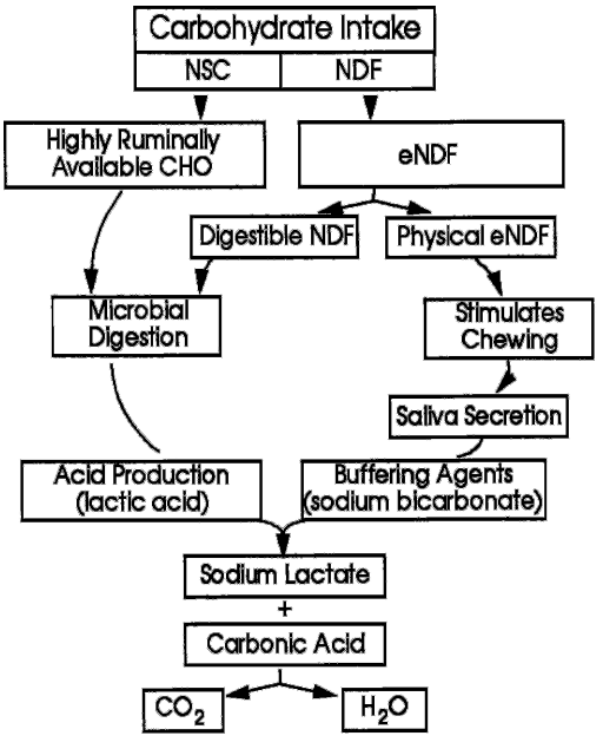


Figure 1.7: Structural (NDF) and non-structural (NSC) carbohydrate buffering effects in the rumen (Nocek, 1997)

Acidosis can occur in dairy cows in the form of acute and subacute, and is induced by loading the rumen with rapidly fermentable carbohydrates which can be common in freshly calved cows due a high dry matter intake (DMI) of diets high in readily fermentable carbohydrates (DeVries *et al*., 2008; Krause and Oetzel, 2005). The cause of acute acidosis is described in Figure 1.8 and can lead to severe symptoms such as keratosis, decreased cardiac outflow and peripheral perfusion, shock, and death (Nocek, 1997). The consumption of large quantities of fermentable carbohydrates such as starch increase microbial activity and VFA production which lowers pH. Lactate utilising bacteria such as

Megasphaera elsdenii are sensitive to low pH (<5.0), while lactate producing bacteria such as *Streptococcus bovis* thrive, rapidly increasing lactate concentration in the rumen, further decreasing pH in a positive feedback loop (Owens *et al*., 1998). If the rate of VFA production exceeds the rate of VFA absorption, due to rapid production or inhibited absorption, VFA accumulation can reduce pH to below 5.0 without the presence of lactate (Owens *et al*., 1998).

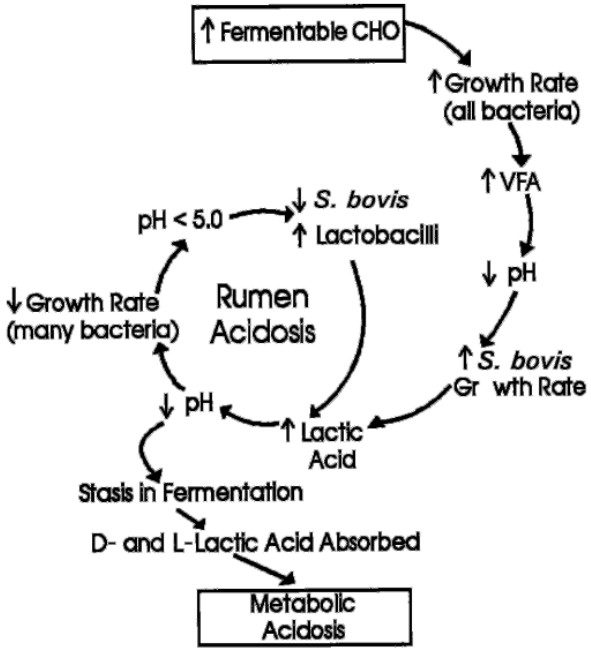


Figure 1.8: Sequence of events associated with induction of acute ruminal acidosis (CHO= carbohydrate; Nocek, 1997)

Subacute ruminal acidosis (SARA) is a subclinical form of the disease and is not associated with the accumulation of lactate in the rumen (McCann *et al*., 2016). There is confusion regarding the precise definition, based on pH threshold and the period of time pH spends below it (Plaizier *et al*., 2008). Duffield *et al*. (2004) reported that the threshold indicating SARA should be varied depending sampling site due to the difference in microbial activity, as pH was recorded to be pH 0.3 higher when sampled from ventral region via rumen cannula, than from rumenocentesis collection. Due to the diurnal variation of pH in the rumen, there were recommendations to define SARA as periods of pH depression lasting more than 5 to 6 h/d during which rumen pH is <5.8 (Zebeli *et al*., 2012). Subclinical symptoms include decreased milk yield, DMI and body condition score (BCS) but the only diagnostic test for SARA is monitoring rumen pH (Nocek, 1997). At herd level, a high prevalence of SARA can result in high culling rates and decreased milk production, resulting in substantial economic loss on farm (Nocek, 1997).

1.3.1 Fibre degradation

Structural polysaccharides in feed are mainly available in the form of cellulose and hemicellulose (Schwarz, 2001). Cellulose is a major component of the plant cell wall and is composed of unbranched linear chains of thousands of glucose units with β-1, 4 glucosidic linkages (Dhingra *et al*., 2012). The linkages are resistant to break down from endogenous digestive enzymes but can be hydrolysed by cellulolytic bacteria in the rumen (Van Soest, 1994). Hemicellulose has a backbone of glucose with β-1, 4 glucosidic linkages but molecules are smaller than cellulose and are often branched with a variety of sugars including xylose and galactose (Dhingra *et al*., 2012).

Bacteria and fungi are responsible for 80% of the degradation of these structural polysaccharides, with protozoa accounting for the remainder (Dijkstra and Tamminga, 1995). The majority of fibre degradation occurs while the cellulolytic bacteria are attached to the solid feed particles (Bowen *et al*., 2018). The main families of cellulolytic bacteria include *Ruminococcaceae, Christensenellaceae* and *Lachnospiraceae* (de Mulder *et al*., 2017). Many cellulolytic bacteria are sensitive to low pH therefore during SARA the relative abundance of these bacteria can decrease, compromising the ability of the rumen microbiota to digest fibre (Li *et al*., 2017; McCann *et al*., 2016). Ciliates *Diplodinium* and *Eupdiplodinium* are able to digest cellulose by phagocytosis (Coleman, 1992). Their mechanism for physically digestion is different to that of bacteria, as protozoa are weakly associated with plant particles while fibrolytic bacteria grow in close association to the plant cell wall (Chesson and Forsberg, 1997). Factors that affect fibre degradation include rumen pH, fractional outflow rate of digesta, and the amount and activity of fibrolytic microorganisms in the rumen (Bannink and Tamminga, 2005).

In order to degrade complex polymers many hydrolytic enzymes must work together simultaneously (Chesson and Forsberg, 1997). More than 85% of cellulase, hemicellulase and glycosidase activities occur when bacteria are bound to the solid digesta fraction (Chesson and Forsberg, 1997). Latham *et al*. (1978) observed that when *Fibrobacter succinogenes* and *Ruminococcus flavefaceins* were bound to ryegrass, *F. succinogenes* adhered to the cut edges of most of the plant cells except the xylem, and some uncut surfaces including meophyll, epidermis and phloem. Meanwhile, *R. flavefaceins* predominantly adhered to the uncut surfaces of the epidermis, phloem and schlerenchyma cell walls. The two bacteria demonstrated uniquely different specificities for binding which would serve to reduce competition (Latham *et al*., 1978).

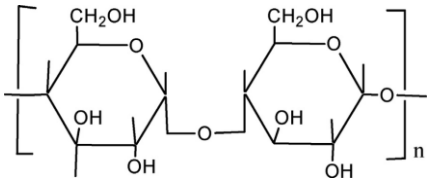
Acetate is a by-product of fibre fermentation (Figure 1.6), with pyruvate being the predominant intermediate product for many cellulolytic bacteria (Van Houtert, 1993; Van

Soest, 1994). Acetate is the main energy yielding source that is oxidised in most body tissue, and is a precursor for milk fat production (Van Houtert, 1993). Butyrate is another by-product of fibre fermentation but only contributes approximately 10% to total VFA production (France and Dijkstra, 2005). Approximately 72% of acetate is absorbed across the rumen wall, and the remainder can be converted into butyrate by butyryl-CoA transferase (Wang *et al*., 2020; Kristensen, 2001). A majority of butyrate is converted into beta hydroxybutyrate (BHB), which is utilised as an energy source (Kristensen, 2001; Dijkstra, 1994a). Butyrate is also involved in lipid synthesis therefore increased supply can have positive effects on milk fat (Van Houtert, 1993). Branched chain fatty acids such as iso-valerate and iso-butyrate are collectively called iso-acids (Andries *et al*., 1987). Iso-acids are derived from oxidative deamination and oxidative decarboxylation of branched-chain amino acids such as valine and leucine (Andries *et al*., 1987). Iso-acids enhance growth of cellulolytic bacteria as they can be synthesised into essential amino acids by rumen microbiota (Andries *et al*., 1987).

1.3.2 Starch degradation

Starch is a major component of grain based concentrates in ruminant feed as it is high in energy (Svihus *et al*., 2005). Starch is composed of two polysaccharides, amylose and amylopectin which vary in proportion depending on the source, which form a granular structure in the endosperm (Mills *et al*., 1999). Both amylose and amylopectin contain glucose molecules however they differ in their structure; amylose has linear α-1, 4 linkages while amylopectin has a branched structure due to α-1, 4 and α-1, 6 linkages (Figure 1.9). Different sources of starch have different rates of degradation with for example, starch from wheat being more readily fermented than starch from maize (Svihus *et al*., 2005). The processing of starch can affect the rate and extent of degradability in the rumen, with grinding breaking down the outer layers of the grain exposing the endosperm, and pelleting decreasing the size of the feed particles which increases the surface area, enhancing the rate of degradation (Svihus *et al*., 2005). Furthermore, heat treatment (>80°C) with the presence of water can result in gelatinisation whereby the hydrogen bonds in the crystalline part of the granule are broken by swelling (50 – 70°C) of the molecule causing the irreversible loss of the structure (Mills *et al*., 1999). Heat treatment above 120°C can reduce starch degradation due to the Maillard reaction, where amino acids react with carbohydrates and decrease their digestibility (Van Boekel, 2001).

A) Amylose



B) Amylopectin

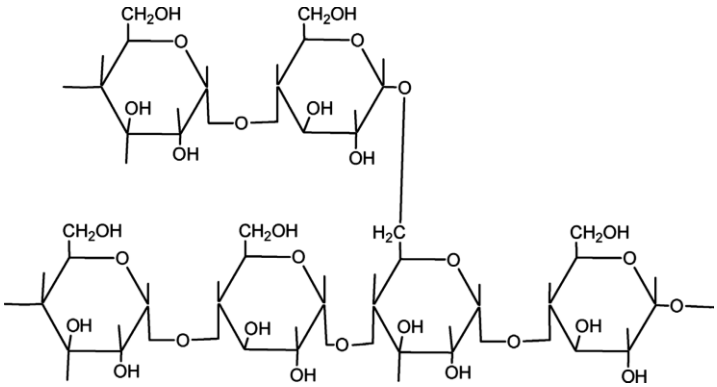


Figure 1.9: Chemical structure of A) amylose and B) amylopectin (Hassan *et al*., 2018)

Between 60 – 90% of starch is degraded in the rumen, and therefore up to 40% escapes rumen fermentation and is degraded enzymatically in the small intestine (Quin *et al.*, 2012; Bannink and Tamminga, 2005). The starch which escapes rumen fermentation provides a source of glucose for viscera such as intestines which have a high glucose demand, and can account for up to 28% of total glucose supply to lactating dairy cows (Mills *et al*., 1999; Allen, 2000).

Virtually all protozoa and fungi possess amylolytic properties, however, it is bacteria which predominantly degrades starch in the rumen (Chesson and Forsberg, 1997; Huntington, 1997). Starch is hydrolysed by extracellular microbial enzymes; α-amylase which degrades amylose and the linear regions of amylopectin, and β-amylase which cleaves starch chains at their end points and degrades both amylose and the peripheral regions of amylopectin (Mills *et al*., 1999). Maltase, maltose phosphorylase and 1,6-glucosidase degrade the subsequent maltose and iso-maltose fragments to glucose or glucose-1-phosphate (Figure 1.9; Mills *et al*., 1999).

Streptococcus bovis and *Ruminobacter amylophilus* are examples of solely amylolytic bacteria, while *Prevotella ruminicola* degrades starch in addition to structural polysaccharides (de Mulder *et al,.* 2017; Dehority, 2003). Entodiniomorph protozoa have been reported to manipulate rate of starch fermentation by engulfing large numbers

bacteria, or decreasing accessibility to the substrate. As a result, the rate of rapid starch fermentation by bacteria can decrease, and consequently aid in stabilising rumen conditions (Qin *et al*., 2012).

Propionate is the main by-product of starch fermentation and is formed by two major pathways in the rumen, pyruvate and succinate metabolism (Figure 1.6; Van Houtert, 1993). Complex carbohydrates are hydrolysed to pyruvate and consequently to succinate which is then converted to propionate. Alternatively, pyruvate can be converted to lactate and then propionate, and this pathway takes prominence when ruminants are fed diets with high levels of concentrates and rumen pH is below pH 6.0 (Van Houtert, 1993). Unlike acetate and butyrate, propionate is a precursor for glucose synthesis, and contributes 46 to 73% of total glucose supply from hepatic gluconeogenesis, while lactate provides 12% (Dijkstra, 1994a; Huntington, 1997).

Lactose is synthesised by glucose and galactose, however glucose cannot be synthesised by the udder due to the lack of glucose-6-phosphatase (Zhao, 2014). Glucose uptake in the mammary gland is dependent on passive or facilitated transport from the blood supply and accounts for up to 60 – 85% of the total glucose that enters the blood (Zhao, 2014). Glucose levels are directly affected by level of feed intake, as intake affects arterial glucose concentrations (Guinard-Flament *et al*., 2006).

1.3.3 Water soluble carbohydrates

Water soluble carbohydrates (WSC) can make up to 15% of a forage based diet and 25-30% of the diet when grazing grass and consist of monosaccharides (glucose and fructose) and disaccharides (sucrose and maltose). Sucrose plays a key role in the transport of sugars round plants and is the main carbon form found in the phloem in the translocation process from the source, where the sugar is produced by photosynthesis, to the sink, areas of active growth or sugar storage (Lemoine *et al*., 2013). Upon ingestion WSC are fermented almost immediately, therefore it is common for only low concentrations of WSC to be measured in rumen fluid (Bannink and Tamminga, 2005). Russell *et al*. (1992) predicted that WSC had a fractional degradation rate of 300% per hour, and as a result high levels in the diet can have negative effects on fibrolytic bacteria and protozoa (Bannink and Tamminga, 2005) due to a depression in rumen pH.

1.4 Protein metabolism in the rumen

In ruminants, dietary protein can be categorised as RDP or undegradable dietary protein (UDP; Bach *et al*, 2005). As Figure 1.10 shows, RDP can be sourced from true protein or NPN sources, and is susceptible to microbial degradation, with true protein being broken

down to amino acids and ammonia (NH_3; Bach *et al*., 2005). Meanwhile UDP bypasses the rumen to the small intestine where any digestible undegradable protein (DUP) is digested by the host animal's enzymes and absorbed (McDonald *et al*., 2011; Bach *et al*., 2005). Metabolisable protein (MP) is defined as the quantity of true protein or amino acids (digestible microbial protein and DUP) which are absorbed following digestion in the small intestine (Van Soest, 1994). The remaining indigestible bypass protein is excreted in faeces by the animal (McDonald *et al*., 2011).

The three catabolic processes which occur during protein degradation are proteolysis,

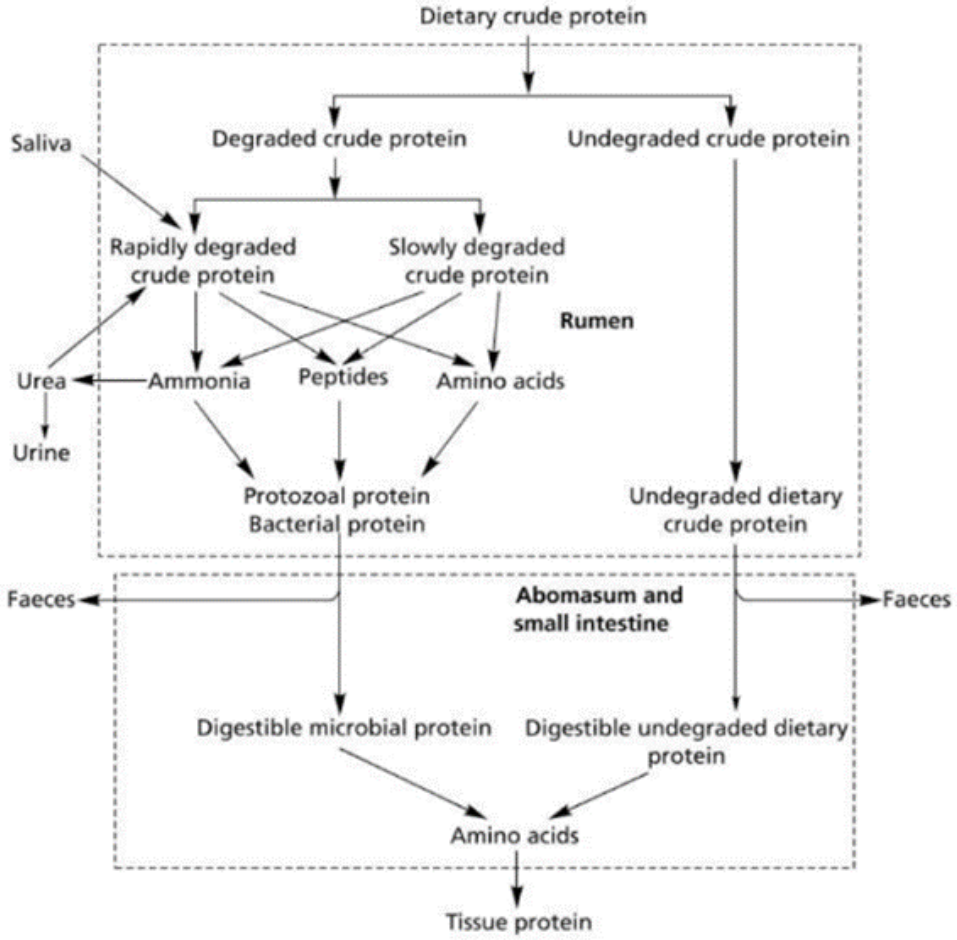


Figure 1.10: Dietary protein metabolism in the rumen (McDonald et al., 2011)

peptidolysis and deamination, to produce peptides and amino acids (Rodríguez *et al*., 2007). If energy supply is low, amino acids are deaminated and their carbon skeleton fermented into VFA (Bach *et al*., 2005). When dietary protein enters the rumen the bacteria attach to the feed particles, followed by microbial activity from cell bound proteases, bacterial proteases and peptidases are both endo-enzymes and exo-enzymes which bind to cells (Bach *et al*., 2005). Of the 70 – 80% of microorganisms that adhere to feed particles in the rumen, 30 – 50% of these have proteolytic activity (Wallace *et al*.,

1997). However, no specific species are considered to be responsible for the majority of proteolytic activity (Nolan and Dobos, 2005).

In the absence of an exogenous source of N, holotrichs will breakdown endogenous protein and digest rumen bacteria (Wallace *et al*., 1997). Ciliates are able to degrade insoluble protein and release large amounts of soluble protein into the rumen, although they are unable to utilise NH_3 (Dijkstra, 1994b). Protozoa contribute less than 20% of the microbial protein which passes through to the small intestine as part of total crude protein flow as it is selectively retained in the rumen (Newbold *et al*., 2015).

During MPS the rumen microbiota ferment carbohydrates such as starch to generate ATP to be used to synthesise peptide bonds (Nolan and Dobos, 2005). Rumen microbes are able to synthesise essential amino acids via transamination which supplies the ruminant with the majority of their amino acid requirement (Figure 1.10; Rodríguez *et al*., 2007). Thus approximately 50% of protein that reaches small intestine is of microbial origin (NRC, 2001; Schwab and Broderick, 2017; McCann *et al*., 2014), although this can vary on the degradability of the dietary protein. Factors which effect the rate of protein degradability include type of protein, ruminal dilution rate, ruminal pH, and substrate/ nutrient interactions (Bach *et al*., 2005).

1.4.1 Nitrogen recycling and excretion

Dietary sources of N include amino acids, nucleic acids, peptides, nitrates, urea and NH_3 (Nolan and Dobos, 2005). Endogenous sources include sloughed cells and recycled urea that enters the rumen via the rumen epithelium or saliva (Huntington and Archibeque, 1999). Ammonia can enter the rumen by proteolysis of dietary protein or microbial protein, including the ingestion of rumen bacteria by protozoa (Reynolds, 1992; Russell *et al*, 1999). Cellulolytic bacteria require NH_3 as their source of N for fibre degradation (Russell *et al*., 1992). Parker *et al*. (1995) predicted that 50% of dietary N passes through the NH_3 pool in the rumen and the proportion of N absorbed as NH_3 can equate to up to 73% of total N intake. Excess NH_3, which is toxic to the host animal in high amounts, is absorbed across the rumen epithelium and transported to the liver via the bloodstream, where it is converted to urea and later excreted in urine (Huntington and Archibeque, 1999). Urea can also be transported back to the rumen as a source of NPN either by diffusion into saliva or directly absorbed across the gut wall (Reynolds and Kristensen, 2008; Huntington and Archibeque, 1999). Bacteria have a high affinity for NH_3 and are able to survive on ruminal concentrations below 50 μM, so fermentation of fibre is able to function during periods of low N in the rumen (Huntington and Archibeque, 1999). When dietary crude protein (CP) levels are deficient (<5%) the N that is recycled in the rumen can make

up to 70% of the total dietary amino acid supply (NRC, 2001). Recycling urea therefore improves N efficiency with decreased dietary N as it lowers the amount that is excreted into the environment (Mutsvangwa *et al*., 2016).

Ammonia emissions contribute to the formation of fine particle matter with a diameter smaller than 2.5 µm (PM2.5) and is associated with several adverse health conditions (Giannakis *et al*., 2019). The UK government launched the Clean Air Strategy to reduce the number of people living in locations above the WHO guideline level of 10 µg/m^3 by half by 2025 (GOV, 2019). The policy covers pollution produced by industry, transport, farming, and households. Agriculture accounts for 88% of UK NH_3 emissions which can occur during slurry application, cattle housing, slurry storage, and grazing, and therefore reducing NH_3 losses requires a whole farm system approach (Guthrie *et al*., 2018; Bussink and Oenema, 1998). Between 65 – 80% of dietary N is excreted from cattle in the form of urine and faeces, and is converted to NH_3 via bacterial degradation (Nolan and Dobos, 2005). Excretion of urinary N is considered to be the most significant environmental polluter of N as urea is rapidly hydrolysed to NH_3 or to nitrate (NO_3) in soil, and urinary derivatives (allantoin, uric acid and creatine) also have a high potential for NH_3 volatilisation (Totty *et al*., 2013; Kebreab *et al*, 2001).

1.5 Physically effective fibre

The physical properties of feed can be affected by a range of factors including forage to concentrate ratio, proportion of non-forage fibre sources, ration mixing and particle size (Mertens, 1997). Mertens (1997) defined the term physically effective fibre (peNDF) as dietary neutral detergent fibre (NDF) which is long enough to avoid passage through to the abomasum and remains in the rumen. The definition relates to the physical characteristics of fibre and its ability to stimulate chewing activity for rumination and saliva production (Kononoff and Heinrichs, 2003), and the formation of the rumen mat, which is a mass of large particles in a pool of liquid and small particles within the rumen (Mertens, 1997). The physical effectiveness of particles in the diet can have an impact on DMI, MPS, nutrient digestibility and performance (Yang and Beauchemin, 2005; Allen, 1997). Additionally, if forage chop length is too long cattle are more likely to sort through the diet and select concentrates over forage (Kononoff *et al*., 2003; Tayyab *et al*., 2018). Sorting behaviour is discussed in Section 1.6.

The peNDF of a diet is measured as the sum of its NDF content and the proportion of particles in the diet longer than 4 mm, commonly measured using a Penn State Particle Separator (Allen, 1997; Mertens, 1997; Zebeli *et al*., 2012). Ewing and Wright (1918) were the first to establish that digesta did not leave the rumen until it decreased below a certain

particle size. This has since developed into the concept of a critical particle size which aided the development of models of digesta flow (Banakar *et al*., 2018). The critical particle size is the minimum size of the feed particle that is able to remain in the reticulorumen before being passed through to the abomasum (Oshita *et al*., 2004). Poppi *et al.* (1980) concluded that after particles had left the rumen there was almost no further reduction in particle size during further digestion in the gastro-intestinal tract and therefore mean particle size for digesta leaving the rumen can be measured from faecal samples. This was measured using a 1.18 mm sieve screen and as the sieve retained 5% of faecal particles (DM) it was suggested the critical particle size was 1.18 mm (Poppi *et al*., 1980; Mertens, 1997). However, there has been some dispute as to whether the true value is greater than 1.18 mm (Teller *et al*., 1990; Shaver *et al*., 1988). This is supported by Oshita *et* al. (2004) who found that when using a larger screen (4 mm) on the Penn State Particle Separator, that less than 5% of faecal DM was retained, suggesting that coarser particles can pass from the rumen. Other studies have also been conducted using a critical particle size greater of 4 or 8 mm (DeVries *et al*., 2007; Yang and Beauchemin, 2005; Plaizier, 2004).

1.5.1 Dry matter intake

There is much evidence from the literature that decreasing forage particle size (FPS) affects DMI, with meta-analyses having been conducted by Tafaj *et al*. (2007), Zebeli *et al*. (2012) and Nasrollahi *et al*. (2015). However, there is no clear conclusion as to whether the effect is positive or negative (Table 1.4). Tayyab *et* al. (2019), Haselmann *et al.* (2019) and Maulfair and Heinrichs (2013) agreed that decreasing FPS increased DMI as a % of bodyweight (BW) by a maximum of 12%, especially in diets with a low forage to concentrate ratio (F:C). Allen (2000) reported that diets with a high inclusion of forage can limit DMI due to distention of the reticulo-rumen. This was supported by Leonardi *et al*. (2005) who concluded that for every 1 mm decrease in mean FPS, DMI increased by 0.09 kg/d. In contrast, Kononoff *et al*. (2000) concluded that increasing FPS from 4.8 mm to 9 mm increased DMI by 5.7% which is supported by Allen (2000) who concluded that when diets contain a high inclusion of concentrates they can become the restricting factor to DMI due to metabolic constraints. Beauchemin *et al*. (2003), Yang and Beuchemin (2005) and Soita *et al*. (2005) reported that decreasing FPS had no effect on DMI. Despite the variation in results most studies agreed that the F:C ratio had a strong influence on DMI.

Table 1.4: Dry matter intake of dairy cows fed forages of varying particle size

		Long forage PS		Short forage PS			
Reference	F:C	PS (mm)	DMI (kg/d)	PS (mm)	DMI (kg/d)	SEM	P value
Kononoff *et al*. (2000)	45:55	9.0	26.2	4.8	24.7	0.26	<0.050
Beauchemin *et al*. (2003)	50:50	10.0	23.5	4.0	23.9	2.10	NS
Yang and Beauchemin (2005)	42:58	19.1	21.0	Fine (<11)	20.3	3.80	NS
Soita *et al*. (2005)	55:45	19.1	24.5	9.5	24.9	1.02	NS
Maulfair and Heinrichs (2013)	42:58	47.1	29.4	6.35	31.4	1.08	<0.01
Haselmann *et al*. (2019)	80:20	52.0	21.0	7.0	22.8	0.23	<0.001
Tayyab *et al*. (2019)	54:46	44.0	22.8	15.0	24.0	0.56	0.035

F:C= Forage to concentrate ratio, PS= Particle size, DMI= Dry matter intake, NS= Not significant

1.5.2 Rumen fermentation and the microbiota

Beauchemin *et al*. (2003), Kononoff and Heinrichs (2003) and Leonardi *et al.* (2005) were all in agreement that increasing FPS increased ruminating time. The increase in saliva production, a result of rumination of forage, can act as a buffer against a decline in rumen pH (Zebeli *et al*., 2012). This is supported by Beauchemin *et al*. (2003), who concluded that increased FPS increased mean rumen pH. In both treatments rumen pH decreased post feeding, however pH remained higher in animals that were fed forage with a longer particle size (Figure 1.11). Tafaj *et al*. (2007) also reported that increased FPS and NDF content were positively correlated to an increased rumen pH (R^2 = 0.55), stating that 14% of this was accounted for by FPS and another 41% by the NDF content. Alternatively, there is much evidence to suggest that rumen pH was not affected by forage particle size (Einarson *et al*., 2004; Leonardi *et al*., 2005; Tayyab *et al*., 2018). Einarson *et al*. (2004) suggested that peNDF only affected rumen pH when it was below a 'threshold pH' (i.e. pH 5.6, with the inducement of SARA), and therefore effects were more likely to be seen more in fine than coarse diets.

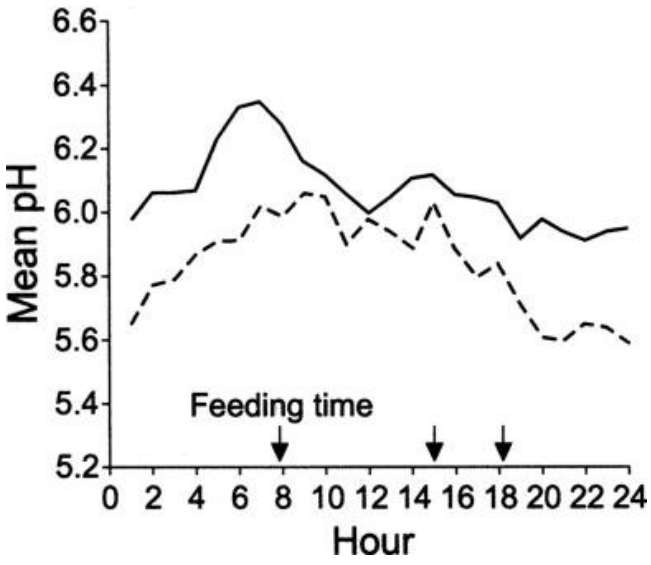


Figure 1.11: Effect of forage particle size on diurnal rumen pH (– diet with chopped forage; - - - diet with ground forage; Beauchemin *et al*., 2003)

Kononoff and Heinrichs (2003) concluded that total VFA concentration increased by up to 9.8% as FPS decreased. Diets with a short FPS had a larger surface area therefore more was availability for cellulolytic activity, increasing VFA production (Zebeli *et al*., 2012; Kononoff and Heinrichs, 2003). In contrast there are studies which have reported that changing FPS had no effect on rumen metabolism (Leonardi *et al*. 2005; Maulfair and Heinrichs, 2013). Einarson *et al*. (2004) argued that the rumen VFA concentration may not reflect VFA production as it is regulated by the balance between production and absorption. Furthermore, variation between studies may be affected by other factors such as F:C, forage source and rumen outflow rate (Einarson *et al.*, 2004).

Long FPS can encourage cellulolytic activity, however it can also reduce DMI and encourage sorting activity. Zebeli *et al.* (2008a; 2008b) reported that medium length (8 – 19 mm) grass and maize silage were able to promote ruminal degradation without compromising cellulolytic microbiota. Zebeli *et al.* (2012) also suggested that reducing FPS increased the surface area available for fibrolytic attachment. When diets contain a short FPS there can be increased risk of developing acidosis due to a reduction in rumen buffering (Zebeli *et al.,* 2012). The microbial population can also shift substantially during clinical acidosis, with cellulolytic bacteria declining and acid tolerant bacteria such as *Streptococcus bovis* and *Lactobacillus* spp. proliferating and increasing lactate production, further decreasing ruminal pH (Khafipour *et al*., 2009; de Mulder *et al*., 2017).

1.5.3 Production and health

Rations with short FPS have been reported to increase DMI and fibre digestibility due to decreased rumen fill and retention time (Tayyab *et al*., 2018; Zebeli *et al*., 2012). However, when FPS is too short (<1.18 mm; Zebeli *et al*., 2012) cattle are at an increased risk of developing acidosis due to reduced rumination and saliva production (Tafaj *et al*., 2007). Increasing peNDF in the diet can mitigate the risk of SARA, however, digesta rate of passage can be decreased compromising DMI (Zebeli *et al*., 2012).

Nasrollahi *et al*. (2015) conducted a meta-analysis and concluded that decreasing FPS increased milk production by 0.54 kg/day, while milk fat decreased by 0.06%. Zebeli *et al.* (2006) agreed that there was a negative correlation between FPS and milk yield and suggested it was a consequence of decreased DMI, as DMI is directly correlated to milk yield (Guinard-Flament *et al*., 2006). Einarson *et al.* (2004) and Zebeli *et al*. (2006) reported that milk fat increased with FPS (R^2 = 0.11), as when rumen pH and the A:P increased the cows were at a lower risk of developing milk fat depression (Yang and Beauchemin, 2005; Bauman and Griinari, 2001). In contrast, Maulfair and Heinrichs (2013), Tafaj *et al*. (2007) and Alamouti *et al*. (2014) concluded that FPS had little effect on either milk yield or composition. Yang and Beauchemin (2006) suggested that many studies rarely showed a treatment effect on milk yield because they were too short (21 days) or too few cows were used. According to Beauchemin *et al*. (1994) effects of FPS on milk fat content may only be seen in cattle fed diets with NDF levels lower than recommended requirements (250 g/kg DM; NRC, 2001). Additionally in early lactation, cows are in negative energy balance and mobilise body fat (NRC, 2001), and as a result they can increase milk fat without a dietary influence (Zebeli *et al*., 2006). Consequently, Mertens (1997) recommended that rumen pH was a better indication of ruminal health and optimal function rather than milk fat production in early lactation.

1.6 Effect of sorting behaviour on performance and rumen function

Total mixed rations provide dairy cattle with their complete nutritional requirements (Greter and DeVries, 2011; Bargo *et al*., 2002b). However, it is not uncommon for cows to select the more palatable parts of the ration, favouring short particles high in starch and protein over long forage particles (DeVries *et al*., 2008; Zebeli *et al*., 2012). When cows express sorting behaviour they change both the level and pattern of concentrate intake (Leonardi and Armentano, 2003), which can alter rumen fermentation and performance (DeVries *et al*., 2008). Competition at the feed face can vary nutrient intake within the herd, as dominant cows access their feed first, and peak feeding activity occurs in the first 90 minutes after delivery when cattle are able to maximise concentrate intake (DeVries *et al*.,

2005). In contrast, less dominant cattle eat later and therefore may be faced with a different ration which is lower in energy and protein and higher in NDF (Miller-Cushon and DeVries, 2017a). This poses less of a health risk but may be reflected by a drop in performance (Rabelo *et al*., 2003).

The probability of cattle selecting their diet increased when rations were poorly mixed as the ingredients were not uniformly distributed (Kononoff *et al*., 2003; Maulfair and Heinrichs, 2010). A study concluded that in the UK that 58% of dairy farms provided the cows with suboptimal or poorly mixed rations, with 66% having significant diet selection 4 h after feed delivery (Tayyab *et al*., 2018). Zebeli *et al*. (2012) reported that reducing FPS improved ration uniformity therefore cattle were less likely to display sorting behaviour. Across all treatments, cows selected against particles >19.0 mm and for particles that were <8.0 mm. When fed a lower forage diet (45:55 DM basis) cows selected against NDF more than when fed a higher forage diet (60:40 DM basis), as concentrates were more easily accessible due to the increased proportion (Zebeli *et al*., 2018). Tayyab *et al*. (2018) reported similar results when feeding cattle grass and maize silage based diets. Of the 50 dairy herds sampled, 82% selected against particles >19.0 mm while there was preferential consumption of particles <8.0 mm. However, on 46% of farms there was no sorting activity of the <8.0 mm fraction. This was considered to be due to the comparatively high moisture content of the ration which caused the smaller particles to adhere to the larger particles making them more difficult to sort (Tayyab *et al*., 2018). The effects of varying FPS in the diet on performance and rumen metabolism are discussed in Section 1.5.

Other factors which can affect sorting behaviour include DM content of the diet and number of meals delivered to the cows per day (Miller-Cushon and DeVries, 2017a). DeVries and Gill (2012) reported that increasing the moisture content of the ration by adding 4.1% of a molasses based liquid feed, altered the particle size distribution of the TMR, with the proportion of small particles selected for reduced by 12.5% and the extent of sorting against long particles reduced by up to 20%. Molasses was considered to be more effective than water as cattle prefer sweet flavours (Nombekela *et al.*, 1994). These results were supported by Firkins *et al*. (2008) who suggested that increased diet palatability may help stimulate DMI. Sova *et al*. (2013) concluded that when cattle were fed twice a day that there was less evidence of sorting against particles >19.0 mm, which increased DMI by 1.42 kg/d compared to when fed once per day. This finding is in agreement with Endres and Espejo (2010) who reported that there was a greater degree of sorting behaviour in cows fed their ration once a day compared to twice or three times. Hart *et al*. (2014) also concluded that when fed three times per day there was little to no

evidence of sorting for or against short particles. It was hypothesised that cows were better able to sort through their ration when fed less frequently as they had access to larger amounts of feed with more time to select before the feed was replenished (Greter and DeVries, 2011). More recently Tayyab *et al*. (2022) reported that there was little effect of dietary starch content on rumen pH or milk fat content, which was partially attributed to the cows being fed individually and several times a day, which reduced the opportunity for selection.

1.6.1 Effect of forage particle size on diet selection

Greter and DeVries (2011) reported an association between reduced DMI and sorting both against long particles (R^2 = 0.23) or for short particles (R^2 = 0.26). It could therefore be concluded that when cows spend more of their time sorting through the ration they limit their ability to maximise intake, which may compromise performance (Sova *et al*. 2013). Sova *et al*. (2013) reported that for every percentage increase of group level overconsumption of fine particles (<1.18 mm) efficiency of milk production (defined as milk yield/ average DMI) decreased by 3%. Miller-Cushon and DeVries (2017b) were in agreement with these findings as they reported that milk fat increased by 0.1% for every 10% increased selection of long particles in the ration. Favouring short particles can increase the risk of depressed rumen pH thus potentially reducing the efficiency of nutrient utilisation, which may reduce milk yield (DeVries *et al*., 2008). Sova *et al*. (2013) and Ingvartsen *et al*. (2001) reported that sorting against long particles was associated with a decrease in milk fat yield, by up to 2.9 g/kg. This is supported by Mertens (1997) who concluded that longer FPS was associated with greater acetate production in the rumen which could subsequently lead to a positive effect on milk fat content.

Miller-Cushon and DeVries (2017a) reported that when cattle express sorting behaviour they often favour concentrates over forage, and subsequently rumination time can decrease as the cattle consume a diet with a higher proportion of short particles. As a consequence, rate of passage in the rumen would increase and the efficiency of fibre digestibility may be reduced (Zebeli *et al*., 2012). DeVries *et al*. (2008) reported that when cattle selected for longer (19 mm) particles from a TMR there was a positive correlation with maximum rumen pH (R^2=0.46) due to increased rumination and buffering from saliva (Krause and Oetzel, 2006). When cattle favour short particles in their diet rumen pH can be depressed which may limit microbial activity, compromising the efficiency of nutrient utilisation (DeVries *et al*., 2008). Furthermore DeVries *et al*. (2008) concluded that selecting for small particles (<8 mm) had a positive correlation with the nadir in pH (R^2=0.57 and R^2=0.68, respectively) due to the reduced intake of peNDF and increased intake of starch. The two mechanisms, decreased rumination resulting in reduced

buffering capacity, and increased VFA production (Stone, 2004) when combined can increase the susceptibility to SARA in dairy cattle (Stone, 2004; Penner *et al*., 2007).

DeVries *et al*. (2008) conducted a study to determine if sorting behaviour changed following acidosis exposure and reported that initially when fed a high starch diet cattle selected for medium and short particles (Table 1.5). When fed the same diets again following the induction of acidosis, cattle showed a significant change in eating behaviour by favouring medium and long particles and eating 4.4% fewer short and fine particles. Meanwhile, cattle fed a low starch diet (low acidosis risk) continued to consume the fractions of their ration consistently between both periods (DeVries *et al*., 2008). As a result, the cattle fed the high starch diet had a higher mean rumen pH in period two (after SARA was induced) than period one, and it was suggested that these animals selected for longer particles to increase the level of peNDF in the diet, increasing rumination and buffering effect (DeVries *et al*., 2008).

Table 1.5: Effects of repeated rumen acidosis challenges (Period 1 and 2) on feed sorting at high and low risk of experiencing acidosis in lactating dairy cows (DeVries *et al*., 2008)

Forage	High risk		Low risk			P Value		
particle size	1	2	1	2	SEM	R	P	R x P
Long	91.8	92.5	89.3	87.9	2.60	0.3	0.8	0.5
Medium	105.3	107.8	102.1	103.3	0.55	<0.001	0.002	0.2
Short	99.0	97.7	101.6	101.4	0.56	<0.001	0.06	0.14
Fine	93.6	86.5	99.2	96.1	2.30	0.004	0.005	0.2

R= Rumen acidosis risk, P= Period, x= Interactions

1.6.2 Effect of pattern of concentrate intake on performance and rumen function

The majority of studies that have fed concentrates in discrete meals (using with in- or out-of parlour feeders) compared to feeding a TMR have reported little effect on DMI (Little *et al*, 2018; Purcell *et al*., 2016; Table 1.6). In contrast, Agnew *et al.* (1996) concluded that feeding the ration as a TMR increased DMI compared to feeding concentrates in multiple meals, although there was no effect on milk yield. When cattle display diet selection, they rarely change just their pattern of concentrate allocation, and the level of concentrate intake is also likely to change, which may further influence DMI (Leonardi and Armentano, 2003). Furthermore, González *et al*. (2012) reported that monitoring DMI as a response to pattern of feeding and SARA was inconsistent due to the extent of multiple factors associated with low rumen pH. On the other hand, Plaizier *et al*. (2008) hypothesised that the reduction in fibre digestibility caused by low rumen pH may result in reduced rate and passage and therefore reduce DMI.

Sutton *et al*. (1985) fed concentrates either in two meals at morning and afternoon feeding or in six meals every four hours, and concluded that, mean milk yield increased by 1.2 kg per cow per day as the number of meals increased. When the level of concentrates fed remained the same but the frequency of concentrate allocation increased milk fat and protein content and yield were unaffected (Sutton *et al*., 1985). It was only when cattle were fed diets with a low F:C ratio (30:70 or lower) that milk fat content increased when the frequency of meals fed was higher, suggesting that the cows fed a low F:C were at increased risk of milk fat depression (Sutton *et al*., 1985).

Table 1.6: Dry matter intake and milk yield in dairy cows when fed different patterns of concentrate allocation

Reference	Number of meals	DMI (kg/d)		Milk yield (kg/d)	
		TMR	Separate	TMR	Separate
Yang and Varga (1989)	One	20.5	20.6	21.1	20.5
Agnew *et al*. (1996)	Four	13.9	12.8*	20.0	19.8
Purcell *et al*. (2016)	> Four	23.0	22.2	40.7	39.3
Little *et al*. (2018)	> Four	22.4	22.2	39.3	38.0

* = $P < 0.05$, TMR= Total mixed ration, Separate= Concentrate fed in separate meals

Reducing the frequency of concentrate meal provision has been shown to alter rumen pH (Yang and Varga, 1989). Sutton *et al.* (1986) reported that when cattle were fed concentrates more frequently (24 meals per day), mean rumen pH was 0.3 – 0.6 units lower than when fed in two or fewer meals, however the minimum pH was higher. As shown in Figure 1.12, when meal frequency increased rumen pH was more consistent despite being lower on average (Sutton *et al*., 1986). With 24 concentrate meals rumen pH did not increase above pH 6.0 in the first 12 hours after feeding due as the rate of starch fermentation in the rumen being more constant throughout the day (Yang and Varga, 1989). Both Sutton *et al*. (1986) and Yang and Varga (1989) were in agreement that changing the frequency of concentrate meals had little effect on total or individual VFA concentration.

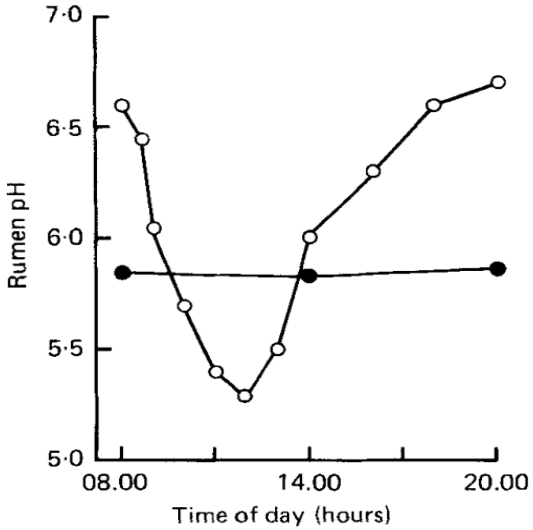


Figure 1.12: Diurnal variation of rumen pH in dairy cattle fed 24 meals a day (●), and twice a day (○), (Sutton *et al*., 1986)

A high intake of rapidly degradable starch can quickly increase the rate of microbial fermentation and VFA concentration in the rumen, decreasing rumen pH and increasing the risk of developing SARA (Plaizier *et al*., 2017). Krause and Oetzel (2006) reported that during SARA, when rumen pH was below pH 5.8, both lactate producing bacteria such as *Streptococcus bovis*, and lactate-utilizing species *Megasphaera elsdenii* and *Selenomonas ruminantium* were present, suggesting SARA was not a result of lactate build up, but total VFA accumulation (Goad *et al*., 1998). As rumen pH declined further to pH <5.0, lactate-producing bacteria began to outnumber the lactate-utilizing species, leading to an accumulation of lactate in the rumen and the onset of acute acidosis (Chaucheyras-Durand *et al*., 2008). Ingvartsen *et al*. (2001) reported that when cows were fed a complete diet, 6.3% of lactating cattle developed metabolic diseases. When fed forage and concentrates separately, 34.4% of the herd had developed metabolic disease due to increased stress (Ingvartsen *et al*., 2001).

1.6.3 Effect of level of concentrate intake on performance and rumen function

The literature on the effect of altering the level of concentrates in the ration of high yielding dairy on performance cows is not always consistent, and can be affected by the difference in the level and composition of the concentrate fed (Table 1.7). For example, Henriksen *et al*. (2019) and Ingvartsen *et al*. (2001) concluded that the level of concentrate intake had no effect on either DMI or milk yield, although the differences in concentrate intake were small. In contrast, Andersen *et al*. (2003), Jiao *et al*. (2014) and Lawrence *et al*. (2015) all reported both an increase in DMI and milk yield with increasing level of concentrate fed. Andersen *et al*. (2003) determined that while overall DMI was 1 kg higher with a high

concentrate diet (25:75 F:C) compared to a low concentrate diet (75:25 F:C) the difference in energy level of the diet was 1.57 MJ/kg DM higher. Furthermore, when cows were fed diets high in NDF, DMI can decrease with gut fill being a limiting factor (Allen, 2000). On the other hand, diets high in energy can reduce pH and consequently decrease fibre digestibility increasing the retention time of particles in the rumen (Allen, 2000).

Table 1.7: Dry matter intake and milk yield in dairy cows when fed different levels of concentrate allocation

	Level of concentrates		DMI (kg/d)		Milk yield (kg/d)	
Reference	Low	High	Low	High	Low	High
Andersen *et al*. (2003)	75:25 F:C	25:75 F:C[1]	16.3	17.2*	34.1	40.2**
Jiao *et al*. (2014)	2 kg	6 kg	14.5	15.5*	19.6	25.9**
Lawrence *et al*. (2015)	4 kg	7 kg	17.1	19.7**	25.8	28.4**
Henriksen *et al*. (2019)	2.2 kg	3.2 kg	20.1	20.1	35.3	35.2

* = P < 0.100, ** = P < 0.05, Low = Low concentrate allocation, High = High concentrate allocation
[1] Fed as a total mixed ration

When concentrate intake is increased, rumen pH often drops due to an increase in VFA production, and therefore inhibition of fibrolytic bacteria such as *Bacteroidetes* (Van Wyngaard *et al*., 2018; Wang *et al*., 2020). Wang *et al*. (2000) reported that pH decreased when cows were fed a diet with high levels of concentrates (35:65 F:C ratio) due to an increase in VFA concentration, particularly propionate and butyrate which is most likely due to an increase in relative abundance of *Prevotella.* This can then reduce the efficiency of fibre digestion and decrease the ruminal production of acetate, which is the precursor for milk fat synthesis (Guinard-Flament *et al*., 2006; Van Soest, 1994). This is supported by Anderson *et al*. (2003), Jiao *et al*. (2014) and Ingvartsen *et al*. (2001) who reported that increasing concentrate intake decreased milk fat concentration. In contrast, Lawrence *et al*. (2015) and Henriksen *et al*. (2019) concluded that increasing the level of concentrates had no effect on milk composition.

1.7 Effect of feed restriction on performance and rumen function

Short-term feed restriction, up to six hours, can be relatively common on commercial dairy farms due to management, physiological or environmental factors (Zhang *et al*., 2013). Feed restriction can occur due to feeding equipment failure, or insufficient allocation of feed or pasture resulting in cattle not being provided with their full daily requirements of nutrients (Thomson *et al*., 2018; Zhang *et al*., 2013). Additionally, cattle have access to feed removed for a number of hours each day due to milking, health checks, or when feed is not frequently pushed up to the feed barrier (Thomson *et al*., 2018). Tayyab *et al*. (2018) reported that 34% of UK dairy farms had no feed available in the morning, and so

the cattle experienced a period of short-term restriction overnight. Transporting cattle can not only deprive them from feed for prolonged periods of time, but also exposes them further stress with increased risk of illness and disease (Zhang *et al*., 2013; Millman, 2016). Heat stress and diseases such as metabolic, infectious and digestive disorders can also reduce DMI and have deleterious consequences on health and performance in dairy cattle (Pragna *et al*., 2017; Goldhawk *et al*., 2009).

1.7.1 Dry matter intake

During feed restriction DMI is reduced, and when feed is reintroduced cows can enter a period of overeating, rapidly increasing their intake which can cause problems for both rumen health and the microbiome (Thomson *et al*., 2018; Oetzel, 2007). Thomson *et al*. (2018) reported that when feed was withheld for six hours from 0830 until 1430 h DMI did not vary between the baseline day and the day of fasting, however as the restriction period occurred on the same day that feed was returned there was no daily distinction between the baseline, restriction and recovery period in terms of DMI (Thomson *et al*., 2018). Rumination time remained the same during the baseline and challenge days, however, upon re-feeding the cattle spent more time eating in the first three hours than at the baseline, with cows spending 57% of their time eating compared to 29%, respectively (Thomson *et al*., 2018). Abdelatty *et al*. (2017) and Velez and Donkin (2005) were in agreement that DMI dropped during the restriction period, then upon re-feeding DMI increased and returned to the baseline level during the recovery period (Table 1.8). Patterson *et al*. (1998) concluded that when the duration of fasting period increased the rate of DMI (kg/hour) also increased up to 6h following re-feeding, after this time DMI remained consistent.

Table 1.8: Dry matter intake in cattle during baseline, period of feed restriction and recovery

Reference	Baseline	Restriction	Recovery	SEM	P value
Velez and Donkin (2005)	25.3	12.3	23.9	-	<0.05
Zhang *et al*. (2013)	11.7	8.20	-	0.375	<0.001
Abdelatty *et al*. (2017)	24.8	15.2	23.8	0.03	0.01
Thomson *et al*. (2018)	23.4	24.3	23.0	1.44	>0.05

1.7.2 Rumen fermentation and the microbiota

Thomson *et al*. (2018) reported that during a six-hour fasting period rumen pH steadily increased from the baseline value of pH 6.36 to pH 7.2, and within the first two hours after re-feeding pH dropped rapidly (Thomson *et al*., 2018). Krause and Oetzel (2005) supported these findings, (Figure 1.13), with a steady increase in rumen pH during the fasting period followed by a steep, rapid decline by approximately pH 0.5 in the first 2 h when feed was reintroduced. Zhang *et al*. (2013) evaluated changes to rumen metabolism when cattle were fed diets restricted to 75, 50 and 25% of the baseline ration. During the fasting period mean rumen pH increased from the baseline pH of pH 6.14 to 6.62 and 6.97 for 75 and 25% feed restriction respectively, suggesting that as degree of restriction increased rumen pH also increased (Zhang *et al*., 2013). Thomson *et al.* (2018) concluded that rumen pH increased during the fasting period as the rumen VFA were absorbed and not being replaced due to lack of substrate fermentation. Furthermore, due to the presence of forage in the rumen cows continued to ruminate during the fasting period and the flow of saliva entering the rumen had a positive buffering effect (Thomson *et al*., 2018).

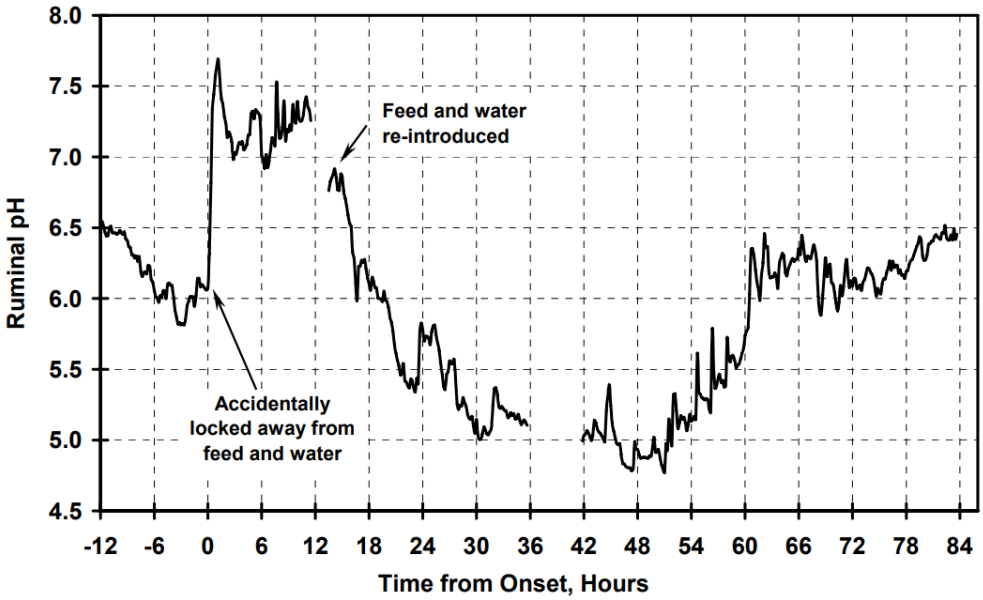


Figure 1.13: Rumen pH during period of feed restriction in a Holstein steer (Krause and Oetzel, 2006)

Zhang *et al*. (2013) reported when beef cattle were subject to feed restriction that not only did VFA concentration in the rumen decrease but as the level of feed restriction increased from 75% to 50%, and 25% of voluntary intake, VFA concentration decreased to a greater extent, by an average of 27.5% per 25% drop in intake. Volatile fatty acids are a stimuli promoting epithelial function, and therefore a decrease in VFA production can reduce the absorptive capacity of the epithelium (Zhang *et al*., 2013), and chronically fasted sheep

were shown to have 32% less VFA absorption than the control group (Doreau *et al*., 1997). Mechanisms of VFA removal are predominantly sodium bicarbonate transport and passive diffusion (Beauchemin *et al*., 2018). After feed restriction rumen VFA concentrations are low, and therefore there can be an insufficient gradient to transport across the rumen epithelium (Thomson *et al*., 2018). As a result, a prompt decline in rumen pH may be seen shortly after re-feeding as microbial productivity increases (Mason and Stuckey, 2016) combined with an initial failure to absorb VFA from the rumen (Thomson *et al*., 2018).

High rumen pH, as seen during feed restriction, may also inhibit populations of lactate utilising bacteria, which are sensitive to a higher ruminal pH, and can result in SARA (Krause and Oetzel, 2005). McCann *et al*. (2016) reported that in the recovery period following up to 50% feed restriction there was a reduction in the specie richness of the liquid fraction, with the abundance of Firmicutes decreasing while Bacteroidetes increased by 23% in the solid fraction. Additionally, the relative abundance of *Streptococcus* and *Lactobacillus* increased following feed restriction from the liquid fraction due to an increased intake of starch (McCann *et al*., 2016).

1.7.3 Production and health

When cattle refeed following the fasting period, starch intake usually increases which increases the risk of developing SARA (Thomson *et al*., 2018). As a result, it is common for researchers to monitor SARA by conducting feed restriction studies on dairy cows (McCann *et al*., 2016; Krause and Oetzel, 2006).

Lactose is synthesised by glucose and galactose in the mammary gland, and is the principal component of milk yield as it regulates the osmotic potential in milk (Guinard-Flament *et al*., 2006). Glucose levels in the blood are directly affected by level of feed intake and therefore when cattle undergo feed restriction there is a reduction in arterial glucose concentrations (Guinard-Flament *et al*., 2006). As a consequence, as the degree of feed restriction increases, milk yield and composition quality decreases (Table 1.9; Thomson *et al*., 2018; Abdelatty *et al*., 2017). When cattle underwent a fasting period of six hours milk yield dropped by 10.6%, and when feed was withheld for 48 hours milk yield dropped by over 60% (Table 1.9; Thomson *et al*., 2018; Chelikani *et al*., 2004). Abdelatty *et al*. (2017) and Velez and Donkin (2005) reported that following restriction milk yield did not return to its baseline level for up to four days despite the increased intake of energy following feed reintroduction, potentially due to the disruption to rumen metabolism (Figure 1.14).

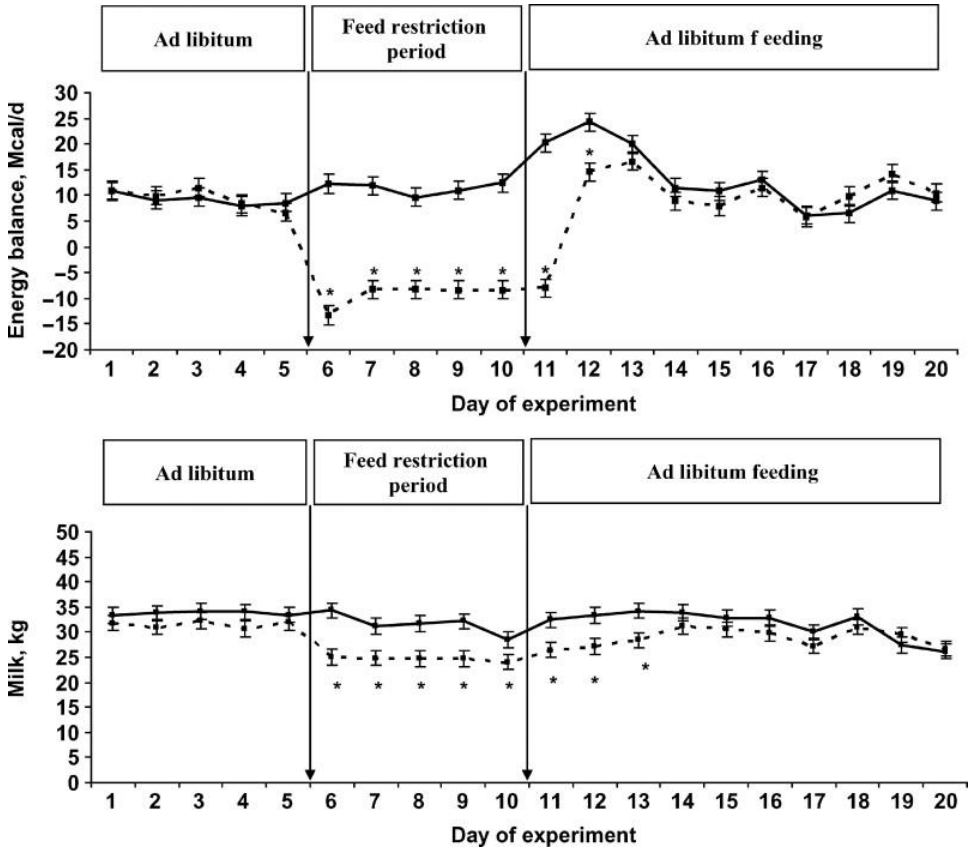


Figure 1.14: Net energy balance and daily milk production for control cows (—) and cows subjected to feed restriction (- - -) (Velez and Donkin, 2005)

Table 1.9: Effect of feed restriction on milk yield and composition

		Milk yield		Milk protein		Milk fat	
Reference	Duration of fasting period	Baseline (kg/d)	Restriction (% change)	Baseline (kg/d)	Restriction (% change)	Baseline (kg/d)	Restriction (% change)
Chelikani *et al*. (2004)	48 hours	21.8	-60.4	0.59	-54.2	0.77	-15.6
Velez and Donkin (2005)	50% of ration, for 5 d	31.5	-24.8	0.89	-22.5	1.41	-31.9
Abdelatty *et al*. (2017)	60% of ration, for 5 d	41.2	-19.9	1.19	-23.4	1.39	-13.0
Thomson *et al*. (2018)	Six hours	29.7	-10.6	0.91	-11.8	1.02	-5.64

1.8 Effect of active dry yeast on performance and rumen function

Saccharomyces cerevisiae is the most common active dry yeast (ADY) used in ruminant nutrition, in live yeasts the biomass is dried to preserve cell viability and metabolic activity (Chaucheyras-Durand *et al*., 2008). Yeast is aerobic and cannot survive long in the rumen, and as a result supplements have to be continually provided in the diet (Jouany, 2006). Chaucheyras-Durand *et al*. (2008) defined the main three effects of yeast on rumen microbiota as improvement of rumen maturity by favouring microbial establishment, stabilisation of ruminal pH and interactions with lactate metabolising bacteria, and increasing fibre degradation and interactions with plant cell wall degrading microbes. Live yeasts metabolise sugars in the rumen and produce ethanol, glycerol, peptides and amino acids which can be utilised by microbes (Jouany, 2006). Yeast has been shown to produce organic acids such as malic acid which stimulate the growth of the lactate utilising bacteria *Selenomonas ruminantium*, and therefore contribute to stabilising rumen pH (Kumprechtová *et al*., 2019; Barrera *et al*., 2019). Additionally, ADY can improve fibre degradation by scavenging oxygen due to its high respiratory activity, resulting in rumen conditions that are more favourable for fibre degrading bacteria (Newbold *et al*., 1996). Active dry yeasts are well accepted to have beneficial effects on livestock performance, including increasing DMI and milk production (Barrera *et al*., 2019; Chaucheyras-Durand *et al*., 2008).

1.8.1 Dry matter intake

There are mixed conclusions regarding the effect of ADY on DMI (Table 1.10). Bach *et al*. (2007) concluded that yeast had no effect on DMI, a finding that was supported by Dias *et al*. (2018) and Jiang *et al*. (2017). Meanwhile, Desnoyers *et al.* (2009) conducted a meta-analysis which concluded that yeast inclusion increased DMI by 0.4% of total BW. Similarly, Erasmus *et al*. (1992) and Williams *et al*. (1991) reported that the inclusion of yeast tended to increase DMI by 1.4 and 1.2 kg/d respectively. Jouany (2006) concluded that DMI increased due to the positive effect of yeast on fibre digestibility, especially in high starch diets (Desnoyers *et al*., 2009). Yeast can effectively scavenge oxygen from the rumen improving rumen conditions for anaerobic fibrolytic bacteria to grow and function (Fonty and Chaucheyras-Durand, 2006). Increased fibrolytic activity can improve organic matter (OM) digestibility, and therefore DMI may increase due to an increased rate of passage (Jouany, 2006). Jiang *et al*. (2017) suggested that there were several factors which may cause contradictory results between studies including: stage of lactation, stress level, diet composition, and yeast species, viability and dose.

Table 1.10: Effect of live yeast on dry matter intake in dairy cows

	DMI (kg/d)			
Reference	Control	Yeast	SEM	P value
Erasmus *et al*. (1992)	21.8	23.2	0.70	<0.100
Bach *et al*. (2007)	18.3	18.5	4.24	0.590
Desnoyers *et al*. (2009)	34.6 (% of BW)	35.0 (% of BW)	1.10	< 0.05
Jiang *et al*. (2017)	25.0	25.5	0.64	0.350
Dias *et al*. (2018)	22.6	22.9	0.70	0.700

1.8.2 Rumen fermentation and microbiome

Ambriz-Vilchis *et al*. (2017) and Erasmus *et al*. (1992) reported that yeast supplementation had no effect on rumen pH, while other studies have concluded that supplementation had a positive effect (Desnoyers *et al*., 2009; Dias *et al*., 2018; Bach *et al*., 2007; Table 1.11). Yeast provides a supply of nutrients to the rumen microbiota which may stimulate the growth of microbial populations (Jouany, 2006; Chaucheyras-Durand *et al*., 2016). Furthermore, yeast has been reported to stimulate the growth of lactate utilising bacteria and decrease lactate concentration, which is supported by results shown in Table 1.11 (Kumprechtová *et al*., 2019). Alternatively, ADY may compete with lactate producers such as *Streptococcus bovis* for sugar for metabolism, reducing their capacity to produce lactate (Chaucheyras *et al*., 1996). Dias *et al*. (2018) reported that the inclusion of yeast increased VFA concentration by 8.5%. A review conducted by Desnoyers *et al*. (2009) concluded that despite an increased VFA concentration the A:P ratio remained consistent. Fibrolytic bacteria activity was reported to increase with yeast supplementation which would increase acetate concentration in the rumen (Dias *et al*., 2018). However, the A:P remained unchanged as rumen conditions improved propionate concentration at an equal rate (Desnoyers *et al*., 2009).

Table 1.11: Effect of live yeast supplementation on rumen pH, VFA and lactate concentration in dairy cattle

	Mean rumen pH		Total VFA concentration (mM)		Lactate concentration (mM)	
Reference	Control	Treatment	Control	Treatment	Control	Treatment
Erasmus *et al*. (1992)	5.99	6.00	-	-	1.63	1.40
Bach *et al*. (2007)	5.49	6.05*	-	-	-	-
Desnoyers *et al*. (2009)	6.31	6.34*	95.2	97.3*	1.21	1.13
Ambriz-Vilchis *et al*. (2017)	6.30	6.34	-	-	-	-
Dias *et al*. (2018)	6.02	6.41*	116	126	2.37	1.56*
Kumprechtová *et al.* (2019)	6.37	6.59*	105	112*	16.40	9.30*

* = $P < 0.05$

One of the most important effects of yeast on fibre degradation is the ability to scavenge oxygen, as up to 16 L of oxygen can enter the rumen per day from feeding, rumination and salivation (Barrera *et al*., 2019). Yeast cells are aerobic so they have the ability to utilise oxygen for metabolism, and lower the oxidation-reduction potential inside the rumen improving anaerobic conditions (Newbold *et al*., 1995; Dehority and Orpin, 1997). Many rumen microbes are strict anaerobes and therefore the presence of yeast can reverse the damaging effects of oxygen and stimulate the growth of bacteria and protozoa (Ghazanfar *et al*., 2017). The two main fibrolytic bacteria *Fibrobacter succinogenes and Ruminococcus flavefaceins* populations both increased upon the inclusion of yeast in the diet (Figure 1.15; Chaucheyras-Durand *et al*., 2016). Similarly, Fonty and Chaucheyras-Durand (2006) concluded that the fibre degrading bacteria *F. succinogenes, R. albus* and *R. flavefaceins* increased two to four fold upon the inclusion of yeast.

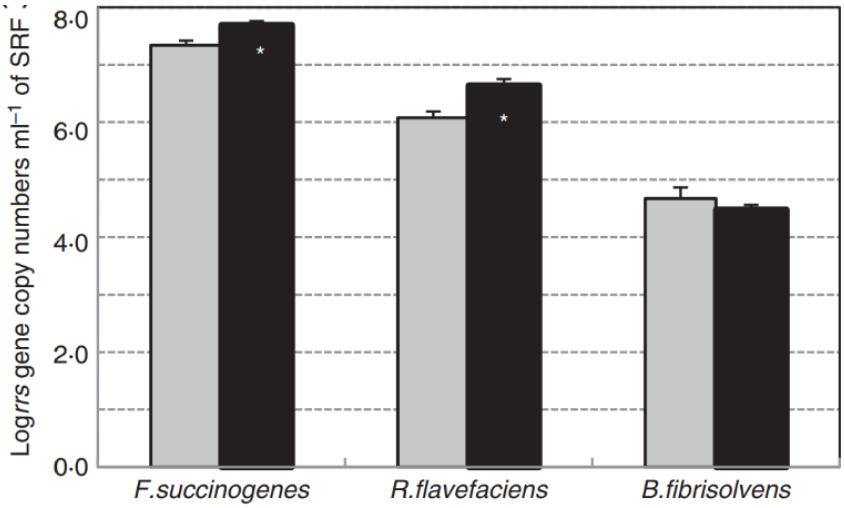


Figure 1.15: Abundance of fibrolytic bacteria in rumen following two feeding periods: control (□) and yeast supplement (■) (Adapted from Chaucheyras-Durand *et al*., 2016)

1.8.3 Health and production

Live yeast has been shown to improve rumen conditions due to an increase rumen pH (Desnoyers *et al*., 2009), increased growth and metabolism of fibrolytic bacteria (Chaucheyras-Durand *et al*., 2016) and reduced lactate concentration (Dias *et al*., 2018). These factors improve rumen and cattle health by increasing digestion, and reducing the risk of developing SARA (Chaucheyras-Durand *et al*., 2008).

There are many studies which have reported increased milk yield with yeast supplementation (Table 1.12). Dias *et al*. (2018) reported that despite no change to DMI, milk yield increased by 3.3 kg with yeast supplementation. This is supported by Desnoyers *et al*. (2009) who concluded that yeast supplementation increased milk yield by 1.2 kg. In addition to the positive correlation between milk yield and DMI, Kumprechtová *et al.* (2019) suggested that milk yield may also increase due to an increased VFA production in the rumen. Dias *et al*. (2018) hypothesised that the rumen stabilising effects of yeast would be most effective with high starch diets, however they concluded that the effects of yeast were independent to dietary starch level.

Table 1.12: Milk yield in dairy cows fed diets supplemented with live yeast

References	Control (kg/d)	Treatment (kg/d)	SEM	P value
Yalcin *et al*. (2011)	23.5	25.0	1.89	0.038
Jiang *et al*. (2017)	29.6	30.0	0.71	0.730
Dias *et al*. (2018)	26.4	29.7	1.30	0.010
Rossow *et al*. (2018)	32.3	33.0	0.41	0.001
Kumprechtová *et al*. (2019)	37.4	39.0	0.55	0.019

Desnoyers *et al.* (2009) reported that the effects of yeast on milk fat content or yield were inconsistent, whilst Kumprechtová *et al.* (2019), Ambriz-Vilchis *et al*. (2017) and Erasmus *et al*. (1992) were in agreement that yeast supplementation had little effect on milk fat content or yield. In contrast, Desnoyers *et al*. (2009) and Dias *et al*. (2018) concluded that milk fat yield and content increased with yeast supplementation by 0.15 kg/d and 0.05%, respectively. Milk fat content can increase with yeast supplementation as a result of the increase in fibrolytic activity as acetate, the by-product of fibre degradation is a precursor for milk fat production (Van Houtert, 1993). Dias *et al*. (2018) concluded that milk protein yield increased by 0.12 kg/d due to an increased amount of amino acids available in the small intestine due to an increased flow of non-microbial, non-ammonia nitrogen (NAN). However, on the whole many studies were in agreement that yeast supplementation had little or no effect on milk protein content or yield (Kumprechtová *et al*., 2019; Ambriz-Vilchis *et al*., 2017; Desnoyers *et al*., 2009).

1.9 Effect of *Yucca schidigera* extract on performance and rumen function

Yucca schidigera is a desert plant native to Mexico, that when harvested can be pressed and the resultant juice concentrated by evaporation (Cheeke, 2000). *Yucca schidigera* extract contains steroidal saponins which have natural detergent properties, and can commonly be used as a feed additive (Lovett *et al*., 2006). Steroidal saponins are a group of high molecular weight glycosides, with the saccharide chain units (1-8 residues) linked to a steroidal aglycone moiety (Patra and Saxena, 2009). The glycofraction has demonstrated NH_3 binding capabilities while the steroidal saponins have been recognised for their anti-protozoal and anti-bacterial properties (Wallace *et al*., 1994). Steroidal saponins form complexes with the cell walls of amylolytic and cellulolytic bacteria, disrupting membrane function and cell growth, reducing their numbers in the rumen (Wang *et al*., 2000). Saponins can also form irreversible complexes with cholesterol in protozoal cell membrane resulting in the breakdown of the cell membranes and ultimately cell lysis and death (Cheeke, 2000).

Wallace *et al*. (2004) reported that *Y. schidigera* supplementation increased the growth of *Prevotella ruminicola* while *Streptococcus bovis* growth was suppressed, and concluded that the antibacterial properties were most pronounced against gram positive bacteria. The defaunation properties of saponins are of interest as 25% of rumen methanogens live in association with protozoa (Newbold *et al*., 1997). The production of CH_4 is negatively correlated to energy utilisation and can increase greenhouse gas (GHG) pollution (Holtshausen *et al*., 2009). Holtshausen *et al*. (2009) reported conflicting results regarding the effect of saponins on protozoal activity and CH_4 production, with CH_4 production decreasing during *in vitro* experiments, while no change was found when carried out *in vivo*. It was suggested that these differences were due to changes in the basal ration, saponin dosage, and potential adaption of the microbiota in vivo (Lu and Jorgensen, 1985).

1.9.1 Dry matter intake and performance

There is little evidence to suggest that supplementing the diet with *Y. schidigera* has a positive effect on DMI. This is supported by Singer *et al*. (2008) and Śliwiński *et al.* (2004) who concluded that *Y. schidigera* supplementation had no effect on DMI in dairy cattle. In contrast Lovett *et al*. (2006) reported that the inclusion *Y. schidigera* decreased DMI by up to 1.2 kg DM/day, however, there was no effect on performance in dairy cows. It was suggested that *Y. schidigera* supplementation increased the duodenal flow of OM and therefore increased the proportion of OM digested post ruminally which may have maintained animal productivity (Lovett *et al*., 2006). Singer *et al*. (2008) and Śliwiński *et al.* (2004) also reported that performance was not affected by *Y. schidigera* supplementation.

1.9.2 Nitrogen metabolism and rumen volatile fatty acid production

Yucca schidigera has been reported to decrease the concentration of NH_3 in the rumen (Holtshausen *et al*., 2009; Singer *et al*., 2008; Śliwiński *et al.,* 2004). Wallace *et al*. (1994) reported that the effect of *Y. schidigera* supplementation on rumen NH_3 concentration may be twofold. The glyco-component of the saponin molecules bind to NH_3 therefore reducing the proportion available for ruminal interaction, and the high anti-protozoal activity can decrease the degree of proteolysis in the rumen (Wallace *et al*., 1994). A reduction in NH_3 may have subsequent negative effects on the rumen microbial population, particularly fibre degrading bacteria, due to a decrease in NH_3 available as substrate for microbial protein synthesis (Singer *et al*., 2008; Wang *et al*., 2000).

There have been several reports that supplementation of *Y. schidigera* had no effect on rumen VFA production, as it is commonly reported that DMI was not affected (Holtshausen

et al., 2009; Hristov *et al*., 1999). However, Lovett *et al*. (2006) concluded that *Y. schidigera* supplementation decreased total VFA and propionate concentration, despite no change in DMI, which was associated with a decrease in nutrient digestibility.

1.9.3 Nutrient digestibility

There are mixed reports regarding the effect of *Y. schidigera* supplementation on nutrient digestibility (Table 1.13). Śliwiński *et al*. (2004), Holtshausen *et al*. (2009) and Hristov *et al*. (1999) concluded that supplementation had no effect on DM, NDF or N whole tract digestibility. There may be a reduction in fibre digestibility in the rumen due to a decrease in cellulolytic bacteria populations, either by a decrease in rumen NH_3 concentrations or the antimicrobial effects of *Y. schidigera* (Singer *et al*., 2008; Wang, 2000). However, Patra and Saxena (2009) suggested that supplementation can change the site of digestion, and stated that while fibre digestibility decreased in the rumen it may increase in the hindgut. Goetsch and Owens *et al*. (1985) reported that *Y. schidigera* supplementation increased OM whole tract digestibility by 3.9%. Increasing the rate of passage can have a negative effect on the extent of digestion in the rumen by limiting the opportunity for particle degradation. Goetsch and Owens (1985) reported that these effects were not apparent during their study, and instead suggested OM digestibility was directly proportional to rumen fluid rate of passage. It was postulated that the results may however be different for cattle in peak lactation with a higher DMI, compared to the late lactation, restricted fed animals in their study (Goetsch and Owens, 1985).

Table 1.13: Effect of *Yucca schidigera* on nutrient digestibility in dairy cows

Reference	DM	OM	NDF	N
Goetsch and Owens (1985)	-	+ 3.1	-	NS
Hristov *et al*. (1999)	NS	-	NS	NS
Śliwiński *et al*. (2004)	-	NS	NS	NS
Holtshausen *et al*. (2009)	NS	-	NS	-

DM= Dry matter, OM= Organic matter, NDF= Neutral detergent fibre, N= Nitrogen

1.10 Knowledge gaps

A study carried out in the UK identified that 66% of dairy farms had poorly mixed rations and 58% displayed sorting behaviour 4 h after feed-out. Sorting can occur due to poorly mixed rations, a low forage to concentrate ratio or a long forage chop length. When cattle sort through their diet they generally favour concentrates over the forage portion of the ration, changing both the level and the pattern of intake of concentrates. When cattle increase their number of meals their DMI tends to remain the same, however, milk yield can increase by up

to 1.2 kg. With increased meals, mean rumen pH has been shown to decrease but remain more stable throughout the day. There is a less understood regarding the effect of pattern of concentrate allocation on the microbiome. Short-term feed restriction is relatively common on many dairy farms and has a negative effect on milk yield and rumen metabolism, and there is a knowledge gap surrounding the impact on the microbiome and interactions with rumen metabolism. Supplementation with yeast has been suggested to increase DMI, milk yield, and improve milk composition and rumen conditions, although little work has been undertaken on the effect of yeast supplementation on the microbiome. Reducing environmental emissions on dairy farms is a major priority within agriculture, the efficiency of N utilisation is often low if the diet is high in RDP which causes high levels of NH_3 excretion, and means to improve the efficiency of utilisation require further research. Supplementation with *Yucca schidigera* has been purported to reduce NH_3 emissions due to its antimicrobial properties, and further work needs to be conducted to assess its effects on the microbiome.

Experimental objectives

The main objectives of this thesis were:

1) To determine the effect of pattern of concentrate allocation when fed with or without supplementation of a live yeast on rumen metabolism, the microbiome, whole tract digestibility, and performance in high yielding dairy cows.
2) To determine the effect of short-term feed restriction and re-feeding on rumen metabolism, the microbiome and performance of high yielding dairy cows when fed different concentrate patterns and either with or without a live yeast.
3) To determine the effect of *Yucca schidigera* extract and a live yeast supplementation in diets high in rumen degradable protein on rumen metabolism, the microbiome, nitrogen balance, and performance of high yielding dairy cows.

2.0 General Methods

2.1 Dry matter (DM)

Forage and TMR samples were oven dried at 105°C for 24 hours to a constant weight according to AOAC (2012; 943.01). Faecal samples were oven dried at 60°C for 72 hours. Samples were cooled in a desiccator for 30 minutes and weighed. Dried samples were milled using a cyclone mill (Cyclotec, FOSS, Warrington, UK) through a 1 mm screen prior to subsequent analysis.

$$\text{DM (g/kg)} = \frac{\text{Dried sample weight (g)}}{\text{Initial sample weight (g)}} \text{ X } 1000$$

Equation 1

2.2 Crude protein (CP)

Crude protein of dried forage, TMR, and faecal samples were determined using Leco FP-528 (Leco Corporation, Stockport, UK) with the Dumas method according to AOAC (2012; 988.05). Approximately 0.15 g of dried, ground sample were weighed into aluminium foil cups and placed into the autoanalyser. The CP calculation used was:

$$\text{CP (g/kg DM)} = \text{Total nitrogen (g/kg DM)} \times 6.25$$

Equation 2

2.3 Total nitrogen of urine and faeces

Total nitrogen of urine and slurry samples was determined using the Kjeldahl method, according to MAFF (1986). Samples of 1 ml of thawed urine, or 5 g of slurry, was pipetted into a distillation tube with 2 Kjeldahl tablets (KT-211-A, Missouri Tablet, AMPCS, Essex, UK) that were folded up in filter paper (150 mm Whatman number 1). The tubes were placed in a tube rack and 15 ml of concentrated sulphuric acid was added to each tube. The rack was then placed on a digestion block and the Turbosog suction system (Gerhardt analytical systems, Königswinter, Germany) was placed on top to remove fumes, and heated from 175°C to 425°C, while being carefully observed to ensure the acid did not over boil. Once at 425°C the samples were boiled for 45 minutes and then the rack was left to cool for 10 minutes. Then 75 ml of distilled water was added to each tube and the samples left to cool in the fume cupboard overnight. The following day samples were analysed for nitrogen content (g/kg) on a Kjeltec autoanalyser (FOSS, Warrington, UK).

2.4 Ash and organic matter (OM)

The ash content of forage, TMR and faecal samples was determined by combustion at 550°C (AOAC, 2012; 942.05). Approximately 2 g of dried, ground sample was weighed into a pre-weighed porcelain crucible. Samples were placed in a muffle furnace (Carbolite AAF 1100, Hope Valley, England) at 550°C for 4 hours, then cooled in a desiccator for 30 minutes and weighed. The ash and OM calculations used were:

$$\text{Ash (g/kg DM)} = \frac{\text{Ashed Weight (g)}}{\text{Initial Sample weight (g)}} \times 1000$$

Equation 3a

$$\text{OM (g/kg DM)} = 1000 - \text{Ash (g/kg DM)}$$

Equation 3b

2.5 Neutral detergent fibre (NDF)

Neutral detergent fibre content of forage, TMR and faecal samples was determined using the procedure described by Van Soest *et al.* (1991) using Fibertec (1020, FOSS, Warrington, UK), and expressed exclusively of residual ash. Approximately 0.5 g of dried ground sample was weighed into a glass crucible (porosity 1, Soham Scientific, Ely, UK). The crucibles were tightly fitted into the Fibertec and 25 ml of NDF reagent (described in Appendix 8.1) and 0.5 ml of octan-1-ol were added to each crucible.

Samples were boiled and digested for 30 minutes, and an additional 25 ml of NDF reagent, 2 ml of alpha amylase and 0.5 g of sodium sulphite were added to each crucible. Samples simmered for another 30 minutes, then were filtered and washed three times with 25 ml of hot distilled water (80°C) to remove NDF reagent. An additional 2 ml of alpha amylase and 25 ml of hot distilled water were added and left to stand for 15 minutes. The samples were filtered again three times with hot distilled water. Crucibles were removed from the Fibertec and oven dried at 105°C overnight, then cooled in a desiccator and weighed. Crucibles were then placed in a muffle furnace (Carbolite AAF 1100, Hope Valley, England) at 550°C for four hours and then cooled in a desiccator and reweighed. The NDF calculation used was:

$$\text{NDF (g/kg DM)} = \frac{\text{Dried weight (g)} - \text{Ashed weight (g)}}{\text{Intial sample weight (g)}} \times 1000$$

Equation 4

2.6 Forage pH

Forage pH was determined according to MAFF (1986). Approximately 50 g of forage was placed in a beaker with 125 ml of distilled water; samples were then stirred every 15 minutes for one hour. A Jenway 3505 pH probe and meter (Bibby Scientific Limited, Staffordshire, UK) was calibrated using pH 4 and 7 buffers and the pH of the water extract of the silage measured.

2.7 Milk composition

Milk samples were analysed by National Milk Laboratories (Wolverhampton, UK) by near midinfrared (MIR) for fat, protein, urea N and lactose.

2.8 Blood analysis

Blood samples were collected by venipuncture via the jugular vein using fluoride/oxalate (grey) for glucose and lithium heparin (green) for urea, β-hydroxybutyrate (BHB) and NH_3 vacutainers (BD Vacutainer, Plymouth, UK). Plasma ammonia analysis was conducted within 30 minutes of sampling and the rest of the plasma was stored at -20°C for subsequent analysis (Sinclair *et al*., 2012). Plasma samples were analysed using a Cobas Mira Plus autoanalyser (ABX Diagnostics, Bedfordshire, UK) for glucose, beta hydroxybutyrate (BHB), urea and ammonia. The kits used were: GLUC-HK, ref GU611; RANBUT, ref RB1008; UREA, ref UR221; and NH3, ref AM1015, respectively.

2.9 Diet digestibility

Subsamples from the total faecal collection were dried at 60°C for 72 hours then milled in a coffee grinder. Samples for the five days were bulked within cow per period, and analysed for DM, CP and NDF.

$$\text{DM digestibility (g/kg DM)} = \frac{\text{DMI (kg)} - \text{Daily faecal output (kg DM)}}{\text{DMI (kg)}} \times 1000$$

Equation 5a

$$C\text{P digestibility (g/kg DM)} = \frac{\text{CP intake (kg DM)} - \text{CP output (kg DM)}}{\text{CP intake (kg DM)}} \times 1000$$

Equation 5b

$$\text{NDF digestibility (g/kg DM)} = \frac{\text{NDF intake (kg DM)} - \text{NDF output (kg DM)}}{\text{NDF intake (kg DM)}} \times 1000$$

Equation 5c

2.10 Nitrogen Balance

Nitrogen balance was calculated using the digestibility results measured by Section 2.9.

$$UrineN\ of\ total\ faecal\ N\ (g/kg) = \frac{Urine\ N\ (g/d)}{Urine\ N\ (g/d) + Faecal\ N\ output\ (g/d)} \times 100$$

Equation 6a

$$N\ use\ efficiency\ (g/kg) = \frac{Milk\ N\ (g/d)}{N\ Intake\ (g/d)} \times 100$$

Equation 6b

2.11 Rumen fluid collection

Rumen fluid samples were collected using a method adapted from Martin *et al*. (1999). Four grab samples of digesta were taken from the ventral region of the rumen, by inserting the arm directly down approximately 50 cm through the cannula and grabbing two handfuls of digesta which was then placed into a bucket. Further fluid was collected with a 250 ml glass bottle from the same region of the rumen. The rumen fluid was then strained through four layers of muslin cloth to separate the solid digesta from the liquid. Liquid samples were stored in a 25% HPO_3 solution at -20°C for subsequent analysis of VFA and ammonia (NH_3). Rumen fluid and digesta samples were stored in 15% glycerol solution at -20°C for subsequent microbial community analysis. Liquid samples were stored in a 10% formalin solution at -20°C for subsequent analysis of protozoa identification and counting using a microscope.

2.12 Rumen pH

Rumen pH was recorded immediately from the strained rumen fluid samples after collected from the rumen using a portable pH meter calibrated using pH 4 and 7 buffer (Hanna Instruments, Bedfordshire, UK).

2.13 Volatile fatty acid (VFA) analysis

Volatile fatty acid analysis was carried out on forage and rumen fluid samples. Forage VFA concentrations were prepared as described by Wiseman and Irvin (1957) and rumen VFA concentrations determined using procedures described by Erwin *et al*. (1961) by gas chromatography (GC).

Forage, samples were prepared by weighing 50 g into a beaker with 250 ml of distilled water and stirring every 15 minutes for one hour. The sample was then filtered using filter paper (150 mm Whatman number 1). Five ml of sample and 1 ml of 25% metaphosphoric acid

(HPO_3) were pipetted into a 10 ml centrifuge tube and left to stand for 30 minutes. The samples were then centrifuged at 4,000 x*g* for 20 minutes. Then 1 ml of the supernatant was pipetted into a GC vial with 100 µl of internal standard (IS; 0.2% 2-methylvaleric acid; 2ml of 2-methylvaleric acid mixed with 1000 ml deionised water) and run on the GC.

For rumen fluid analysis, approximately 2 ml of strained rumen fluid was centrifuged at 10,000 x*g* for 10 minutes at 4°C. Then 1000 µl of the supernatant was pipetted into the GC vial with 100 µl of internal standard (IS; 0.2% 2-methylvaleric acid, 2ml of 2-methylvaleric acid mixed with 1 L deionised water).

The VFA analysis was conducted on a 6890 Agilent technologies GC using a DBFFAP column (30 m x 0.250 mm x 0.2 µm; Agilent J and W, GC columns, Cheadle, UK) and a flame ionization detector (Agilent Inc. Wilmington, DE). The GC conditions were: carrier gas nitrogen; flow rate 2.7 ml/min; column pressure 11.72 psi; split ratio 30:1; maximum oven temperature 235°C; temperature programmed on 60 – 200°C (20°C/min, 10 min), injector temperature 250°C; detector temperature 300°C. To remove particles of dirt, a glass wool liner was placed in the injector.

An external standard solution (acetate, propionate, iso-butyrate, butyrate, iso-valerate, and valerate) and internal standard were run before the samples to ensure consistency between readings. The internal response factor was used to quantify VFA with the following equations:

$$\text{Internal response factor (IRF)} = \frac{\text{IS area} \times \text{specific VFA amount (µM)}}{\text{IS amount (µM)} \times \text{specific VFA area}}$$

IS = Internal standard Equation 7a

$$\text{Amount of specific VFA (µM)} = \frac{\text{IS amount (µM)} \times \text{specific VFA area} \times \text{IRF of specific VFA}}{\text{IS area}}$$

Equation 7b

2.14 Lactate analysis

Lactate analysis was conducted on rumen fluid samples by high performance liquid chromatography (Agilent 1100, Germany) using a Rezex ROA-Organic column (Phenomenex, Macclesfield, UK). Strained rumen fluid samples were centrifuged for 10 minutes at 10,000 x*g* and the supernatant filtered through a 0.45 nm syringe filter (Fisher Scientific UK Ltd., Loughborough, UK). The standard solutions of lactic acid (Sigma, Gillingham, UK) used had concentrations of: 0.007, 0.015, 0.0313, 0.0625, 0.125, 0.25, 0.5, 1 mM. One ml of filtered sample or the standard solution was pipetted into each vial and ran

on the HPLC. The column dimensions were 300 x 7.8 mm, and the mobile phase was 0.005N H_2SO_4, with a flow rate 0.5 ml/min, maximum pressure 400 bar, injector temperature of 55°C, detector temperature of 40°C, and the detection level was UV 210 nm.

2.15 Ammonia nitrogen

Ammonia N analysis was conducted on forage, rumen fluid and slurry samples using a method adapted from MAFF (1986) and an auto-titrator (FOSS 1030 auto-titrator, FOSS, Warrington, UK; Buchi Labortechnik AG CH-9230, Flawil, Switzerland).

For the forage analysis samples were prepared by weighing 50 g of forage into a beaker with 250 ml of distilled water and stirring every 15 minutes for one hour. The sample was then filtered using filter paper (150 mm Whatman number 1) and 5 ml of filtrate was transferred to a kjeldahl digestion tube. Additionally, 6 ml of magnesium oxide (17 g of heavy magnesium oxide dissolved in 100 ml distilled water) was added, and the sample analysed by auto-titration.

Rumen fluid samples were prepared by centrifuging strained rumen fluid at 10,000 x*g* for 10 minutes at 4°C, and 5 ml of filtrate transferred to a kjeldahl digestion tube with 6 ml of magnesium oxide, and the sample was analysed by the auto-titrator.

Slurry samples were prepared by centrifuging the slurry at 8,000 x*g* for 12 minutes at 4°C, and 0.5 ml of filtrate and 6 ml of magnesium oxide were added into a kjeldahl digestion tube, and analysed by auto-titration.

The ammonia N content of forage was calculated as:

$$\text{Ammonia N (g/kg DM)} = \frac{7 \times \text{T} \times (120 - (0.02 \times \text{DM g/kg}))}{10 \times \text{DM g/kg}}$$

Equation 8a

The ammonia N content of rumen fluid was calculated as:

$$\text{Ammonia N (mg/L)} = \frac{\text{T}}{0.005} \times 0.14$$

Equation 8b

The ammonia N content of slurry was calculated as:

$$\text{Ammonia N (mg/L)} = \frac{\text{T}}{0.0005} \times 0.14$$

Where T = titre reading – blank Equation 8c

2.16 Measurement of rumen microbial community composition using Illumina MiSeq amplicon sequencing of 16S ssrRNA gene

2.16.1 Solid phase digesta (SPD) sample preparation

Preparation for DNA extraction of the solid phase digesta (SPD) samples was based on the method of Ramos *et al*. (2009). Saline solution (0.9% w/v 0.7 L per sample; Appendix 8.1) was made up using dH_2O. Digesta samples were thawed overnight and transferred into a 500 ml beaker with 300 ml of saline solution. The sample was then gently mixed and strained using a sieve to remove excess microbiota associated with the liquid phase digesta (LPD). This process was repeated again with 300 ml of saline solution. The strained sample was transferred into a Stomacher® bag (Seward, West Sussex, UK) and 100 ml of saline solution added. The sample was then homogenised in a Stomacher® 400 Circulator (Seward, West Sussex, UK) for 5 minutes at 230 rpm. Then 20 ml of the supernatant was pipetted into a 30 ml Nalgene centrifuge tube and was centrifuged at 8,500 x*g* for 20 minutes. The majority of the supernatant was aspirated off leaving approximately 0.5 ml to re-suspend the pellet, which was transferred into a 2 ml screwcap tube containing sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm; Biospec Products, USA), ready for DNA extraction (Section 2.16.3).

2.16.2 Liquid phase digesta (LPD) sample preparation

For LPD samples 5 ml of strained rumen fluid was pipetted into a Nalgene centrifuge tube and was centrifuged at 8,500 x*g* for 20 minutes. The majority of the supernatant was aspirated off leaving approximately 0.5 ml to re-suspend the pellet, which was transferred into a 2 ml screwcap tube containing sterile zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm), ready for DNA extraction (Section 2.16.3).

2.16.3 DNA extraction

Extraction of DNA from both SPD and LPD samples was carried out following a protocol based on Yu and Morrison (2004). One ml of lysis buffer (Appendix 8.1) was added to the 2 ml screwcap tubes containing the pellet of sample and sterile zirconia beads).

Tubes were placed in a Mini-Beadbeater-16 (Biospec Products, USA) for six cycles of 30 seconds bead beating followed by 2 minutes of cooling. Samples were incubated at 70°C for 15 minutes then centrifuged at 13,000 x*g* for 5 minutes. Approximately 800 μl of the supernatant was pipetted into a 2 ml centrifuge tube and an additional 300 μl of lysis buffer

was added to the lysate. The bead beating, incubation and centrifugation process was repeated and an additional 400 μl of supernatant was added to the 2 ml tubes.

To each centrifuge tube 260 μl of 10 M ammonium acetate was added, mixed well then incubated at -20°C for 5 minutes, before being centrifuged at 13,000 x*g* for 10 minutes. The supernatant was then split between two 1.5 ml centrifuge tubes (500 μl in each) and 500 μl of isopropanol was added and the samples were incubated at -20°C for 30 minutes then centrifuged at 13,500 x*g* for 15 minutes at 4°C, and the supernatant removed by aspiration. The remaining nucleic pellet was washed with 500 μl of 70% v/v ethanol/ dH_2O, vortexed and centrifuged at 13,000 *xg* for 5 minutes, then the supernatant was aspirated and discarded and the pellet dried in a hood at room temperature for approximately 30 minutes. Nucleic acid pellets were dissolved in 100 μl of TE buffer and the two aliquots pooled.

The RNA was removed by adding 2 μl of DNase-free RNase (10 mg/ml), vortexing and incubation at 37°C for 15 minutes. Protein was removed by adding 15 μl of Proteinase K and 200 μl of AL buffer from QIAamp DNA Stool Mini kit (QIAGEN Ltd., Manchester, UK) vortexing and incubation at 70°C for 10 minutes. Ethanol (200 μl at 70% v/v) was added and mixed, then the total volume of the sample was transferred into a QIAamp column and centrifuged at 13,000 x*g* for one minute. The flow through was discarded and 500 μl AW1 buffer was added and centrifuged at 13,000 x*g* for one minute. This step was repeated with AW2 buffer. The columns were then placed in a fresh dry collection tube and centrifuged at 13,000 x*g* for one minute. The ATE elusion buffer (100 μl) was added and left at room temperature for two minutes. The columns were then placed in a 1.5 ml centrifuge tube and centrifuged at 13,000 x*g* for one minute to elute the DNA. Samples were quantified using a NanoDrop Spectrophotometer (Thermo Fisher Scientific UK Ltd., Loughborough, UK) to assess yield and quality, then stored at -20°C until further analysis.

2.16.4 Polymerase chain reaction (PCR)

2.16.4.1 PCR optimisation

The PCR amplification of the 16S ss rRNA gene was carried out using a protocol based on Kozich *et al*. (2013). Samples were prepared in 25 μl reaction volumes in triplicate in 200 μl PCR tubes (Sarstedt, Nümbrecht, Germany). DNA polymerase (Q5 ® High-Fidelity DNA polymerase; New England Biolabs Inc., Hitchin, UK), buffers, and dNTP were supplied as part of the Q5 ® High Fidelity DNA polymerase kit. Primers based on Kozich *et al*. (2013) were designed to amplify the V4 hypervariable region of the 16S ss rRNA gene and contained sequences to allow annealing of the amplicons to the flow cell as well as 8 nt

index sequences to identify individual libraries after pooling prior to sequencing (Table 2.1; Table 2.2; Table 2.3).

The generic primer design was:

AATGATACGGCGACCACCGAGATCTACAC <i5><pad><link><16Sf> VX.N5

CAAGCAGAAGACGGCATACGAGAT <i7><pad><link><16Sr> VX.N7

Table 2.1: Forward and reverse primer barcode sequences

i5 Barcode	Sequence	i7 Barcode	Sequence
SA501	ATCGTACG	SA701	AACTCTCG
SA502	ACTATCTG	SA702	ACTATGTC
SA503	TAGCGAGT	SA703	AGTAGCGT
SA504	CTGCGTGT	SA704	CAGTGAGT
SA505	TCATCGAG	SA705	CGTACTCA
SA506	CGTGAGTG	SA706	CTACGCAG
SA507	GGATATCT	SA707	GGAGACTA
SA508	GACACCGT	SA708	GTCGCTCG
SB501	CTACTATA	SA709	GTCGTAGT
SB502	CGTTACTA	SA710	TAGCAGAC
SB503	AGAGTCAC	SA711	TCATAGAC
SB504	TACGAGAC	SA712	TCGCTATA

Table 2.2: Forward and reverse primer pad and link sequences

Pad 16Sf V4 (Forward)	Pad 16Sr V4 (Reverse)	Link V4f (Forward)	Link V4r (Reverse)
GTGCCAGCMGCCGCGGTAA	GGACTACHVGGGTWTCTAAT	GT	CC

Table 2.3: Forward and reverse primer sequence

Name	Sequence
Forward	
v4.SA501	AATGATACGGCGACCACCGAGATCTACACATCGTACGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA502	AATGATACGGCGACCACCGAGATCTACACACTATCTGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA503	AATGATACGGCGACCACCGAGATCTACACTAGCGAGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA504	AATGATACGGCGACCACCGAGATCTACACCTGCGTGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA505	AATGATACGGCGACCACCGAGATCTACACTCATCGAGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA506	AATGATACGGCGACCACCGAGATCTACACCGTGAGTGTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA507	AATGATACGGCGACCACCGAGATCTACACGGATATCTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SA508	AATGATACGGCGACCACCGAGATCTACACGACACCGTTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SB501	AATGATACGGCGACCACCGAGATCTACACCTACTATATATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SB502	AATGATACGGCGACCACCGAGATCTACACCGTTACTATATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SB503	AATGATACGGCGACCACCGAGATCTACACAGAGTCACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
v4.SB504	AATGATACGGCGACCACCGAGATCTACACTACGAGACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA
Reverse	
v4.SA701	CAAGCAGAAGACGGCATACGAGATAACTCTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA702	CAAGCAGAAGACGGCATACGAGATACTATGTCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA703	CAAGCAGAAGACGGCATACGAGATAGTAGCGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA704	CAAGCAGAAGACGGCATACGAGATCAGTGAGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA705	CAAGCAGAAGACGGCATACGAGATCGTACTCAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA706	CAAGCAGAAGACGGCATACGAGATCTACGCAGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA707	CAAGCAGAAGACGGCATACGAGATGGAGACTAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA708	CAAGCAGAAGACGGCATACGAGATGTCGCTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA709	CAAGCAGAAGACGGCATACGAGATGTCGTAGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA710	CAAGCAGAAGACGGCATACGAGATTAGCAGACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA711	CAAGCAGAAGACGGCATACGAGATTCATAGACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA712	CAAGCAGAAGACGGCATACGAGATTCGCTATAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT

To each 200 µl PCR tube 15.75 µl molecular water, 1.25 µl forward i5 primer (Table 2.3), 1 µl template DNA, and 7 µl master mix (Table 2.4). To ensure sufficient reagent for all samples an additional six reactions of master were prepared for every 36 reactions. A negative control containing all reagents except the template DNA was also prepared.

Table 2.4: Mastermix components for 25 µl PCR reaction

Component	Volume/reaction
Buffer	5 µl
dNTP	0.5 µl
Reverse i7 primer	1.25 µl
Taq polymerase	0.25 µl

The PCR tubes were placed in a thermal cycler (BIO-RAD Laboratories, USA) with the conditions shown in Table 2.5. Steps 2 – 4 were repeated for 19 times for a total of 20 cycles.

Table 2.5: Initial PCR cycle program

Step	Temperature (°C)	Time (minutes: seconds)
1	98	2:00
2	98	0:30
3	50	0:30
4	72	1:30
5	72	5:00
6	10	Infinite

Amplicons were visualised by gel electrophoresis using a 1% agarose gel (with Tris Acetate EDTA (TAE) and 3 µl DNA stain (Biotium, USA)) through TAE buffer at 120 v/cm for one hour. To determine the presence of the amplified DNA bands the gel was placed in a trans-illuminator (Carestream, NY, USA) and viewed under UV light.

Initially the results seemed acceptable, then the bands of DNA in the gel became weak and unclear, often showing no bands at all despite accurate pipetting and using fresh reagents. There were two possible reasons why the process was not working: lack of apparent amplification or poor quality gel. The original method was therefore adapted by changing the PCR program to exactly follow Kozich *et al*. (2013) increasing the number of cycles to 30, using freshly prepared primers, using 1% Tris Borate EDTA (TBE) buffer instead of 1% TAE, and with lower voltage (60 v/cm), these changes improved the quality of the results (Figure 2.1). The final method is described in full in Section 2.16.4.2.

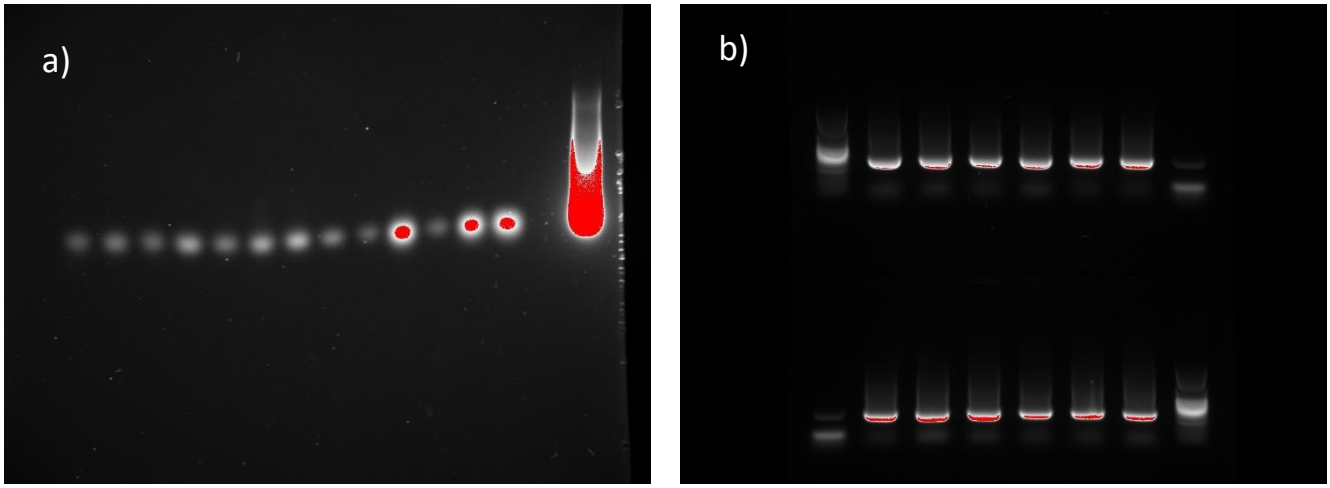


Figure 2.1: Comparison of a) original PCR protocol with b) optimised PCR protocol

2.16.4.2 PCR

Reactions were prepared in triplicate in 200 µl PCR tubes, with the addition of 15.75 µl molecular water, 5 µl Q5 buffer, 1.25 µl forward primer, 1.25 µl reverse primer, 1 µl template DNA, 0.5 µl dNTP, 0.25 µl Q5 ® High-Fidelity DNA polymerase (New England Biolabs Inc., Hitchin, UK). A negative control containing all reagents except the template DNA was also prepared.

The PCR tubes were placed in a thermal cycler (BIO-RAD Laboratories, USA) with the conditions shown in (Table 2.6), steps 2 – 4 were repeated 29 times for a total of 30 cycles.

Table 2.6: Final PCR cycle program

Step	Temperature (°C)	Time (minutes: seconds)
1	95	2:00
2	95	0:20
3	55	0:15
4	72	5:00
5	72	10:00
6	10	Infinite

2.16.4.3 Gel electrophoresis

Amplicons were visualised by gel electrophoresis using a 1% agarose gel (with TBE) through TBE buffer at 6 v/cm for 1 h. For a gel consisting of 8 – 16 wells 0.35 g of agarose was dissolved in 35 ml of TBE buffer and melted by heating briefly in a microwave. Once the gel was cooler 3 µl of DNA stain (Gel Red ®, Biotium, USA) was added and the gel was poured into the mould. When the gel had set it was transferred into a gel tank containing enough TBE buffer to fully cover the gel. The PCR product (8 µl) was combined with 2 µl of

loading dye (New England Biolabs Inc., Hitchin, UK) was pipetted into each well, in addition to the negative control and a 50 kb ladder (Invitrogen, Fisher Scientific UK Ltd., Loughborough, UK). The gel was subjected to electrophoresis, with an electric current (6 v/cm) with the DNA migrating towards the positive terminal. To determine the presence of the amplified DNA bands the gel was placed in a trans-illuminator (Carestream, NY, USA) and viewed under trans UV light (Figure 2.1).

2.16.4.4 DNA precipitation and concentration

For clean-up by ethanol precipitation, 19.5 µl 1M NaCl and 170 µl 70% ethanol were added to each centrifuge tube and stored at -20°C until the next step (minimum of 24 h). Samples were then centrifuged at 14,000 x*g* for 20 minutes, and the supernatant gently aspirated off and discarded. Next, 600 µl of 70% ethanol was added and the samples centrifuged for a further 15 minutes at 14,000 x*g* at 4°C. The supernatant was aspirated off and the samples were left to dry at room temperature for approximately 1 h or until the pellet had dried and turned opaque white. The pellet was then re-suspended in 30 µl of TE buffer and stored at 4°C.

Samples were quantified using the Quant-It PicoGreen high sensitivity dsDNA assay kit (Invitrogen, Fisher Scientific UK Ltd., Loughborough, UK) on a qPCR BIO-RAD C1000 Touch Thermal cycler (BIO-RAD Laboratories, USA). To make the standard curve, concentrations were prepared using the lambda DNA standard (Invitrogen, Fisher Scientific UK Ltd., Loughborough, UK) and TE buffer (Table 2.7), with 1 µl of sample DNA and 99 µl of TE buffer pipetted into the wells on the PCR plate. The plate was run on the qPCR block with the conditions: warmup and equilibrate for 5 minutes at 24°C and run for 30 seconds at 24°C, followed by a scan of fluorophore emissions. Measurements were recorded as relative fluorescent units (RFU) and lambda DNA standard used to calculate concentrations of each of the libraries. Samples were pooled in equimolar concentrations into a centrifuge tube, up to a maximum of 10 µl.

Table 2.7: Standard curve for DNA quantification

Concentration (ng/ml)	Lambda Standard (µl)	TE buffer (µl)
1000	100	0
800	80	20
750	75	25
600	60	40
500	50	50
250	25	75
100	10	90
0	0	100

The pooled sample then underwent clean-up by gel purification using the Wizard ® SV Gel and PCR Clean-up System Kit (Promega UK, Southampton, UK). The pooled amplicon libraries were run on a 1% w/v agarose TBE electrophoresis gel, under the same conditions as described previously, in 8 wells with 12 µl of pooled PCR product added per well. Following electrophoresis, the gel was examined under UV light and the bands containing the amplicons were excised with a sterile scalpel blade and placed into pre-weighed centrifuge tubes. The tubes were reweighed and the difference calculated to determine the weight of the gel. Membrane binding solution from the gel clean up kit was added to each tube at a rate of 10 µl per 10 mg of gel. The samples were then vortexed and incubated on a heat block at 65 °C for 10 minutes, or until the gel had dissolved. Each dissolved sample was pipetted individually into the SV Mini-column assembly (filter column plus collection tube) and left to bind for 1 minute before centrifugation at 16,000 x*g* for 1 minute; the liquid in the collection tube was discarded. This was repeated for every gel sample into the same SV Mini-column assembly. To wash the column, 700 µl of membrane wash solution was added, and centrifuged for 1 minute at 16,000 x*g*. The liquid from the collection tube was discarded. A further 500 µl of Membrane Wash Solution was added then the sample was centrifuged for 6 minutes at 16,000 x*g*. The filter column was placed into a clean tube and 60 µl of Nuclease Free Water was added, incubated at room temperature for 1 minute and then centrifuged for 1 minute at 16,000 x*g* to elute the cleaned DNA. The DNA was split equally between two labelled centrifuge tubes and stored at -20°C. The samples were quantified again using PicoGreen, and were then ready for sequencing.

2.16.5 Sequencing

The libraries were quality assessed using an Agilent 2100 Bioanalyzer System (Agilent Technologies. Santa Clara, CA, US) and sequenced using the Illumina MiSeq v2 250 paired end reagent kit to yield 11M sequences (Illumina UK, Cambridge, UK.).

2.16.6 Bioinformatics quality control

The stages of quality control which were undertaken during the bioinformatics are described in Table 2.8.

Table 2.8: Quality control stages for bioinformatics using mothur v.1.44.0

Stage
Assemble paired end reads from fastq files
Summary statistics of assembled sequences (summary seqs and count groups)
Screening for maximum/minimum sequence length, ambiguities and homopolymer runs
Generate unique sequence file
Align unique sequences
Screen unaligned seqs and filter gaps
Precluster to remove sequences likely formed from pyrosequencing errors
Remove singletons
Removal of chimera (UCHIME; Edgar *et al.*, 2011)
Taxonomic classification (reads) using Silva 132 SEED (Yilmaz *et al*., 2014)
Subsampling to normalise data
Generate operational taxonomic units (OTU)
Cluster (Average neighbour; Westcott and Schloss, 2017)
Taxonomic classification (OTU)
OTU representative sequence
Calculate alpha and beta diversity
Statistical analysis (AMOVA) and LEfSe (Segata *et al*., 2011)

2.16.7 Protozoa

The relative abundance of ciliate protozoa was carried out using a light microscope (Olympus CX31, Olympus, Tokyo) with a 10X objective and 10X/20 eyepiece.

The rumen protozoa sample was mixed briefly to resuspend the pellet, then a drop was placed onto a microscope slide and stained with methyl blue. Identification of the two major taxonomic families: *Isotrichidae* and *Ophryoscolecidae* was based on Imai (1998). Cells were counted at five different locations on the slide to ensure a representative selection of ciliates and the relative abundance was calculated.

Chapter 3: Effect of pattern of concentrate allocation when fed with or without supplementation of a live yeast on the performance, digestibility, rumen metabolism and microbiome in high yielding dairy cows

3.1 Introduction

Dairy cows are commonly fed a total mixed ration (TMR) as the diet composition can be controlled more easily to ensure cows receive the correct balance of nutrients (March *et al*., 2014). Despite one of the benefits of TMR being to provide a consistent diet, 58% of UK dairy farms have been reported to have suboptimal or poorly mixed rations with 66% having significant diet selection (Tayyab *et al*., 2018). When cows sort through the TMR they change both their level and pattern of concentrate intake (Leonardi and Armentano, 2003), therefore changing the forage to concentrate ratio (Yang *et al*., 2001; Lawrence *et al*., 2015; Van Wyngaard *et al*., 2018). As a consequence, some cows may consume excess concentrates, increasing the risk of developing subacute ruminal acidosis (SARA), which has been defined as periods of pH depression to less than pH 5.8 lasting more than 5 to 6 h/d (Zebeli *et al*., 2012). Excess concentrate intake increases microbial activity resulting in increased volatile fatty acid (VFA) and lactate production, causing a decrease in rumen pH (Zebeli *et al*., 2012). At a low pH, lactate utilising bacteria such as *Selenomonas ruminantium* and fibre degrading bacteria such as *Fibrobacter succinogenes* are inhibited, and there is a reduction in nutrient digestibility and rate of passage (DeVries *et al*., 2008). Consequently, dry matter intake (DMI) may be reduced which may compromise performance (Plaizier *et al*., 2008).

The use of active dry yeasts is common in ruminant nutrition as they have been reported to reduce the variability of rumen pH by scavenging excess oxygen and reducing the lactate concentration in the rumen by competing with other lactate producers such as *Streptococcus bovis* for substrate availability. In addition, yeast can stimulate growth of lactate utilising bacteria such as *Megasphaera elsdenii* and *Selenomonas ruminantium*, stabilising conditions for microbial activity, particularly fibrolytic bacteria (Newbold *et al*., 1996; Chaucheyras-Durand *et al*., 2016). Yeast can also increase DMI and milk yield (Barrera *et al*., 2019) but their effects on rumen metabolism and the microbiome when fed with varied patterns of concentrates is unclear.

The objective of the study was to determine the effect of pattern of concentrate allocation when fed with or without supplementation of a live yeast on rumen metabolism, the rumen microbiome, performance, and whole tract digestibility in high yielding dairy cows.

3.2 Materials and Method

The procedures for the animals used were conducted in accordance with the UK Animals Scientific Procedures Act (1986; amended 2012) and were approved by the local ethics committee at Harper Adams University.

3.2.1 Animals and experimental design

Four Holstein-Friesian dairy cows that had previously been fitted with a 10 cm permanent rumen cannula (Bar Diamond, Idaho, USA) were used. The cows were 69 days (SE ±12.1) post-calving, weighed 650 kg (± 26.2), were in their second lactation, and were yielding 40 kg (±2.6) of milk per day at the start of the study. Each cow was randomly assigned to one of four dietary treatments and remained on study for 20 weeks (140 days) with five week periods. For the first four weeks of each period the cows were group housed in a pen bedded with sawdust (10 x 4.5 m) with a rubber matted area (10 x 3 m) in front of the feed barrier which was manually scraped out twice a day. In the fifth week of each period the cows were housed in individual metabolism stalls fitted with mattresses for five days for rumen, faecal and urine sample collection. Cows had continuous access to water at all times.

The experimental design was a 2 x 2 factorial Latin square, with two patterns of concentrate feeding, either with or without the inclusion of yeast (Yea-Sacc®, Alltech UK). During the final two weeks of each period performance, digestibility, rumen metabolism and microbiome measurements were undertaken.

3.2.2 Forages and diets

All cows were fed a partial mixed ration (PMR; Table 3.1) with a forage: concentrate ratio of 60:40 (DM basis), and a grass silage: maize silage ratio of 45:55 (DM basis) via individual Calan gates (American Calan, Northwood, NH, USA) at a rate of 105% of the previous recorded intake at approximately 0730 h, with refusals collected three times a week (Monday, Wednesday and Friday). An additional 4 kg/cow/d of concentrates (

Ingredient	kg DM/d	kg fresh/d
Maize silage	7.5	21.5
Grass silage	6.2	19.7
Concentrate	9.3	10.6
Total	23.0	51.8
Predicted chemical analysis, g/kg DM	Total diet	Partial mixed ration
Forage:concentrate (DM basis)	0.60	0.70
ME, MJ/kg DM	11.7	11.5

Crude protein	162	153
NDF	363	389
Sugar	59	49
Starch	206	195
Rumen degradable starch	178	169
Oil	40	39
MPE1, g/kg DM	105	
MPE2, % requirements	103	
MPN3, g/kg DM	114	
MPN,% requirements	112	

[1]ME, metabolisable energy

[2]MPE, metabolisable protein-rumen energy limited

[3]MPN, metabolisable protein-rumen nitrogen limited

Table 3.2) was provided, to reflect the potential range of concentrate intake due to diet selection (Tayyab *et al*., 2018), in one of two patterns of allocation: uneven (U) with all 4 kg provided at 0600 h, or even (E), with the concentrates provided in four equal meals of 1 kg at 0600, 1000, 1400 and 1700 h. In addition, each diet was either supplemented (+) or unsupplemented (–) with Yea-Sacc® (Alltech UK) at a rate of 1 g/cow/day, which was provided in the concentrates. The diets were formulated according to Thomas (2004) to meet the metabolisable energy and metabolisable protein requirements of a dairy cows yielding 40 kg/d at 32 g/kg protein and 40 g/kg fat content.

The four dietary treatments were:

U– 4 kg concentrates fed in one meal, no supplement

U+ 4 kg concentrates fed in one meal, with Yea-Sacc®

E– 4 kg concentrates in four meals, no supplement

E+ 4 kg concentrates in four meals, with Yea-Sacc®

Table 3.1: Diet composition (DM and fresh weight basis) and predicted chemical composition for a 675 kg cow yielding 37 kg/d

Ingredient	kg DM/d	kg fresh/d
Maize silage	7.5	21.5
Grass silage	6.2	19.7
Concentrate	9.3	10.6
Total	23.0	51.8
Predicted chemical analysis, g/kg DM	Total diet	Partial mixed ration
Forage:concentrate (DM basis)	0.60	0.70
ME, MJ/kg DM	11.7	11.5
Crude protein	162	153
NDF	363	389

Sugar	59	49
Starch	206	195
Rumen degradable starch	178	169
Oil	40	39
MPE[1], g/kg DM	105	
MPE[2], % requirements	103	
MPN[3], g/kg DM	114	
MPN,% requirements	112	

[1]ME, metabolisable energy

[2]MPE, metabolisable protein-rumen energy limited

[3]MPN, metabolisable protein-rumen nitrogen limited

Table 3.2: The composition of concentrates (g/kg DM) fed to dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation and either with (+) or without (–) yeast supplementation, during a short-term feed restriction period

Ingredient	g/kg DM
Wheat	320
Soyhulls	178
Sugarbeet pulp (molassed)	130
Soyabean meal	170
Rapeseed meal	100
Molasses	60
Megalac[1]	8
Limestone	16.5
Sodium chloride	10
Calcined magnesite	5.0
Minerals/vitamins[2]	2.5

[1] A rumen-protected source of fat (Volac, Royston, UK).

[2] Minerals/Vitamins premix (KW Alternative Feeds, Leeds, UK), major minerals g/kg: Ca 220, P 30, Mg 80 Na 80, trace minerals mg/kg: Cu 1000; I 400, Mn 4000; Se 160, Zn 3000; Vitamins (IU): A 1,000,000; D3 300,000; E 4,000; B12 135.

3.2.3 Experimental routine

3.2.3.1 Intake and milk parameters

The experimental routine is shown in Table 3.3. Intake was recorded daily during the sampling period, and on day eight of each sampling period, intake was also measured at four hourly intervals from 0730 to 1930 h. Forage, PMR and concentrate samples were collected daily during the sampling period and stored at -20°C for subsequent analysis. The cows were milked twice daily using a portable milking machine (Milkline, London, UK) at 0600 and 1600 h and the yield measured. During days one to eight of each period milk samples were collected on four occasions (two AM and two PM) for subsequent composition analysis (fat, protein, lactose and urea). Body weight and condition score (Ferguson *et al*.,

1994) were recorded at the same time of day (after morning milking) at the start and end of each period.

Table 3.3: Experimental routine for sampling week of dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation

Sampling	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Milk yield (AM and PM)	X	X	X	X	X	X	X	X
Milk composition	PM	AM			PM	AM		
DMI	X	X	X	X	X	X	X	0730, 1130, 1530, 1930
Behaviour	X							
Blood sampling			X					
Digestibility			Apparatus on at 1400	X	X	X	X	Apparatus off at 1400
Rumen fluid sampling[1]								X

[1] Rumen sampling for pH, VFA, NH_3, microbiome analysis at 0, 3, 6, 9, 12, 15 h post concentrate feed

3.2.3.2 Eating and rumination behaviour

The behaviour of the cows was monitored at five minute intervals for 24 h by visual observation while the cows were group housed starting at 0600h on day one of each sampling period. Location (walkway, bedding, yoke) physical activity (lying, standing, walking) and jaw activity (eating, drinking, ruminating, idle) were recorded, and during milking periods cattle were only observed for jaw movement. Four observers were trained before the start of the study with an inter-observer reliability Pearson coefficient (IRPC) of 0.97 was attained, and observations were conducted for a maximum duration of six hours to minimise fatigue inaccuracy (Martin and Bateson, 2007).

3.2.3.3 Blood sampling

Blood samples were collected on day three of each sampling period by venipuncture via the jugular vein at 0530, 0700, 0900 and 1100 h using fluoride/oxalate (grey) for glucose and lithium heparin (green) for urea, β-hydroxybutyrate (BHB) and NH_3 vacutainers (BD Vacutainer, Plymouth, UK). The samples were centrifuged at 1500 *x*g for 15 min and the plasma extracted. Plasma ammonia analysis was conducted within 30 minutes of sampling and the rest of the plasma stored at -20°C for subsequent analysis.

3.2.3.4 Diet digestion

On day three of each sampling period, total urine was collected daily for five days using a modified catheter bag (Shop Optimum, West Yorkshire, UK) fitted with a pipe (32 mm internal diameter) connected to a barrel (25 L; Figure 3.1; Figure 3.2). Modified catheter bags were held over the vulva of the cow with Velcro® straps which were glued to the cows using EvoStick® compact adhesive. The urine was acidified by the addition of acid to each barrel (1 L of 20% sulphuric acid) to maintain urinary pH below pH 3.0. Total urine output was recorded daily for five days and a 1.25% subsample stored at -20°C for subsequent analysis. Faecal samples were also collected daily for the same five consecutive days by collecting all deposited material from the floor and weighing, with 2.5% subsamples of the daily output stored at -20°C prior to bulking the five sampled days together for each cow per period and subsequent analysis.

Figure 3.1: Urine collection apparatus including storage barrels

Figure 3.2: Modified catheter bag and transfer pipe apparatus

3.2.3.5 Rumen digesta sampling

Rumen fluid samples were collected on days eight of each sampling period, at 0 (immediately before morning feeding), 3, 6, 9, 12, and 15 h post morning feed, using a method adapted from Martin *et al*. (1999). Four grab samples of digesta were collected from the ventral region of the rumen, by inserting an arm directly down approximately 50 cm through the cannula and grabbing a large handful of digesta then placing into a bucket, as described in Section 2.11. Further fluid was collected by inserting a 250 ml glass bottle into the same area. The rumen fluid and digesta was then strained through four layers of muslin cloth to separate the solid digesta phase (SPD) from the liquid digesta phase (LPD). The pH of the strained rumen fluid was recorded immediately after samples were taken using a calibrated portable pH meter (Hanna Instruments, Bedfordshire, UK), and LPD samples were then stored in a 25% HPO_3 solution at -20°C for subsequent analysis of VFA and ammonia (NH_3).

Rumen fluid and digesta collected at three time points (0, 3, 12 h post morning feed) for SPD and LPD samples were stored in 15% glycerol solution at -20°C for subsequent microbial community analysis. The LPD samples were stored in a 10% formalin/saline solution at room temperature for ciliate protozoa identification and counting.

3.2.4 Chemical analysis

Forage, PMR and concentrate samples were bulked between days for each period, and the sub-samples analysed for DM (943.01), CP (990.03; intra-assay CV of 0.147%) and ash (942.05) according to AOAC (2012). Neutral detergent fibre (intra-assay CV of 0.943%) was determined according to Van Soest *et al*. (1991) and expressed exclusive of residual ash. Milk composition was analysed by National Milk Laboratories (NML; Wolverhampton, UK) for fat, protein, lactose and urea using near midinfrared (MIR; Foss, Denmark).

Blood plasma samples were analysed for glucose, BHB, urea and ammonia (with intra-assay CV of 1.72, 4.28, 1.78, and 4.16%, respectively) using a Cobas Mira Plus autoanalyser (ABX Diagnostics, Bedfordshire, UK). The kits used were: GLUC-HK, Ref GU611; RANBUT, Ref RB1008; and UREA, Ref UR221 and NH3, Ref AM1015 respectively (Randox Laboratories, County Antrim, UK).

Volatile fatty acids were analysed in the liquid rumen fluid fraction by GC according to Erwin *et al*. (1961) using a column (DBFFAP, 30 m x 0.250 mm x 0.2 µm; Agilent J and W, GC columns, UK) and flame ionisation detector (Agilent 6890, Stockport, UK) as described in Section 2.13. Lactate analysis was conducted on rumen fluid samples by high performance liquid chromatography (Agilent 1100, Germany) as described in Section 2.14. Rumen NH_3 concentration was measured from the liquid fraction from a method adapted from MAFF (1986) and using an auto-titrator (FOSS 1030 auto-titrator, FOSS, Warrington, UK; Buchi Labortechnik AG CH-9230, Flawil, Switzerland).

Ciliate protozoa identification and counts of relative abundance of *Isotrichidae* and *Ophryoscolecidae* was conducted using a light microscope (Olympus CX31, Olympus, Tokyo) with a 10X objective and 10X/20 eyepiece (Section 2.16.7). Microbial community analysis was undertaken using 16S rRNA gene amplicon sequencing (as described in Section 2.16). The DNA extraction was undertaken at Harper Adams University using the bead beating protocol based on Yu and Morrison (2004; Section 2.16.3). Amplicon libraries were prepared using dual index primers based on Kozich *et al*. (2013; Section 2.16.4), the order of the primers used are described in Appendix 8.2. The libraries were quality assessed using an Agilent 2100 Bioanalyzer System (Agilent Technologies. Santa Clara, CA, US) and sequenced using the Illumina MiSeq v2 250 paired end reagent kit to yield 11M sequences (Illumina UK, Cambridge, UK.).

3.2.5 Statistical analysis

Performance and rumen metabolism parameters were evaluated by repeated measures analysis of variance, and digestibility parameters were evaluated by ANOVA as a 2x2

factorial design as a Latin square design using GenStat Release 18.1 (VSN International Ltd). The repeated measure was time of sampling during the day (h), with main effects of pattern of concentrate allocation (C), addition of yeast (Y) and their interaction (Int). The N balance equations are described in Section 2.10. Results are reported as treatment means with SED, with the level of significance set at $P < 0.05$, and a tendency stated at $P < 0.10$.

Microbial community data was analysed using mothur v1.44.0 (Schloss *et al*., 2011), assembling paired end sequences and removing low quality sequences. Sequence counts from the library were normalised by subsampling to 40,000 sequences per sample prior to statistical analysis. Sequences were clustered into operational taxonomic units (OTU) at 97% identity, and taxonomic classification of the representative sequences was carried out using the SILVA 132 SEED reference database (Yilmaz *et al*., 2014). Microbial OTU richness and diversity were summarised using Chao1, Shannon, inverse Simpson, and number of observed OTU (OBS) indices. Beta diversity was calculated using the Bray Curtis dissimilarity metric, with a significance level from analysis of molecular variance (AMOVA) set at $P < 0.001$. Taxonomic biomarkers associated to the respective treatment groups were determined by comparing relative abundance of OTU using Linear Discriminant Analysis (LDA) Effect Size (LEfSe; Segata *et al*., 2011) with minimum LDA score >2.0 and $P < 0.05$.

3.3 Results

3.3.1 Forages and diets

The DM of the grass silage (GS) was 123 g/kg lower than the maize silage (MS), whilst the CP and NDF content were 103 and 68 g/kg DM higher, respectively in the GS than the MS (Table 3.4). The MS had a higher pH than the grass silage at 4.37, whilst the fermentation acid concentrations were similar in both forages. The two concentrates had a mean DM, CP and NDF values of 881, 204 and 243 g/kg DM respectively. The two PMR had mean DM, CP and NDF values of 320, 150 and 413 g/kg DM respectively.

Table 3.4: Nutritional composition (g/kg DM) and fermentation characteristics of grass silage (GS), maize silage (MS), concentrates (without (–) or with yeast supplementation (+)) and partial mixed ration (PMR; without (–) or with yeast supplementation (+))

	GS	MS	Concentrates (–)	Concentrates (+)	PMR (–)	PMR (+)
DM	233	356	883	879	323	316
CP	183	80.3	198	209	149	150
Ash	129	34.0	78.0	67.0	92	90
OM	845	966	922	933	908	910
NDF	441	373	236	249	423	402
Fermentation characteristics						
pH	4.19	4.37				
NH_3-N, g/kg of total N	27.1	23.6				
Acetate	42.6	43.5				
Propionate	1.13	1.47				
Iso-butyrate	0.1	0.16				
Butyrate	1.99	2.10				
Iso-valerate	0.17	0.23				
Lactate	82.4	67.6				

3.3.2 Intake, production and milk composition

Dry matter intake and milk yield were not affected ($P > 0.05$) by the pattern of concentrate allocation or inclusion of yeast, with mean values of 23.1 kg/day DM and 39.2 kg/d, respectively (Table 3.5). Similarly, milk fat and protein yield (kg/d) were also not affected ($P > 0.05$) by dietary treatment, with mean values of 1.62 and 1.19 kg/d, respectively. Live weight and body condition score (BCS) were also not affected ($P > 0.05$) by pattern of concentrate allocation or inclusion of yeast, with mean values of 697 kg and 2.45, respectively.

Table 3.5: Production performance dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation

	Treatments					P-Value		
	E+	E–	U+	U–	SED	C	Y	C x Y
DMI, kg/d	23.2	22.6	22.9	23.6	0.48	0.367	0.943	0.103
Milk yield, kg/d	39.0	40.0	39.1	38.8	1.30	0.560	0.717	0.512
Milk fat, kg/d	1.65	1.59	1.65	1.57	0.123	0.880	0.453	0.922
Milk fat, g/kg	38.2	41.2	39.1	40.9	5.43	0.933	0.552	0.876
Milk protein, kg/d	1.20	1.21	1.18	1.18	0.041	0.388	0.895	0.810
Milk protein, g/kg	27.8	30.1	27.8	30.6	2.65	0.871	0.219	0.904
Milk lactose, g/kg	45.8	45.9	45.8	45.5	0.10	0.831	0.887	0.767
Milk urea-N, g/kg	0.26	0.25	0.25	0.25	0.002	0.706	0.578	0.920
Live weight, kg	693	689	700	705	10.4	0.154	0.948	0.564
Live weight change, kg[1]	9.00	12.5	37.0	18.5	14.05	0.138	0.479	0.331
Body condition score	2.38	2.44	2.44	2.56	0.084	0.168	0.168	0.620
Body condition score change[1]	0.06	0.00	0.00	0.19	0.153	0.585	0.585	0.292

C= Concentrate allocation, Y= inclusion of yeast, C x Y= interaction between C and Y
[1] = change over 35 day period

3.3.3 Eating and rumination behaviour

Pattern of concentrate allocation or yeast supplementation had no effect on eating, ruminating, drinking or idling time when expressed as, h/d, min/kg DMI, min/kg NDF intake, or min/kg forage NDF intake (Table 3.6).

Table 3.6: Eating and ruminating behaviour of dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation

	Treatments				SED	P-value		
	E+	E–	U+	U–		C	Y	C x Y
Eating								
h/d	4.44	4.77	4.33	4.44	0.429	0.498	0.498	0.718
Min/kg DMI	11.7	12.5	11.0	10.8	0.71	0.057	0.598	0.398
Min/kg NDFI	28.3	30.2	26.6	26.1	1.73	0.057	0.598	0.398
Min/kg fNDFI	40.5	43.1	38.0	37.3	2.47	0.057	0.598	0.398
Ruminating								
h/d	9.75	10.3	10.2	9.85	0.278	0.959	0.581	0.084
Min/kg DMI	25.7	26.7	25.9	24.2	2.31	0.497	0.841	0.435
Min/kg NDFI	1.04	1.08	1.04	0.974	0.2286	0.497	0.841	0.435
Min/kg fNDFI	1.48	1.54	1.49	1.39	0.134	0.497	0.841	0.435
Drinking								
h/d	0.73	0.58	0.62	0.56	0.133	0.531	0.310	0.673
Min/kg DMI	1.97	1.50	1.52	1.42	0.407	0.386	0.358	0.546
Min/kg NDFI	0.080	0.061	0.061	0.057	0.0164	0.386	0.358	0.546
Min/kg fNDFI	0.114	0.087	0.088	0.082	0.0234	0.386	0.358	0.546
Idling								
h/d	9.08	8.38	8.90	9.15	0.308	0.227	0.33	0.070
Min/kg DMI	24.1	21.8	22.5	23.0	2.06	0.932	0.565	0.368
Min/kg NDFI	0.970	0.878	0.908	0.929	0.0830	0.932	0.565	0.368
Min/kg fNDFI	1.39	1.25	1.30	1.33	0.118	0.932	0.565	0.368

C= Concentrate allocation, Y= inclusion of yeast, C x Y= interaction between C and Y

3.3.4 Blood metabolites

Plasma ammonia tended to decrease by 2.8 mmol/L (P = 0.072) in cows that received yeast supplementation (Table 3.7). Pattern of concentrate allocation or yeast supplementation had no effect on mean plasma glucose, BHB or urea concentration with mean values of 3.80, 0.689 and 3.74 mmol/L, respectively. There was no interaction between dietary treatment and time (Figure 3.3). There was however an effect of time on all the plasma metabolites, with ammonia, BHB and urea concentrations increasing by 16.2, 0.489 and 0.539 mmol/L, respectively, during the day and glucose concentration decreasing by 0.394 mmol/L.

Table 3.7: Plasma metabolites (mmol/L) in dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation and fed with (+) or without (–) yeast supplementation

	Treatments				SED	P-value		
	E+	E–	U+	U–		C	Y	C x Y
NH_3	43.3	44.1	39.2	44.1	1.83	0.165	0.072	0.170
Glucose	3.89	3.75	3.76	3.79	0.240	0.812	0.748	0.653
BHB	0.65	0.61	0.74	0.76	0.102	0.161	0.920	0.694
Urea	3.34	2.97	3.52	3.27	0.301	0.295	0.199	0.773

C= Concentrate allocation, Y= inclusion of yeast, C x Y= interaction between C and Y

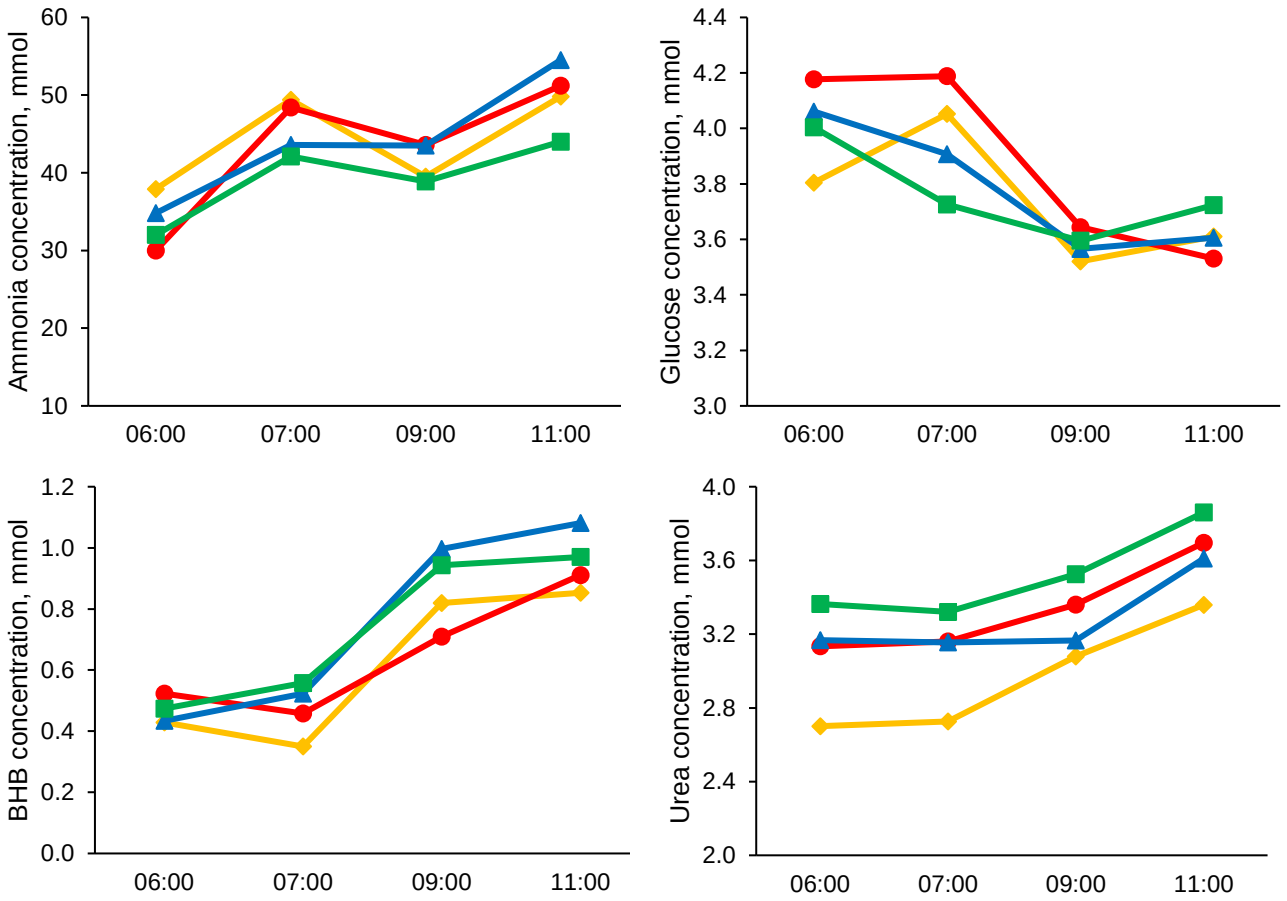


Figure 3.3: Plasma beta hydroxybutyrate (BHB), plasma urea, plasma ammonia (NH_3), plasma glucose of dairy cows receiving an even pattern of concentrates with (E+; ●) or without (E-; ♦) yeast supplementation, or uneven pattern of concentrate allocation either with (U+; ■) or without (U-; ▲) yeast supplementation. For plasma NH_3, SED= 6.08; Time, P = <0.001; Time x Diet, P = 0.814, Time x Yeast, P = 0.847; Time x Diet x Yeast, P = 0.572; for plasma glucose, SED= 0.0.319; Time, P < 0.001; Time x Diet, P = 0.365; Time x Yeast, P = 0.888; Time x Diet x Yeast, P = 0.582; for plasma BHB, SED= 0.155; Time, P = <0.001; Time x Diet, P = 0.385; Time x Yeast, P = 0.608; Time x Diet x Yeast, P = 0.868; for plasma urea, SED= 0.402; Time, P = 0.003; Time x Diet, P = 0.893; Time x Yeast, P = 1.000; Time x Diet x Yeast, P = 0.939.

3.3.5 Diet digestion and nitrogen balance

Pattern of concentrate allocation or yeast supplementation had no effect (P > 0.05) on DM or OM digestibility with mean values of 0.710 and 0.735 kg/kg, respectively (Table 3.8). There tended to be an interaction between concentrate allocation and yeast on NDF digestibility (P = 0.089) which decreased by 0.03 kg/kg in cows fed U+ compared to U–, and increased by 0.013kg/kg in cows fed E+ compared to E–. Pattern of concentrate allocation or yeast supplementation had no effect on N intake, faecal N output or N digestibility with mean values of 553 g/d, 215 g/d and 0.611 kg/kg, respectively (Table 3.9). Milk N and milk use efficiency were also not affected by pattern of concentrate allocation or yeast supplementation with mean values of 191 g/d and 345 g/kg, respectively.

Table 3.8: Diet digestibility in dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation

	Treatments					P-value		
	E+	E–	U+	U–	SED	C	Y	C x Y
DM, kg/d								
Intake	22.9	22.8	23.1	23.6	0.48	0.367	0.943	0.103
Output	6.65	6.70	6.56	6.86	0.185	0.814	0.224	0.394
Digestibility, kg/kg	0.710	0.705	0.715	0.711	0.0048	0.156	0.247	0.943
OM, kg/d								
Intake	20.9	20.6	21.0	21.4	0.62	0.327	0.887	0.466
Output	5.50	5.52	5.49	5.657	0.109	0.438	0.279	0.352
Digestibility, kg/kg	0.736	0.731	0.738	0.736	0.0050	0.365	0.418	0.702
NDF, kg/d								
Intake	9.73	9.04	9.21	10.1	0.59	0.551	0.818	0.132
Output	3.19	3.20	3.20	3.17	0.082	0.861	0.906	0.715
Digestibility, kg/kg	0.673	0.660	0.653	0.688	0.0151	0.723	0.383	0.089

C= Concentrate allocation, Y= inclusion of yeast, C x Y= interaction between C and Y

Table 3.9: Nitrogen balance in dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation

	Treatments					P-value		
N, g/d	E+	E–	U+	U–	SED	C	Y	C x Y
Intake	560	540	549	563	16.3	0.635	0.808	0.190
Faecal output	214	215	211	220	5.4	0.812	0.226	0.311
Digested	346	326	339	343	12.7	0.612	0.403	0.205
Digestibility, g/g	0.617	0.603	0.615	0.610	0.0078	0.673	0.115	0.444
Urine	84.9	69.5	93.0	91.3	11.44	0.205	0.401	0.485
Urine-N of total faecal N, g/kg	292	249	302	290	27.4	0.315	0.292	0.509
Milk N	187	189	194	192	10.9	0.572	0.980	0.784
N use efficiency, g/kg	333	352	353	342	23.6	0.759	0.811	0.408

C= Concentrate allocation, Y= inclusion of yeast, C x Y= interaction between C and Y

3.3.6 Rumen pH, ammonia and volatile fatty acids

Pattern of concentrate allocation had no effect ($P > 0.05$) on rumen pH with a mean value of pH 5.81 (Table 3.10), although there was a tendency ($P = 0.084$) for the inclusion of yeast to increase mean rumen pH by 0.07 units. Rumen pH of cows fed U declined rapidly between 0 and 3 h from pH 6.3 to 5.7, while pH declined more slowly in cows fed E ($P < 0.001$), and spent less time under pH 5.8 (9.5 h compared to 12.5 h, respectively; Figure 3.4). Maximum and minimum pH were not affected ($P > 0.05$) by pattern of concentrate allocation or yeast supplementation, with mean values of pH 6.30 and 5.47, respectively. Rumen NH_3 concentration in cows fed yeast tended to increase ($P = 0.100$) post feeding by 17.1 mg/L

compared to no supplementation. In the first 3 h post concentrate feeding mean rumen NH_3 concentration approximately doubled from 39.1 to 81.5 mg/L, dropped by 30 mg/L at 6 h post feeding, and remained relatively consistent for the following 14 h (P = 0.03; Figure 3.5). Pattern of concentration allocation had no effect (P >0.05) on total or individual VFA concentration (Table 3.11). Yeast supplementation tended to decrease total VFA (P = 0.074) and acetate concentration (P = 0.068) by 15.5 and 11.5 µM, respectively, compared to no supplementation. Inclusion of yeast also tended (P = 0.060) to alter the diurnal ratio of A: P (Figure 3.6). Acetate to propionate ratio of cattle fed yeast remained consistent throughout the day, with a mean value of 4.28, whilst the A: P for cattle fed the diet without yeast supplementation tended to decline during the day from 4.78 to 3.87 (P = 0.060; Figure 3.6). Pattern of concentrate allocation and yeast supplementation had no effect (P > 0.05) on lactate concentration with a mean value of 0.02 µM.

Table 3.10: Rumen metabolism in dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation

	Treatments					P-value		
	E+	E–	U+	U–	SED	C	Y	C x Y
Mean rumen pH	5.85	5.77	5.83	5.78	0.046	0.890	0.084	0.745
Maximum rumen pH	6.27	6.31	6.31	6.29	0.086	0.948	0.866	0.589
Minimum rumen pH	5.45	5.48	5.47	5.49	0.068	0.737	0.665	0.967
Mean rumen ammonia, mg/L	65.5	40.4	61.4	52.4	12.41	0.669	0.100	0.391

C= Concentrate allocation, Y= inclusion of yeast, C x Y= interaction between C and Y

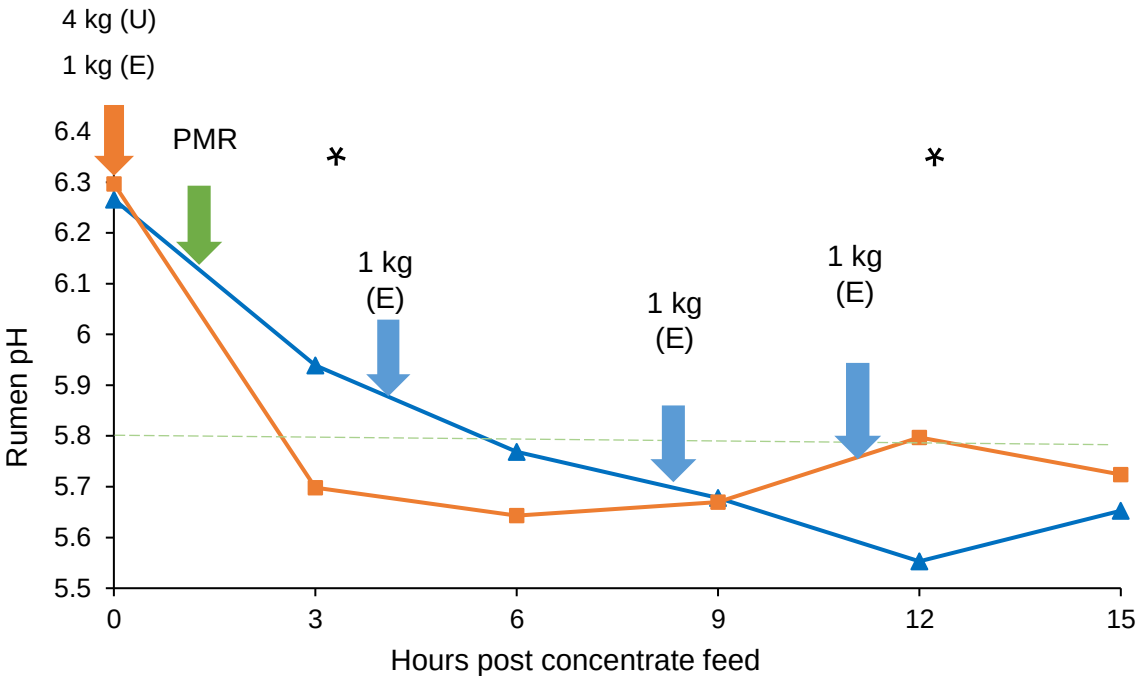


Figure 3.4: Diurnal variation of rumen pH in dairy cows receiving an even pattern of concentrates (▲) and uneven pattern of concentrates (■). (SED= 0.064; Time, P <0.001; Diet x Time, P < 0.001; Yeast x Time, P = 0.559; Time x Diet x Yeast = 0.583). * P <0.05.

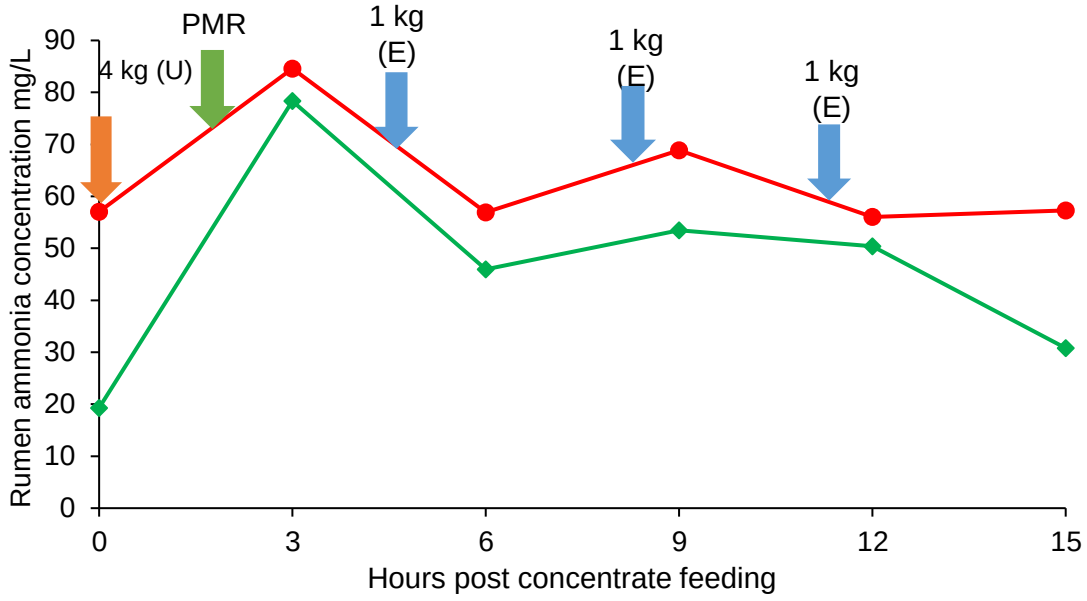


Figure 3.5: Diurnal variation of rumen ammonia concentration (mg/L) of dairy cows fed diets either with (●) and without (♦) yeast supplementation. (SED= 23.08; Time, P = 0.003; Diet x Time, P = 0.542; Yeast x Time, P = 0.068; Time x Diet x Yeast = 0.621).

Table 3.11: Rumen volatile fatty acid content (μM) in dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation

	Treatments					P-value			
	E+	E–	U+	U–	SED	T	C	Y	C x Y
Total VFA									
0600 h	130	173	154	163	21.7	0.010	0.454	0.074	0.986
0900 h	157	196	173	193					
1200 h	152	182	172	182					
1500 h	185	146	146	176					
1800 h	195	202	168	183					
2100 h	127	141	167	177					
Acetate									
0600 h	90.9	125	107	120	17.18	0.060	0.324	0.068	0.905
0900 h	107	137	119	135					
1200 h	104	124	120	126					
1500 h	129	98.6	98.2	120					
1800 h	136	138	115	126					
2100 h	82.2	92.9	119	125					
Propionate									
0600 h	21.2	26.9	26.2	24.0	3.19	<0.001	0.996	0.123	0.800
0900 h	26.8	31.2	29.5	31.4					
1200 h	25.9	31.0	27.7	30.8					
1500 h	29.8	26.2	24.8	30.0					
1800 h	31.7	35.7	27.6	30.9					
2100 h	23.0	27.8	25.1	29.0					
Butyrate									
0600 h	13.3	15.1	15.3	14.1	2.04	<0.001	0.813	0.390	0.873
0900 h	16.8	20.5	18.3	19.9					
1200 h	15.9	20.2	17.9	19.0					
1500 h	19.7	15.9	17.2	19.5					
1800 h	21.2	21.5	19.0	19.3					
2100 h	16.4	15.0	17.5	16.8					
Iso-butyrate									
0600 h	0.99	1.10	1.17	0.97	0.115	<0.001	0.744	0.700	0.812
0900 h	1.14	1.26	1.02	1.14					
1200 h	0.99	1.10	1.08	1.03					
1500 h	1.08	0.82	0.92	1.05					
1800 h	1.04	1.04	0.98	1.09					
2100 h	0.84	0.80	0.95	1.02					
Valerate									
0600 h	1.59	1.89	1.91	1.55	0.286	<0.001	0.977	0.404	0.957
0900 h	2.29	2.78	2.35	2.55					
1200 h	2.18	2.64	2.39	2.72					
1500 h	2.54	1.95	2.20	2.41					
1800h	2.81	2.80	2.42	2.61					
2100 h	1.94	2.02	2.14	2.24					
Iso-valerate									
0600 h	2.32	2.51	2.37	2.01	0.399	<0.001	0.844	0.432	0.840
0900 h	2.93	3.58	2.65	3.04					
1200 h	2.63	3.30	2.85	3.06					
1500 h	3.07	2.37	2.56	3.03					
1800 h	3.08	3.13	2.83	3.16					
2100 h	2.34	2.24	2.61	2.84					
Mean Lactate	0.071	0.00	0.01	0.00	0.032	–	0.229	0.229	0.130

T= Time, C= Concentrate allocation, Y= inclusion of yeast, C x Y= interaction between C and Y

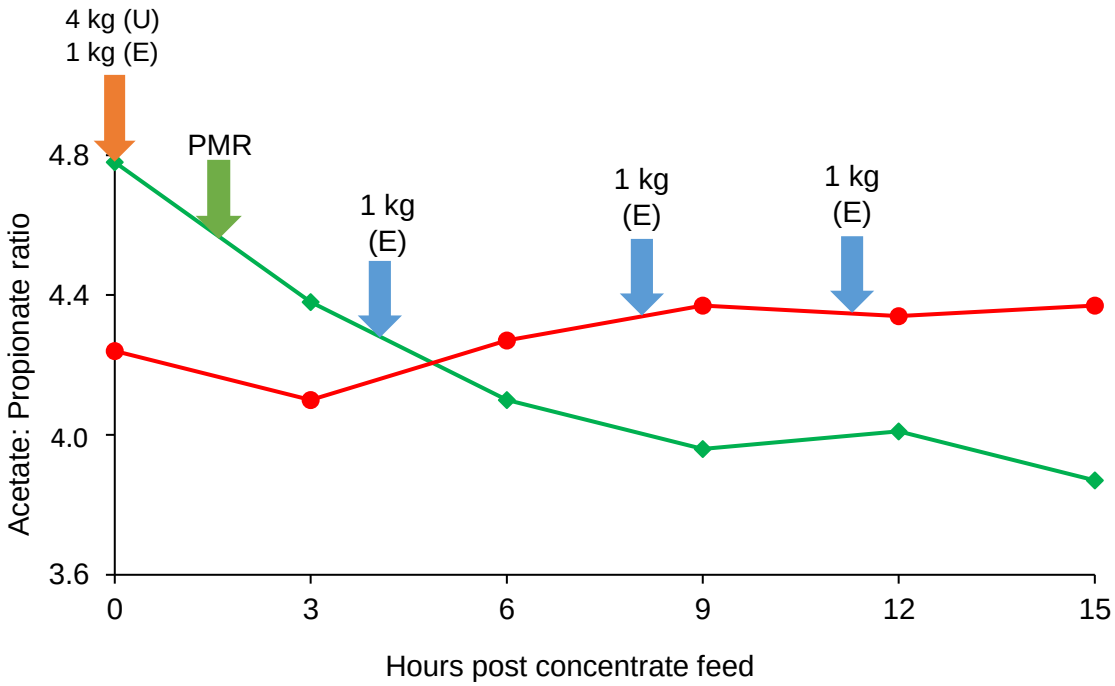


Figure 3.6: Diurnal variation of the rumen fluid acetate:propionate ratio of dairy cows fed diets either with (●) or without (♦) yeast supplementation. (SED= 0.444; Time, P = 0.417; Diet, P =0.352; Yeast, P = 0.617; Time x Diet, P = 0.119; Time x Yeast, P = 0.060; Time x Diet x Yeast = 0.889).

3.3.7 Microbial community analysis amplicon sequencing of 16S rRNA gene

Following sequencing the result was a total of 12.7 million sequences, before quality control subsampling and conducted, and there were a total of 16381 OTU were identified. The libraries were normalised by subsampling to 47,000 reads per sample, and low abundance OTU (total number of reads per OTU <10) were removed from the dataset. As a result there were 3586 OTU in total across all samples. Coverage measured using Good's statistic was between 98.5% and 99.6% per library. The relative abundance of phyla were Bacteroidetes (43%), Firmicutes (32%), Proteobacteria (9%), Spirochetes (5%), Euryarchaeota (4%), and Fibrobacteres (3%); the remaining 4% consisted of unclassified bacteria and low abundance taxonomic groups (Figure 3.7).

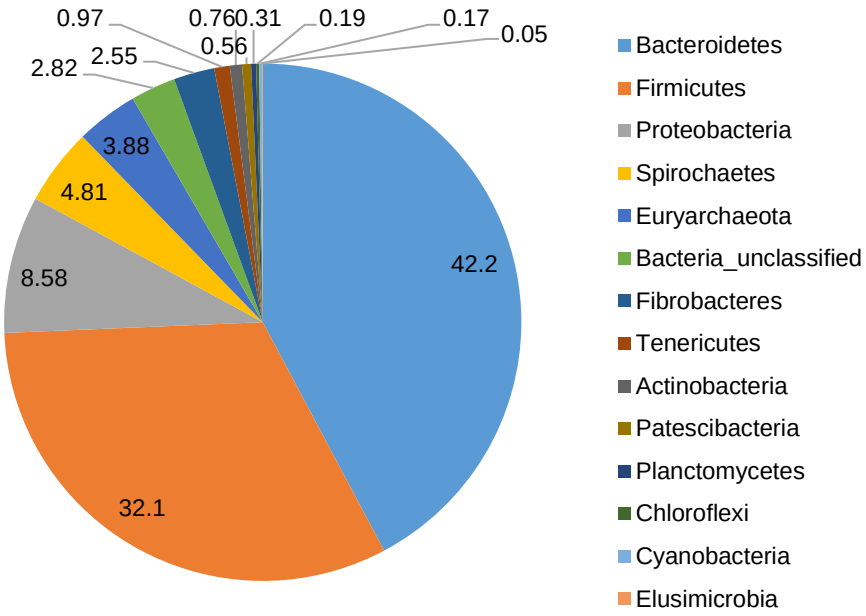


Figure 3.7: Relative abundance (%) of phyla of total dataset

3.3.7.1 Alpha diversity

Pattern of concentrate allocation or yeast supplementation had no effect (P > 0.05) on the alpha diversity of the LPD samples (Table 3.12). Chao1 (OTU richness) and OBS (number of observed OTU) tended to increase in SPD of dairy cattle when fed an uneven pattern of concentrates compared to an even pattern by 166 and 122, respectively. The Inverse Simpson index (OTU evenness) of SPD samples tended to increase (P = 0.073) by 9.1 in cattle fed an even diet compared to an uneven diet, while Shannon (OTU diversity) was unaffected by pattern of concentrate allocation or yeast supplementation.

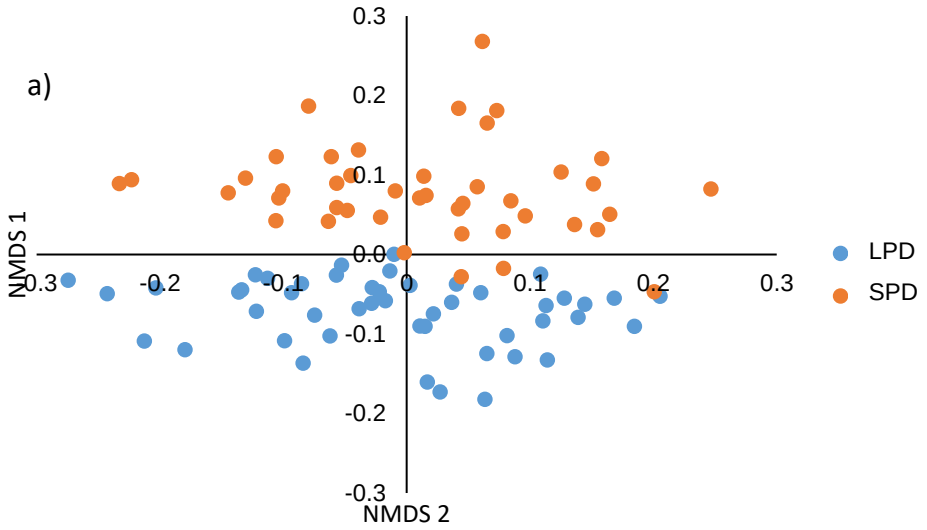
Table 3.12: Alpha diversity of rumen microbial community in liquid digesta phase (LPD) and solid digesta phase (SPD) in dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation

	Treatment				SED	P-value		
	E+	E–	U+	U–		C	Y	C x Y
LPD								
OBS	1492	1557	1577	1552	79.5	0.507	0.730	0.457
Chao1	1745	1823	1870	1825	104.9	0.421	0.834	0.440
Inv. Simpson	53.0	37.5	44.5	48.9	10.96	0.853	0.504	0.245
Shannon	5.22	5.10	5.18	5.13	0.194	0.970	0.543	0.815
SPD								
OBS	1583	1649	1766	1710	72.5	0.063	0.931	0.287
Chao1	1814	1907	2086	1967	102.5	0.071	0.864	0.203
Inv. Simpson	65.2	69.3	56.4	59.9	5.68	0.073	0.387	0.943
Shannon	5.37	5.45	5.33	5.33	0.066	0.168	0.392	0.440

C= Concentrate allocation, Y= inclusion of yeast, C x Y= interaction between C and Y

3.3.7.2 Beta Diversity

Bray Curtis dissimilarity was not affected between even and uneven pattern of concentrate allocation (AMOVA $P > 0.05$; Figure 3.8). Bray Curtis dissimilarity was however affected (AMOVA $P < 0.05$) by yeast supplementation, digesta phase and individual cow. When comparing the individual cow response to yeast supplementation, two of the four cows showed differences in their relative microbial community ($P < 0.0001$) when diets were supplemented with yeast compared to without yeast (Table 3.13). Regardless of yeast supplementation treatment there were differences ($P < 0.0001$) between the microbial communities of all the individual cows (Table 3.14).


a)
0.3
0.2
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0.0
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-0.3
-0.3
-0.2
-0.1
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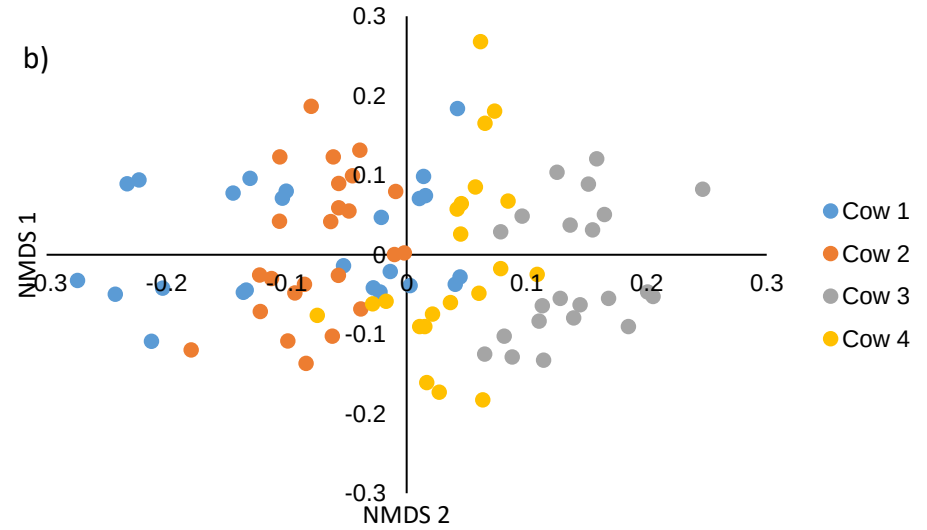
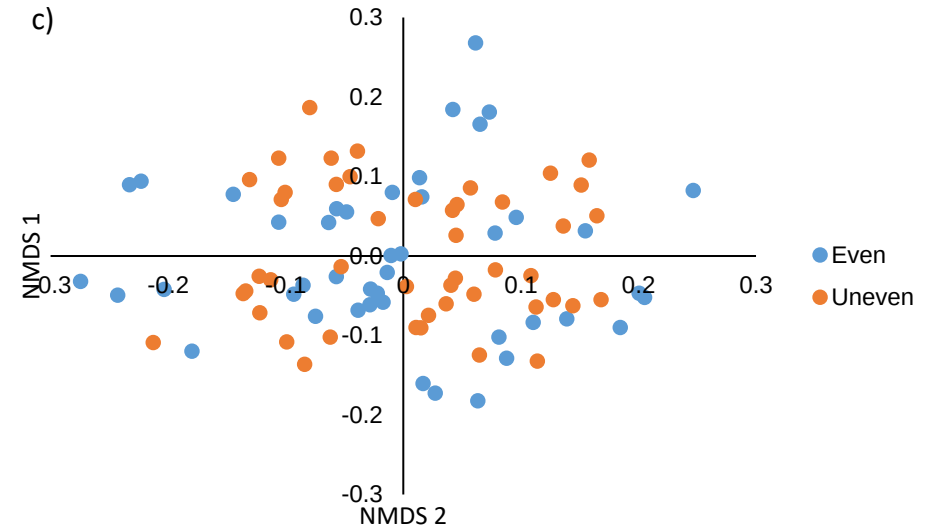
0.3
NMDS 1
NMDS 2
LPD
SPD


b)
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
NMDS 1
NMDS 2
Cow 1
Cow 2
Cow 3
Cow 4


c)
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
NMDS 1
NMDS 2
Even
Uneven

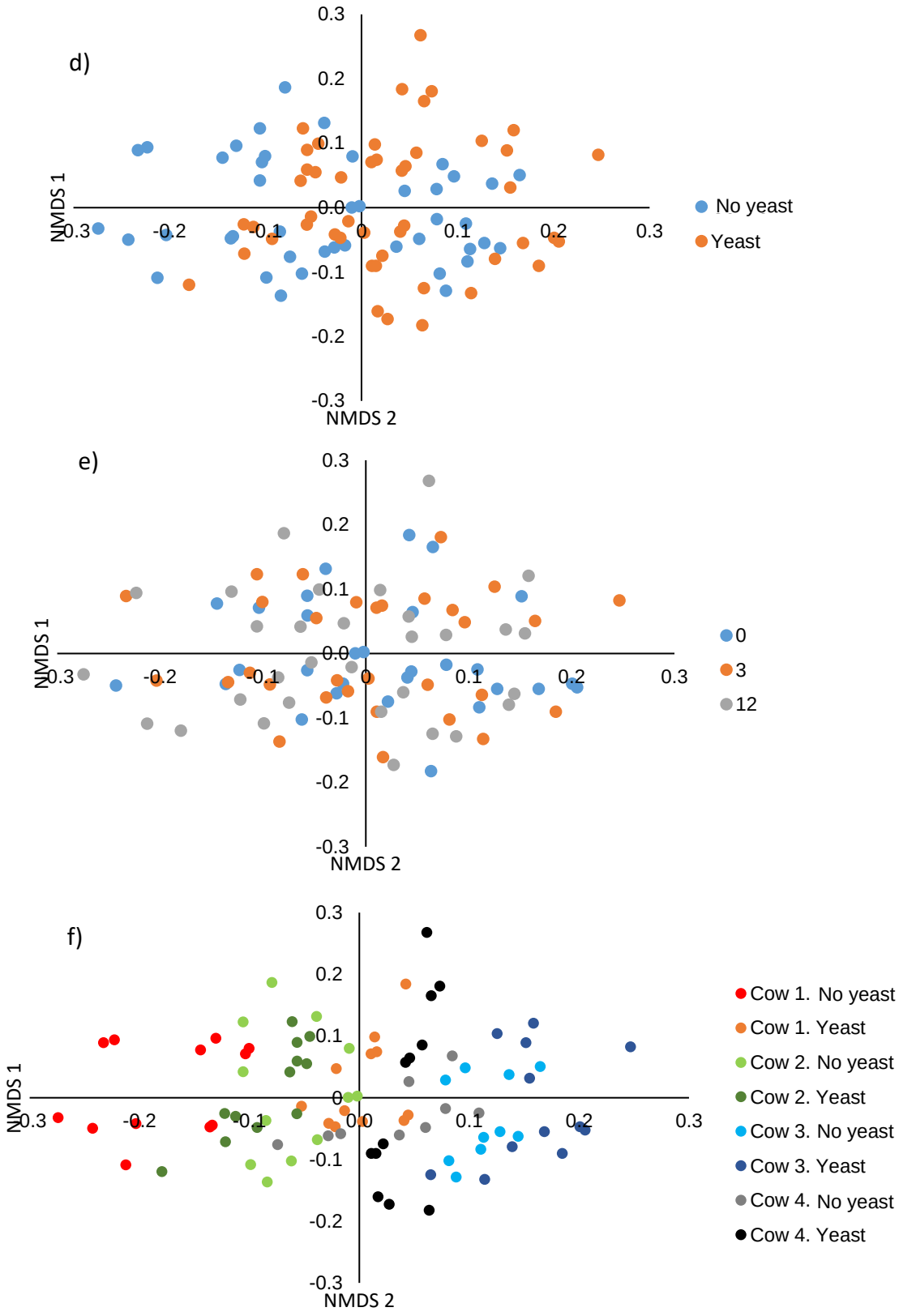


Figure 3.8: NMDS plot (Stress value 0.21556) based on Bray Curtis dissimilarity matrix, for a) Digesta phase ($P < 0.001$) b) Cow ($P < 0.001$) c) Yeast supplementation ($P = 0.012$) d) Pattern of concentrate allocation ($P < 0.224$), e) Sampling time ($P < 0.008$) f) Cow x yeast ($P < 0.001$; AMOVA calculated using mothur software)

Table 3.13: Pair wise comparisons of the effect of yeast supplementation on individual cows, using analysis of molecular variance (AMOVA)

1.Cow	1.Yeast	2.Cow	2.Yeast	P-value
Cow 1	No yeast	Cow 1	Yeast	<0.0001*
Cow 2	No yeast	Cow 2	Yeast	0.0006*
Cow 3	No yeast	Cow 3	Yeast	0.0276
Cow 4	No yeast	Cow 4	Yeast	0.0021

Table 3.14: Pair wise comparisons of cow and yeast supplementation, using analysis of molecular variance (AMOVA)

		Cow 1		Cow 2		Cow 3	
		No yeast	Yeast	No yeast	Yeast	No yeast	Yeast
Cow 1	No yeast	–	–	–	–	–	–
	Yeast	–	–	–	–	–	–
Cow 2	No yeast	<0.0001*	–	–	–	–	–
	Yeast	–	<0.0001*	–	–	–	–
Cow 3	No yeast	<0.0001*	–	<0.0001*	–	–	–
	Yeast	–	<0.0001*	–	<0.0001*	–	–
Cow 4	No yeast	<0.0001*	–	0.0003*	–	0.003	–
	Yeast	–	<0.0001*	–	<0.0001*	–	<0.0001*

3.3.7.3 Linear Discriminant analysis (Identification of Taxonomic Biomarkers; LEfSe)

The differences ($P < 0.05$) between relative microbial communities of the solid and liquid digesta phases were a result of an increase in OTU00002 and OTU00018, OTU00014, and OTU00021 which are assigned to multiple species of *Prevotella* in LPD samples, and an increase in OTU assigned to *Methanobrevibacter olleyae* (OTU00004), *Treponema bryantii* (OTU00005) and *Fibrobacter succinogenes* (OTU00033) in SPD samples (Table 3.15). There were strong differences ($P < 0.05$) between microbial communities of the individual cows, although they all contained at least two OTU which were assigned to the genus *Prevotella* (e.g. OTU00025, OTU00030, OTU00011; Table 3.16). When cows were fed diets without yeast there was an increase in OTU00001 and OTU00019 which are related to *Chelonobacter oris* (87%) *Prevotella copri* (89%), respectively, and when cows were supplemented with yeast there was an increase in OTU00009 which is related to *Gracilibacter thermotolerans* (87%; Table 3.17). At first feed (0600 h) there was increased abundance of OTU00010 and OTU00009 assigned to *Marseillibacter massiliensis* (92%) and *Gracilibacter thermotolerans* (87%), respectively. At 3 h post feeding (0900 h) clustering was associated with the two OTU assigned to *Lactobacillus* (OTU00015, OTU00006). By 12 h post feeding (1800 h) *Chelonobacter oris* (87%; OTU00001) was in high abundance (Table 3.18).

Table 3.15: OTU level taxonomic biomarkers for liquid digesta phase (LPD) and solid digesta phase (SPD) in dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation (Linear discriminant analysis (LDA) score > 2.0, P < 0.05)

	P-value	LDA score	% Seqs	Silva 132 SEED taxonomy (mixed rank)	BLASTn Type (% Ident) (Accessed 10/11/2021)	Reference
LPD						
OTU00001	0.000142	-3.19	6.68	Unclassified *Gammaproteobacteria* (Genus; 100%)	*Chelonobacter oris* (87%)	Kudirkiene *et al*. (2014)
OTU00002	2.85E-05	-2.61	6.47	*Prevotella* (Genus; 100%)	*Prevotella ruminicola* (100%)	Purushe *et al*. (2010)
OTU00012	6.69E-07	-2.46	1.15	Unclassified *Gammaproteobacteria* (Genus; 100%)	*Moraxella catarrhalis* (87%)	Verduin *et al*. (2002)
OTU00018	1.04E-11	-2.25	0.865	Unclassified *Prevotella* (Genus; 100%)	*Prevotella bryantii* (98%)	Fraga *et al*. (2018)
OTU00014	2.18E-13	-2.21	1.10	*Prevotella* (Genus; 100%)	*Prevotella brevis* (93%)	Avguštin *et al*. (1997)
OTU00020	1.90E-08	-2.2	0.829	Unclassified *Bacteroidetes* (Class; 100%)	*Microbacter margulisiae* (87%)	Sanchez-Andrea *et al*. (2014)
OTU00021	1.29E-11	-2.1	0.816	*Prevotella* (Genus; 100%)	*Prevotella brevis* (91%)	Avguštin *et al*. (1997)
OTU00016	0.000648	-2.07	0.909	*Ruminococcaceae* (Family; 100%)	*Ruminococcus bromii* (97%)	Ze *et al*. (2012)
OTU00011	0.000152	-2.05	1.20	*Prevotella* (Genus; 100%)	*Prevotella ruminicola* (93%)	Purushe *et al*. (2010)
SPD						
OTU00004	7.40E-14	2.75	2.03	*Methanobrevibacter* (Genus; 100%)	*Methanobrevibacter olleyae* (97%)	Rea *et al*. (2007)
OTU00005	1.01E-11	2.68	2.00	*Treponema* (Genus; 100%)	*Treponema bryantii* (97%)	Stanton and Canale-Parola (1980)
OTU00006	0.00031	2.67	1.62	*Lactobacillus* (Genus; 100%)	*Lactobacillus fuchuensis* (100%)	Sakala *et al*. (2002)
OTU00007	2.93E-11	2.46	1.45	Unclassified *Acidaminococcaceae* (Genus; 99%)	*Succiniclasticum ruminis* (98%)	Van Gylswyk (1995)
OTU00009	2.84E-05	2.08	1.32	*Clostridiales* (Class; 94%)	*Gracilibacter thermotolerans* (87%)	Lee *et al*. (2006)
OTU00008	0.000303	2.03	1.37	*Rikenellaceae* (Family; 100%)	*Gallalistipes aquisgranensis* (86%)	Zenner *et al*. (2021)
OTU00033	0.000121	2.01	0.504	*Fibrobacter* (Genus; 100%)	*Fibrobacter succinogenes* (97%)	Suen *et al*. (2011)

LPD= Liquid digesta phase, SPD= Solid digesta phase

Table 3.16: OTU level taxonomic biomarkers for individual dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation (Linear discriminant analysis (LDA) score > 2.0, P < 0.05)

	P-value	LDA score	% Seqs	Silva 132 SEED taxonomy (mixed rank)	BLASTn Type (% Ident) (Accessed 10/11/2021)	Reference
Cow 1						
OTU00025	4.33E-07	2.55	0.655	*Prevotella* (Genus; 100%)	*Prevotella mizrahii* (98%)	Wylensek *et al*. (2020)
OTU00019	8.83E-05	2.39	0.829	Unclassified *Prevotellaceae* (Family; 100%)	*Prevotella copri* (91%)	Iljazovic *et al*. (2021)
OTU00070	9.11E-13	2.18	0.250	*Lachnospiraceae* (Family; 100%)	*Clostridium vitabionis* (92%)	Shin *et al*. (2021)
OTU00077	2.76E-10	2.14	0.232	Unclassified *Prevotellaceae* (Family; 100%)	*Metaprevotella massiliensis* (88%)	Ricaboni *et al*. (2017)
Cow 2						
OTU00001	1.34E-05	3.25	6.68	Unclassified *Gammaproteobacteria* (Genus; 100%)	*Chelonobacter oris* (87%)	Kudirkiene *et al*. (2014)
OTU00022	1.48E-07	2.34	0.811	*Fibrobacter* (Genus; 100%)	*Fibrobacter succinogenes* (95%)	Suen *et al*. (2011)
OTU00078	1.87E-07	2.11	0.226	*Prevotella* (Genus; 100%)	*Prevotella dentalis* (92%)	Willems and Collins (1995)
OTU00075	2.47E-12	2.03	0.233	*Prevotella* (Genus; 100%)	*Prevotella multisaccharivorax* (92%)	Sakamoto *et al*. (2005)
Cow 3						
OTU00027	1.22E-13	2.41	0.571	Unclassified *Bacteroidia* (Class; 100%)	*Pontibacter russatus* (87%)	Maeng *et al*. (2020)
OTU00071	1.55E-07	2.36	0.244	*Prevotella* (Genus; 100%)	*Prevotella ruminicola* (96%)	Purushe *et al*. (2010)
OTU00046	1.82E-09	2.28	0.350	*Bifidobacterium* (Genus; 100%)	*Bifidobacterium castoris* (99%)	Duranti *et al*. (2019)
OTU00057	3.59E-05	2.21	0.289	*Prevotella* (Genus; 100%)	*Prevotella ruminicola* (93%)	Purushe *et al*. (2010)
OTU00030	1.33E-07	2.19	0.543	*Prevotella* (Genus; 100%)	*Prevotella brevis* (92%)	Avguštin *et al*. (1997)
OTU00091	4.44E-09	2.15	0.194	*Ruminococcaceae* (Family; 97%)	*Ruminococcus bromii* (96%)	Ze *et al*. (2012)
OTU00047	1.35E-05	2.09	0.349	*Methanobacteriaceae* (Family; 100%)	*Methanosphaera stadtmanae* (97%)	Fricke *et al*. (2006)
OTU00097	6.24E-08	2.03	0.180	*Prevotella* (Genus; 100%)	*Prevotella ruminicola* (92%)	Purushe *et al*. (2010)
OTU00069	2.48E-08	2.01	0.258	*Prevotella* (Genus; 100%)	*Prevotella ruminicola* (95%)	Purushe *et al*. (2010)
Cow 4						
OTU00011	4.14E-05	2.33	1.20	*Prevotella* (Genus; 100%)	*Prevotella ruminicola* (93%)	Purushe *et al*. (2010)
OTU00026	8.39E-09	2.13	0.583	*Prevotella* (Genus; 100%)	*Prevotella ruminicola* (90%)	Purushe *et al*. (2010)

Table 3.17: OTU level taxonomic biomarkers in dairy cows fed diets either with (+) or without (-) yeast supplementation (Linear discriminant analysis (LDA) score > 2.0, P < 0.05)

	P-value	LDA score	% Seqs	Silva 132 SEED taxonomy (mixed rank)	BLASTn Type (% Ident) (Accessed 10/11/2021)	Reference
No yeast						
OTU00001	0.044721	-2.77	6.68	Unclassified *Gammaproteobacteria* (Genus; 100%)	*Chelonobacter oris* (87%)	Kudirkiene *et al*. (2014)
OTU00019	0.008804	-2.18	0.83	Unclassified *Prevotellaceae* (Family; 100%)	*Prevotella copri* (89%)	Iljazovic *et al*. (2021)
Yeast						
OTU00009	0.003309	1.99	1.32	*Clostridiales* (Class; 100%)	*Gracilibacter thermotolerans* (87%)	Lee *et al*. (2006)

Table 3.18: OTU level taxonomic biomarkers at 0, 3, 12 h post morning feed in dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation (Linear discriminant analysis (LDA) score > 2.0, P < 0.05)

	P-value	LDA Score	% Seqs	Silva 132 SEED taxonomy (mixed rank)	BLASTn Type (% Ident) (Accessed 10/11/2021)	Reference
O h						
OTU 00010	6.81E-04	2.14	1.21	Unclassified *Ruminococcaceae* (Family; 100%)	*Marseillibacter massiliensis* (92%)	Ndongo *et al*. (2017)
OTU00009	0.026737	2.05	1.32	*Clostridiales* (Class; 94%)	*Gracilibacter thermotolerans* (87%)	Lee *et al*. (2006)
3 h						
OTU00015	0.00349	2.69	1.08	*Lactobacillus* (Genus; 100%)	*Lactobacillus nangangensis* (99%)	Liu and Gu (2019)
OTU00006	0.006828	2.62	1.62	*Lactobacillus* (Genus; 100%)	*Lactobacillus fuchuensis* (100%)	Sakala *et al*. (2002)
12 h						
OTU00001	0.014761	3.12	6.68	Unclassified *Gammaproteobacteria* (Genus; 100%)	*Chelonobacter oris* (87%)	Kudirkiene *et al*. (2014)

3.3.7.4 Protozoa

Pattern of concentrate allocation or yeast supplementation had no effect (P < 0.05) on the relative abundance of the protozoa *Isotrichidae* (Table 3.19). The relative abundance of *Isotrichidae* was highest at 0600 h and then decreased by 11.5 cells per 1000 in the first three hours post feeding, and remained consistent until 1800 h.

Table 3.19: Relative abundance (*Isotrichidae* cells per 1000 cells of *Ophryoscolecidae*) of rumen protozoa in dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation

	Treatments					P-value						
	E+	E–	U+	U–	SED	T	C	Y	C x Y	T x C	T x Y	T x C x Y
0600 h	23.3	30.2	25.0	24.1	2.94	0.006	0.855	0.424	0.243	0.317	0.564	0.523
0900 h	14.3	17.3	10.3	14.4								
1800 h	12.6	16.1	15.8	21.8								

T= Time, C= Concentrate allocation, Y= inclusion of yeast, C x Y= interaction between C and Y

3.4 Discussion

3.4.1 Forage and diet composition

The current study was conducted to determine the effect of varying pattern of concentrate allocation on performance, digestibility, rumen metabolism and the microbiome in high yielding dairy cows. The basal PMR was the same for all treatments, and all cows received the same proportion of forage and concentrates. The DM content of the grass silage was lower than described by Sinclair *et al*. (2015) however the mean DM content of the PMR was consistent with results reported by Tayyab *et al*. (2019) for rations containing GS and MS in the UK. The NDF content of GS and MS were also comparable to those reported by Tayyab *et al*. (2019). Forage pH and VFA concentration and ratios (including lactate) were similar to those of Little *et al*. (2018) and Purcell *et al*. (2016).

One of the primary objectives of the study was to determine the effect of altering pattern of concentrate allocation on rumen metabolism and the microbiome in high yielding dairy cows. In preliminary work 6 kg of concentrates was fed, however, when monitoring the rumen parameters pH was consistently low (pH< 5.8) and the cows were at an increased risk of both SARA and acute rumen acidosis. Therefore the decision was made to reduce the portion of concentrates to 4 kg/d, to avoid compromising the health of the cows, as it is a commonly accepted amount fed in one meal in parlour feeders (Lawrence *et al*., 2015).

3.4.2 Performance

Pattern of concentrate allocation or yeast supplementation had no effect on DMI, a finding in agreement with Lawrence *et al.* (2015) and Little *et al*. (2018) who reported similar findings when altering the pattern of concentrate allocation in dairy cows and Dias *et al*. (2018) who supplemented the diet with yeast in dairy cows. When fed varied patterns of concentrate allocation in the current study cows still received the same daily allocation of concentrates and forage, whereas when cows select concentrates from a TMR the amount of concentrates and the ratio of concentrates to forage can differ (Tayyab *et al*., 2018). Yeast can have beneficial effects on rumen metabolism when conditions are compromised, and Ferreira (2019) suggested that when the nutritional composition of the diet was not challenging to the rumen performance may not show any benefit with yeast supplementation, which may be the case in the current study. There was no treatment effect on milk yield or composition, which is in accordance with the results reported by Sutton *et al*. (1985) for pattern of concentrate allocation, and Kumprechtová *et al.* (2019) and Ambriz-Vilchis *et al*. (2017) with yeast supplementation. Milk yield is directly correlated to DMI

(Guinard-Flament *et al*., 2006) and both DMI and nutrient digestibility were unaffected by pattern of concentrates or yeast supplementation in the current study.

3.4.3 Diet digestibility and nitrogen balance

Pattern of concentrate allocation or yeast supplementation had no effect on DM or OM digestibility or N balance. These findings are consistent with Ferreira (2019) for yeast supplementation, and Sutton *et al*. (1985) and Robinson and Sniffen (1985) for pattern of concentrate allocation while Ghazanfar *et al.* (2015) reported that yeast supplementation increased DM digestibility. There was no change in the microbial beta diversity in the current study with pattern of concentrates, suggesting that there was minimal change to the microbial community, and supports the lack of an effect on whole tract digestibility. However, there tended to be an interaction between pattern of concentrate allocation and yeast supplementation on NDF digestibility in the current study, with cows supplemented with yeast having a lower digestibility when fed an uneven but not even pattern of allocation.

3.4.4 Rumen and plasma ammonia concentrations

Cellulolytic bacteria use NH_3 as their primary N source during fibre degradation (Hristov *et al*., 2010), therefore the improvement to fibre degradation in the rumen facilitated by yeast supplementation may cause rumen NH_3 concentration to decrease. However, Thrune *et al*. (2009) and Hristov *et al*. (2010) found no change in rumen NH_3 concentration with yeast supplementation at 140 and 56 mg/L, respectively, suggesting that the effect of change in fibre degradation on NH_3 concentration may be less consequential than other factors such as nitrogen recycling. However, in the current study NH_3 increased with yeast supplementation, which may have been due to a decrease in acetate concentration therefore reducing the amount of NH_3 that was utilised during microbial activity. This is supported by the findings that plasma NH_3 concentration decreased with yeast supplementation, suggesting that excess N was not being removed from the rumen for excretion. Therefore it could be theorised that any excess rumen nitrogen was recycled back into the rumen from the liver and not excreted in urine or faeces.

3.4.5 Rumen metabolism and the microbiota

Supplementation of yeast tended to increase mean rumen pH by 0.07 units compared to without yeast. Yeast has many mechanisms which can increase rumen pH, including scavenging oxygen which can encourage more favourable conditions for fibre degrading bacteria (Chaucheyras-Durand *et al*., 2008). Additionally, yeast can stimulate lactate utilising bacteria, such as *Megasphaera elsdenii* reducing lactate concentration in the rumen and

therefore reducing diurnal variation of rumen pH (Kumprechtová *et al*., 2019), this is supported by the increase in relative abundance of OT00009 from the Firmicutes phyla with yeast supplementation in the current study, which while being most closely related to *Gracilibacter thermotolerans* (87%), is most likely a novel species due to the low % identity.

For rumen pH, there was an interaction between the pattern of concentrate allocation and time in the current study. When cattle were fed an uneven pattern, they received 4 kg of concentrates in the first feed resulting in rumen pH dropping by 0.6 units in the first three hours post feeding compared to a drop of 0.327 units when fed an even pattern of concentrates. Furthermore, at three hours post feeding there was an increase in OTU associated with multiple species of *Lactobacillus,* which are linked to the fermentation of concentrates (Yang *et al*., 2018), although there was no visible change in rumen lactate concentration with pattern of concentrates. At six hours post feeding, rumen pH remained constant at approximately pH 5.7 in cows fed U, for the remainder of the day cows received the forage based PMR, encouraging a lift in pH at 12 h post feeding. In contrast when cattle were fed E, rumen pH declined at a steadier rate, these findings are similar to those found by Sutton *et al.* (1986) who reported that when cows were fed frequent meals rumen pH was more consistent while fewer meals resulted in a rapid drop in rumen pH in the first three hours after the initial feed, then increased within 12 h of feeding.

Pattern of concentrate allocation had no effect on rumen VFA concentration, a finding in agreement with Yang and Varga (1989). In contrast, in the current study yeast supplementation tended to decrease total VFA and acetate concentration, findings in contrast that of Desnoyers *et al*. (2009) who reported that with yeast supplementation increase total VFA concentration from 95.2 µM to 97.3 µM. It is important however to take into account that VFA concentration does not equate to VFA production, as the rumen is dynamic and VFA are constantly being produced and absorbed (Dijkstra, 1994a). Yeast can stabilise rumen conditions and has been reported to promote fibre degradation and potentially increase acetate production (Chaucheyras-Durand *et al*., 2008; Dias *et al*., 2018). Despite the decrease in VFA concentration in cows fed yeast, nutrient digestibility and performance were not affected. Additionally, the A:P ratio remained consistent throughout the day in cows supplemented with yeast, and decreased in cows when diets were unsupplemented. The effects of dietary addition of yeast in rumen A:P ratio in the current study are inconsistent, with Desnoyers *et al*. (2009) reporting that yeast supplementation had no effect on the ratio while Dias *et al*. (2018) reported that when dairy cows were supplemented with yeast A:P ratio decreased.

In the current study there was a higher alpha and beta diversity in microbial communities associated with SPD than LPD samples, and in SPD there was a higher OTU richness in cows fed an uneven pattern of concentrates. When cows were fed an uneven pattern of concentrates they received a diet which had a high proportion of concentrates in the morning, and then the more forage based PMR for the rest of the day, which may have encouraged the growth and activity of both amylolytic and cellulolytic bacteria during the different times of the day. In contrast, when cattle were fed the even pattern of concentrates the diet was more consistent throughout the day, which may have encouraged a more stable microbial community. Furthermore, the effect of concentrate allocation was only observed on OTU richness in the SPD group which contained more fibre degrading bacteria such as *Treponema bryantii* (OTU00005) as they have a very specific function and adhere to forage particles. In contrast, amylolytic bacteria such as *Prevotella*, are present mainly in the LPD group and are much more diverse in their function and are able to degrade both starch and fibre. As a consequence, an uneven pattern of concentrates may not cause such a large shift in the microbial population associated with the liquid phase (Tapio, *et al*., 2017; Bowen *et al*., 2018).

Clustering of samples from cows fed yeast supplementation was due to an increased relative abundance in the OTU most closely related to *Chelonobacter oris* and *Prevotella copri* compared to without yeast, although with both OTU sharing <90% similar identity to the assigned taxa they are likely to belong to different genera which are currently unidentified. The NCBI BLASTn type strain search in the current study of the representative sequence identified OTU00001 as *Chelonobacter oris*, and SILVA 132 SEED identified OTU00001 as unclassified Gammaproteobacteria, Snelling *et al*. (2018) conducted phylogenetic analysis and identified that the OTU is likely a close relation of the family *Succinivibrionaceae*. *Succinivibrionaceae* is associated with producing succinate, and combined with the relative increase in *Prevotella* also seen in cows fed diets without yeast supplementation which has been known to convert succinate to propionate in the rumen, which when present in high quantities, can increase the risk of SARA (Reichardt *et al*., 2014). There was an increased relative abundance of OTU0009 assigned to *Gracilibacter thermotolerans* with yeast supplementation, although at <90% it may also be a novel and uncultured species. However, SILVA 132 SEED identifies OTU00009 as a member of the Clostridiales class which is associated with fibre degradation, and suggests that the yeast supplementation may have a beneficial effect on promoting fibrolytic bacteria activity which can increase rumen pH, which supports findings in the current study.

Pattern of concentrate allocation and yeast supplementation had no effect in the current study on the relative abundance of *Isotrichidae* and *Ophryoscolecidae* protozoa. Ishaq *et al*.

(2017) also reported that yeast supplementation had no effect the protozoal community when diets that were low or high in starch were fed. However, the effect of altering pattern of concentrate meals has not been researched as thoroughly.

3.5 Conclusions

Pattern of concentrate allocation and yeast supplementation had no effect on the performance, digestibility (DM, OM and N), plasma metabolites (BHB, glucose and urea) concentration, or eating behaviour in dairy cows. Yeast supplementation tended to increase rumen pH, rumen and plasma NH_3 concentrations, whilst total rumen VFA and acetate concentration tended to decrease. There was an increase in OTU associated with Clostridiales when cows were supplemented with yeast and increase in unclassified *Gammaproteobacteria* and *Prevotella copri* when fed diets without yeast supplementation indicating that yeast promoted fibrolytic bacteria. Diurnal variation of rumen pH and A:P ratio changed with pattern of concentrate allocation, whilst the OTU richness in SPD tended to increase when cows were fed the uneven diet indicating that an uneven pattern of concentrate allocation was associated with increased variation, while an even pattern of concentrates encouraged a more stability in the rumen microbiota.

Chapter 4: Effect of short-term feed restriction and re-feeding on the rumen microbiome, metabolism and performance of high yielding dairy cows fed different concentrate patterns and either with or without a yeast

4.1 Introduction

Tayyab *et al*. (2018) reported that periods of short-term feed restriction (FR) occurs on over a third of dairy farms in the UK. Feed restriction can occur due to many reasons, including feeding equipment failure or insufficient allocation of feed or pasture, and as a result cows are not provided with their full daily requirements of nutrients (Thomson *et al*., 2018; Zhang *et al*., 2013). Additionally, cattle may have access to feed removed for a few hours each day due to milking, health checks, or when feed is not frequently pushed up to the feed barrier (Thomson *et al*., 2018).

During the FR period rapidly degradable carbohydrates are digested, leaving a high proportion of fibrous material (Thomson *et al*., 2018). Rumen VFA continue to be absorbed but the rate of synthesis is reduced due to the lack of substrate for fermentation, which can result in an increase in rumen pH (Thomson *et al*., 2018). Furthermore, cows may continue to ruminate and produce saliva during the fasting period which can increase rumen pH (Thomson *et al*., 2018). Upon re-feeding following FR cows can enter a period of overeating, rapidly increasing their intake of concentrates in a short period of time. Thomson *et al*. (2018) reported that following feed deprivation cattle spent more time eating in the first three hours compared to the baseline, spending 57% of their time eating compared to 29%, respectively. Concentrates often contain rapidly degradable starch and when cattle increase their concentrate intake in larger, fewer meals microbial activity is altered resulting in an increase in amylolytic bacteria such as *Prevotella*, and lactate producing bacteria such as *Streptococcus bovis* increasing VFA and lactate production (Krause and Oetzel, 2006). This can increase the risk of developing SARA (Oetzel, 2007), defined as periods of pH depression lasting more than 5 to 6 h/d during which rumen pH is <5.8 (Zebeli *et al*., 2012). Furthermore, at low pH fibrolytic bacteria such as *Fibrobacter* activity and growth is inhibited which can compromise fibre degradation in the cow (Allen, 2000).

The use of active dry yeasts is common in ruminant nutrition as they have been reported to reduce the variability of rumen pH by reducing the lactate concentration in the rumen by competing with other lactate producers such as *Streptococcus bovis* for substrate availability. Additionally, yeast can stimulate growth of lactate utilising bacteria such as *Megasphaera elsdenii* and *Selenomonas ruminantium*, stabilising conditions for microbial activity, particularly fibrolytic bacteria (Newbold *et al*., 1996; Chaucheyras-Durand *et al*.,

2016). Yeast can also improve performance including increased dry matter intake (DMI) and milk yield (Barrera *et al*., 2019) but their effects on rumen metabolism and the microbiome following a period of FR is unclear. The objective of the study was to determine the effect of short-term feed restriction and re-feeding on rumen metabolism, the microbiome and performance of high yielding dairy cows when fed different concentrate patterns and either with or without a live yeast.

4.2 Materials and Methods

This experiment formed part of the study reported in Chapter 3. The procedures for the animals used in this experiment were conducted in accordance with the UK Animals Scientific Procedures Act (1986; amended 2012) and were approved by the local ethics committee at Harper Adams University.

4.2.1 Animals, diets and experimental design

The same cows used in Chapter 3 were kept under the same conditions and feed the same diets for the current study, with a short-term FR and re-feeding period occurring at the end of each period. Briefly, four Holstein-Friesian dairy cows that had previously been fitted with a 10 cm permanent rumen cannula (Bar Diamond, Idaho, USA) were used. The cows were 69 days (SE ±12.1) post-calving, weighed 650 kg (± 26.2), were in their second lactation, and were yielding 40 kg (±2.6) of milk per day at the start of the study. Each cow was randomly assigned to one of four dietary treatments in five week periods as part of a 2 x 2 factorial Latin square design study and remained on study for 20 weeks (140 days) with five week periods.

All cows were fed a partial mixed ration (PMR; Table 4.1) with a forage: concentrate ratio of 60:40 (DM basis), and a grass silage: maize silage ratio of 45:55 (DM basis) via individual Calan gates (American Calan, Northwood, NH, USA) at approximately 0730 h at a rate of 105% of the previous recorded intake, with refusals collected three times a week (Monday, Wednesday and Friday). An additional 4 kg/cow/d of concentrates (Table 4.*1*) was provided, to reflect the potential range of concentrate intake due to diet selection (Tayyab *et al*., 2018), in one of two patterns of allocation: uneven (U) with all 4 kg provided at 0600 h, or even (E), with the concentrates provided in four equal meals of 1 kg at 0600, 1000, 1400 and 1700 h. In addition, each diet was either supplemented (+) or unsupplemented (–) with Yea-Sacc® (Alltech UK) at a rate of 1 g/cow/day, which was provided in the concentrates. The diets were formulated according to Thomas (2004) to meet the metabolisable energy and metabolisable protein requirements of a dairy cows yielding 40 kg/d at 32 g/kg protein and 40 g/kg fat content.

The four dietary treatments were:

U– 4 kg concentrates fed in one meal, no supplement

U+ 4 kg concentrates fed in one meal, with Yea-Sacc®

E– 4 kg concentrates in four meals, no supplement

E+ 4 kg concentrates in four meals, with Yea-Sacc®

Table 4.1: Diet composition (DM and fresh weight basis) and predicted chemical composition for a 675 kg cow yielding 37 kg/d

Ingredient	kg DM/d	kg fresh/d
Maize silage	7.5	21.5
Grass silage	6.2	19.7
Concentrate	9.3	10.6
Total	23.0	51.8
Predicted chemical analysis, g/kg DM	Total diet	Partial mixed ration
Forage:concentrate (DM basis)	0.60	0.70
ME, MJ/kg DM	11.7	11.5
Crude protein	162	153
NDF	363	389
Sugar	59	49
Starch	206	195
Rumen degradable starch	178	169
Oil	40	39
MPE[1], g/kg DM	105	
MPE[2], % requirements	103	
MPN[3], g/kg DM	114	
MPN,% requirements	112	

[1]ME, metabolisable energy

[2]MPE, metabolisable protein-rumen energy limited

[3]MPN, metabolisable protein-rumen nitrogen limited

On sampling day eight of each period, performance was recorded (intake and milk yield) with milk samples collected for the subsequent determination of composition (Table 4.2). Rumen samples were also collected for subsequent determination of metabolism (pH, VFA, NH_3, lactate) and microbiome. On sampling day nine, cows received their PMR at a rate of 75% of their recorded intake from the previous week, with any feed remaining at midnight removed, and rumen pH and performance measured. The PMR was reintroduced at 0600 h, the following day (day 10, recovery day 1; rec d1) at a rate of 125% of their recorded intake from the previous week, and rumen metabolism and performance measurements recorded. Additional intake, milk yield and rumen pH measurements were also recorded on sampling

day 11 (recovery day 2; rec d2). Allocation of concentrates (even/uneven) remained the same each day.

Table 4.2: Experimental routine for sampling week of dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation, during a short-term feed restriction period (FR)

	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13
Day	Baseline	Feed restriction	Rec d1	Rec d2	Rec d3	Rec d4
Allocation of ration for cows, based off previously recorded intake	105%	75%, access to feed removed from midnight to 0600 (day 10)	125%	105%	105%	105%
Milk yield (AM and PM)	X	X	X	X	X	X
Milk composition	PM	AM	PM	AM		
Intake (Weighed feed at 0, 4, 8, 12, 24 h)	X	X	X	X	X	X
Rumen fluid sampling[1]	X	pH only	X	pH only		

Rec d= Recovery day

[1] Rumen sampling for pH, VFA, NH_3, microbiome analysis at 0, 3, 6, 9, 12, 15 h post concentrate feed

4.2.2 Experimental routine

4.2.2.1 Intake and milk parameters

Intake was recorded daily during the sampling week of each period, and from days eight to 11 of each sampling period intake was also measured at four hourly intervals from 0730 to 1930 h. Forage, PMR and concentrate samples were collected daily during the sampling period and stored at -20°C for subsequent analysis. Cows were milked twice daily using a portable milking machine (Milkline, London, UK) at 0600 and 1600 h with milk yield recorded at each milking from day 8 to 13 and samples collected on four occasions (two morning and two evening milkings) during week five of each period for subsequent analysis (fat and protein).

4.2.2.2 Rumen digesta sampling

Rumen fluid samples were collected on days 8 to 11 of each sampling period, at 0 (immediately before morning feeding), 3, 6, 9, 12, and 15 h post morning feed, using a method adapted from Martin *et al*. (1999). Four grab samples of digesta were taken from the ventral region of the rumen, by inserting an arm directly down approximately 50 cm through the cannula and grabbing a large handful of digesta, then placing into a bucket. Further fluid was collected by inserting a 250 ml glass bottle into the same area. The rumen fluid and digesta was then strained through four layers of muslin cloth to separate the solid digesta from the liquid. The pH of the strained rumen fluid was recorded immediately after samples were taken using a calibrated portable pH meter (Hanna Instruments, Bedfordshire, UK), and samples collected on days 8 and 10 were stored in a 25% HPO_3 solution at -20°C for subsequent analysis of VFA and ammonia.

Rumen fluid and digesta collected on sampling days 8 and 10 at three time points (0, 3, 12 h post morning feed) for SPD and LPD samples were stored in 15% glycerol solution at -20°C for subsequent microbial community analysis. Liquid samples were stored in a 10% formalin/saline solution at room temperature for analysis of ciliate protozoa identification and counting.

4.2.3 Chemical analysis

Forage, PMR and concentrate samples were bulked between days for each period, and sub-samples analysed for DM (943.01), CP (990.03; intra-assay CV of 0.147%) and ash (942.05) according to the Association of Official Analytical Chemists (2012). Neutral detergent fibre (using heat stable α-amylase; Sigma, Gillingham, UK) was determined according to Van Soest *et al*. (1991) and expressed exclusive of residual ash (intra-assay CV of 0.943%). Milk composition was analysed by National Milk Laboratories (Wolverhampton, UK) for fat, and protein using near midinfrared (MIR; Foss, Denmark).

Volatile fatty acids were analysed in the liquid rumen fluid fraction, by GC according to Erwin *et al*. (1961) using a column (DBFFAP, 30 m x 0.250 mm x 0.2 µm; Agilent J and W, GC columns, UK) and flame ionisation detector (Agilent 6890, Stockport, UK), as described in Section 2.13. Lactate analysis was conducted on rumen fluid samples by high performance liquid chromatography (Agilent 1100, Germany), as described in Section 2.14. Rumen ammonia concentration was measured from the liquid fraction from a method adapted from MAFF (1986) and using an auto-titrator (FOSS 1030 auto-titrator, FOSS, Warrington, UK; Buchi Labortechnik AG CH-9230, Flawil, Switzerland).

Ciliate protozoa identification and counts of relative abundance of *Isotrichidae* and *Ophryoscolecidae* was conducted using a light microscope (Olympus CX31, Olympus, Tokyo) with a 10X objective and 10X/20 eyepiece (Section 2.16.7). Microbial community analysis was undertaken using 16S rRNA gene amplicon sequencing (as described in Section 2.16). The DNA extraction was undertaken at Harper Adams University using the bead beating protocol based on Yu and Morrison (2004; Section 2.16.3). Amplicon libraries were prepared using dual index primers based on Kozich *et al*. (2013; Section 2.16.4), the order of the primers used are described in Appendix 8.2. The libraries were quality assessed using an Agilent 2100 Bioanalyzer System (Agilent Technologies. Santa Clara, CA, US) and sequenced using the Illumina MiSeq v2 250 paired end reagent kit to yield 11M sequences (Illumina UK, Cambridge, UK.).

4.2.4 Statistical analysis

Performance and rumen metabolism parameters were evaluated by repeated measures analysis of variance as a Latin Square design with a 2 x 2 factorial treatment structure using GenStat 18.1 (VSN International Ltd, Oxford, UK). The repeated measure to monitor feed restriction was measured by day (baseline, FR, rec d1 and rec d2), with main effects of feed restriction and re-feeding (FR), pattern of concentrate allocation (C), addition of yeast (Y), time of sampling during the day (h) and their interactions (Int). Results are reported as treatment means with SED, with the level of significance set at $P < 0.05$, and a tendency stated at $P < 0.10$.

Microbial community data were analysed using mothur v1.44.0 (Schloss *et al*., 2011), assembling paired end sequences and removing low quality sequences. Sequence counts from the library were normalised by subsampling to 40,000 sequences per sample prior to statistical analysis. Sequences were clustered into operational taxonomic units (OTU) at 97% identity, and taxonomic classification of the representative sequences was carried out using the SILVA 132 SEED reference database (Yilmaz *et al*., 2014).

Microbial species richness and diversity were summarised using Chao1, Shannon, inverse Simpson, and number of observed OTU (OBS) indices. Beta diversity was calculated using the Bray Curtis dissimilarity metric, with a significance level from analysis of molecular variance (AMOVA) set at $P < 0.001$. Taxonomic biomarkers associated to the respective treatment groups were determined by comparing relative abundance of OTU using Linear Discriminant Analysis (LDA) Effect Size (LEfSe; Segata *et al*., 2011) with minimum LDA score >2.0 and $P < 0.05$.

4.3 Results

4.3.1 Forages and diets

The forage and diets used are described in Table 3.2, Section 3.3.1.

4.3.2 Intake, production and milk composition

The pattern of concentration allocation or yeast supplementation had no effect ($P > 0.05$) on performance (Table 4.3). Relative to the baseline, DMI was 5.14 kg lower ($P < 0.001$) during the FR period, and then 4.96 kg higher on rec d1 before returning to the baseline level on rec d2. On rec d1 DMI was consistently higher throughout the day compared to the other days (Figure 4.1). Milk yield decreased ($P < 0.001$) by 3.14 kg/d on rec d1 and remained on average 1.89 kg/d below the baseline until rec d4. There was an interaction ($P = 0.007$) between FR and pattern of concentrate allocation, with cows receiving E having a 1.56 kg/d higher milk yield than when fed U during the baseline, but during the recovery period (rec d1, rec d2, rec d3) milk yield decreased to a mean of 37.3 kg/d in all treatments ($P > 0.05$) until milk yield returned to baseline level on rec d4. There tended to be an interaction between FR and yeast supplementation ($P = 0.064$), on the FR day and rec d1 milk yield tended to be 2.56 kg/d lower in cows fed diets supplemented with yeast compared to without yeast, there was no difference between treatments during the baseline and the other recovery days (rec d2, rec d3, rec d4). There was no treatment effect ($P > 0.05$) on milk fat composition or yield, with mean values of 42.2 g/kg and 1.61 kg/d, respectively. Milk protein content increased ($P < 0.001$) during rec d1 by 1.36 g/kg compared to the baseline.

Table 4.3: Performance of dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation, during a short-term feed restriction period (FR)

	Treatments					P-value						
Item[1]	E+	E–	U+	U–	SED	C	Y	C x Y	FR	FR x C	FR x Y	FR x C x Y
DMI, kg												
Baseline	21.8	22.3	22.3	22.4	1.2	0.260	0.432	0.211	<0.001	0.291	0.091	0.127
FR day	17.7	16.6	17.0	16.9								
Rec d1	28.0	25.5	26.9	28.2								
Rec d2	22.5	22.1	21.4	22.8								
Rec d3	21.1	23.9	23.6	25.1								
Rec d4	22.7	22.0	22.3	24.2								
Milk yield, kg/d												
Baseline	39.3	40.7	38.3	38.5	1.85	0.792	0.280	0.679	<0.001	0.007	0.064	0.883
FR day	37.9	41.1	36.5	38.4								
Rec d1	33.9	37.1	35.6	37.6								
Rec d2	37.0	38.6	37.3	38.0								
Rec d3	37.8	37.7	38.3	38.6								
Rec d4	37.2	39.3	38.2	38.4								
Milk fat, g/kg												
Baseline	42.5	41.0	41.2	40.8	1.76	0.988	0.317	0.409	0.102	0.434	0.625	0.546
Rec d1	44.4	40.9	43.5	43.3								
Milk fat, kg/d												
Baseline	1.66	1.55	1.65	1.55	0.126	0.826	0.992	0.560	0.806	0.726	0.065	0.298
Rec d1	1.53	1.74	1.60	1.59								
Milk protein, g/kg												
Baseline	30.0	30.6	29.7	30.6	0.49	0.901	0.716	0.064	<0.001	0.409	0.303	0.867
Rec d1	32.1	30.9	31.5	31.9								
Milk protein, kg/d												
Baseline	1.19	1.22	1.19	1.16	0.043	0.516	0.490	0.304	0.066	0.614	0.193	0.574
Rec d1	1.11	1.20	1.15	1.15								

FR= Feed restriction and re-feeding (in days), C= Concentrate allocation, Y= Inclusion of yeast, x= interaction between treatments

[1] Baseline data were collected on d 30, the deprivation day was d 31, during which animals were offered 75% of their previously recorded PMR intake and spent 6 h of the day fasting. Recovery days 1 and 2 were the subsequent 24-h periods (d 32 and 33, respectively), on rec d 1 cows were offered the PMR at a rate of 125% of their previously recorded PMR intake.

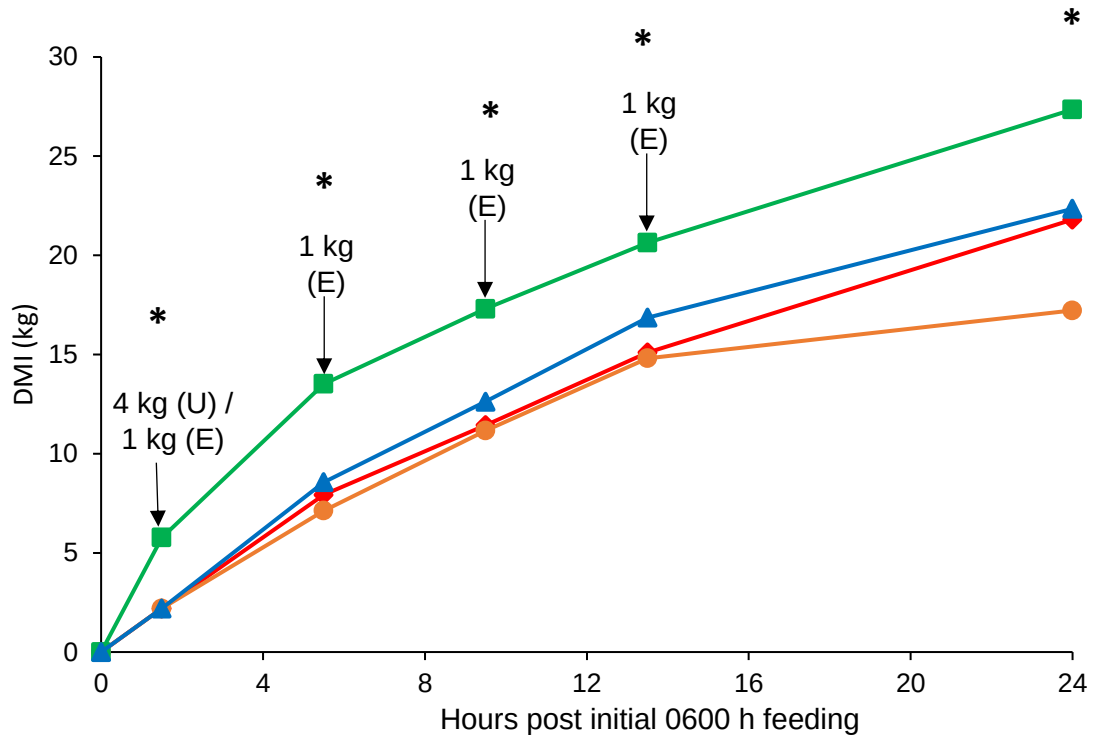


Figure 4.1: Mean dry matter intake (kg) in dairy cows during the baseline (♦), feed restriction (FR; ●) and recovery periods (day one (■) and two (▲)). (SED = 0.651; Time (h), P <0.001; FR, P <0.001; Time x FR, P = <0.001).

4.3.3 Rumen pH and ammonia

Pattern of concentrate allocation or yeast supplementation had no effect on rumen pH and ammonia concentration (Table 4.4). On rec d1 mean rumen pH not different (P > 0.05) from the baseline, however, was lower (P < 0.001) during rec d2 by 0.088 units compared to the baseline. There was an interaction (P = 0.004) between FR period and yeast supplementation on rumen pH during the baseline day, with cows fed yeast having a rumen pH that was 0.103 units higher than the baseline, but during rec d2 yeast supplementation decreased rumen pH by 0.056 units. Maximum rumen pH was 0.416 units higher (P < 0.001) during rec d1 than the baseline. There were no interactions (P > 0.05) between FR, pattern of concentrate allocation or yeast supplementation on maximum or minimum rumen pH, and rumen NH_3 concentration. Rumen lactate concentration was higher (P = 0.03) on rec d1 with a mean value of 0.73 µM compared to 0.02 µM in the baseline. On rec d1 rumen pH was 0.379 higher at 0600 h (immediately before re-feeding) than the baseline (Figure 4.2), and by 6 h post concentrate feeding mean rumen pH dropped to pH 5.52, and increased to pH 5.73 by 2100 h. On rec d2 pH was 0.176 higher than the baseline at 0600 h then decreased to pH 5.57 between 3 and 15 h post concentrate feed. Mean NH_3 concentration was 19.1 mg/L higher (P < 0.001) on rec d1 than the baseline, and was 66.4 and 41.5 mg/L higher at 3 and 6 h post concentrate feeding, respectively, than the baseline level (Figure 4.3).

Table 4.4: Rumen metabolism in dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation, during a short-term feed restriction period (FR)

	Treatments					P-value						
Item[1]	E+	E–	U+	U–	SED	C	Y	C x Y	FR	FR x C	FR x Y	FR x C x Y
Mean rumen pH												
Baseline	5.86	5.77	5.89	5.77	0.029	0.784	0.472	0.381	<0.001	0.687	0.004	0.688
Rec d1	5.85	5.85	5.84	5.84								
Rec d2	5.69	5.79	5.71	5.72								
Maximum rumen pH												
Baseline	6.29	6.38	6.37	6.29	0.031	0.019	0.569	0.612	<0.001	0.266	0.950	0.498
Rec d1	6.70	6.61	6.82	6.85								
Rec d2	6.53	6.52	6.56	6.55								
Minimum rumen pH												
Baseline	5.44	5.43	5.40	5.46	0.068	0.729	0.372	0.410	0.163	0.309	0.718	0.973
Rec d1	5.45	5.43	5.37	5.46								
Rec d2	5.32	5.35	5.35	5.47								
pH range												
Baseline	0.85	0.95	0.97	0.83	0.062	0.289	0.225	0.263	<0.001	0.158	0.873	0.658
Rec d1	1.25	1.18	1.45	1.39								
Rec d2	1.22	1.18	1.22	1.08								
Rumen NH_3 mg/L												
Baseline	65.5	40.4	60.3	52.4	12.64	0.544	0.155	0.258	<0.001	0.578	0.369	0.629
Rec d1	81.3	58.9	76.5	78.1								

FR= Feed restriction and re-feeding (in days), C= Concentrate allocation, Y= inclusion of yeast, x= interaction between treatments

[1] Baseline data were collected on d 30, the deprivation day was d 31, during which animals were offered 75% of their previously recorded PMR intake and spent 6 h of the day fasting. Recovery days 1 and 2 were the subsequent 24-h periods (d 32 and 33, respectively), on rec d 1 cows were offered the PMR at a rate of 125% of their previously recorded PMR intake.

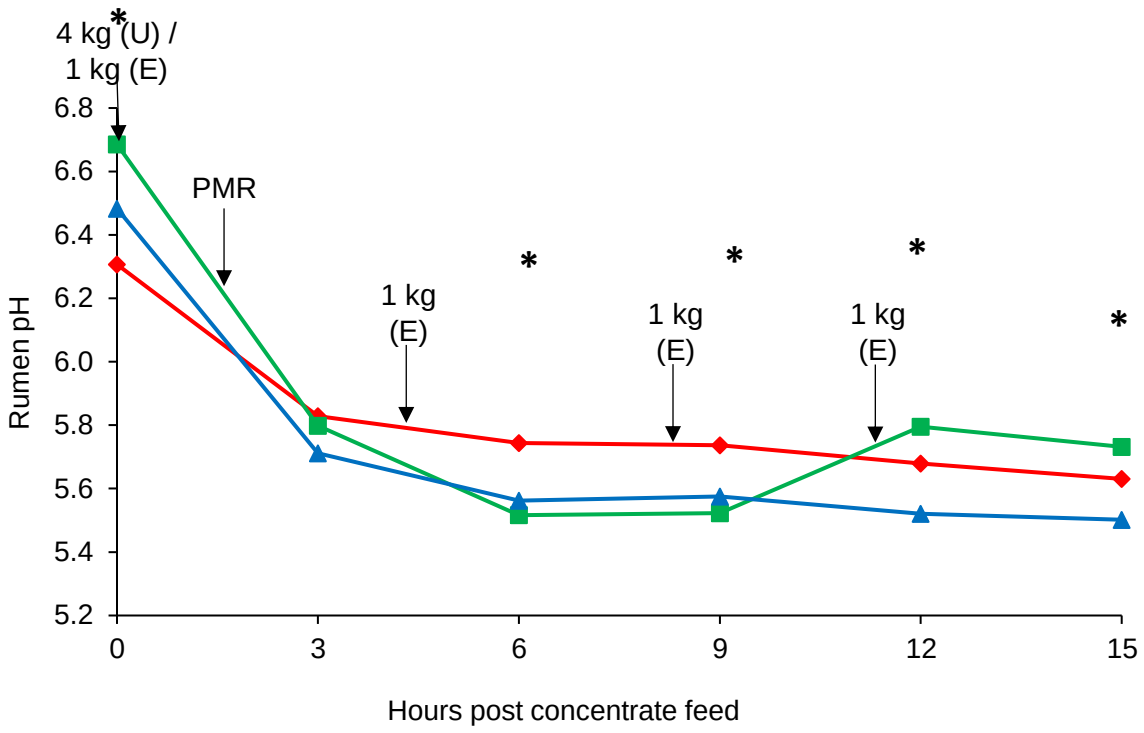


Figure 4.2: Mean rumen pH in dairy cows during the baseline (♦), and recovery periods (day one (■) and two (▲)). Meal times for even (E) and uneven (U) diets and partial mixed ration (PMR) are shown with an arrow (↓; SED = 0.066; Time (h), P < 0.001; FR, P < 0.001; Time x FR, P < 0.001). * P < 0.05.

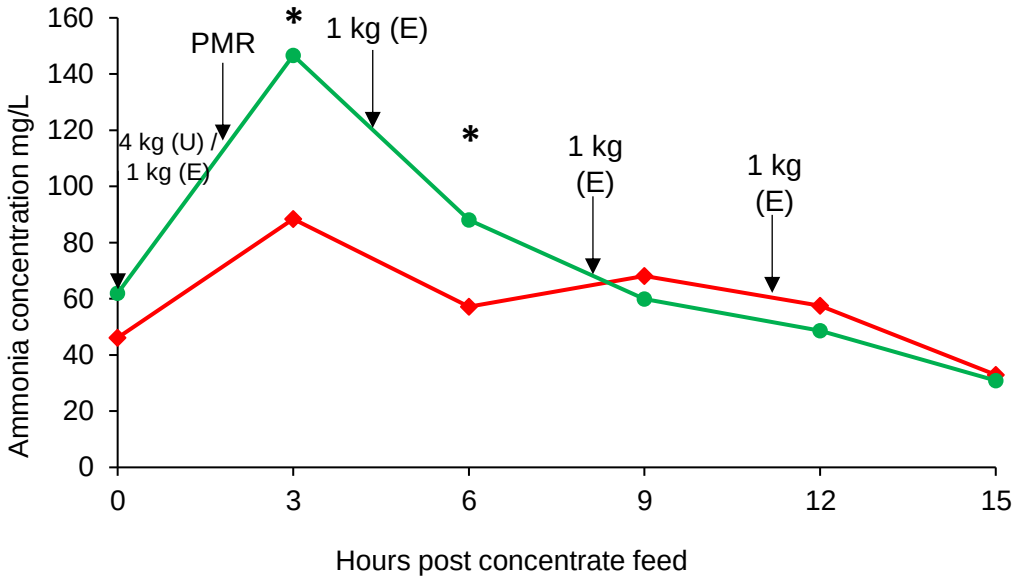


Figure 4.3: Mean rumen ammonia (NH_3) concentration (mg/L) in dairy cows during baseline (♦), and recovery day one (■). Meal times for even (E) and uneven (U) diets and partial mixed ration (PMR) are shown with an arrow (↓; SED =10.60; Time (h), P < 0.001; FR, P < 0.001; Time x FR, P < 0.001). * P < 0.05.

4.3.4 Rumen volatile fatty acids

Pattern of concentrate allocation, yeast supplementation and FR had no effect ($P > 0.05$) on total VFA, acetate, butyrate, iso-butyrate or valerate concentration, with mean values of 174, 123, 18.1, 1.04, 2.28 µM, respectively (Table 4.5). Mean propionate concentration decreased ($P = 0.012$) by 1.47 µM in cows on rec d1 compared to the baseline. There was a tendency for an interaction ($P = 0.057$) between FR and pattern of concentrate allocation for propionate concentration which had a mean value of 28.32 µM during the baseline, while on rec d1 was 2.73 µM higher when cows were fed E compared to U. The acetate to propionate ratio tended to increase ($P = 0.082$) on rec d1, and there was an interaction ($P = 0.012$) between FR and yeast supplementation on iso-butyrate concentration, which was higher during rec d1 in cows not supplemented with yeast (–) while the concentration decreased in cows fed diets supplemented with yeast. There was also a tendency for an interaction ($P = 0.061$) between FR and yeast supplementation on iso-valerate concentration; there was no effect of FR in cows not fed yeast while iso-valerate concentration decreased by 0.201 µM in those that were supplemented. Rumen lactate concentration was higher ($P = 0.03$) on rec d1 with a mean value of 0.73 µM on rec d1 compared to 0.02 µM in the baseline. There was an interaction ($P < 0.05$) between FR and time (h); total VFA concentration was lowest at 0600h compared to the baseline (Figure 4.4).

Table 4.5: Mean rumen volatile fatty acid content (µM) in dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation, either with (+) or without (–) yeast supplementation, during a short-term feed restriction period (FR)

	Treatments					P-value						
Item [1]	E+	E–	U+	U–	SED	C	Y	C x Y	FR	FR x C	FR x Y	FR x C x Y
Total VFA												
Baseline	168	179	167	187	10.1	0.617	0.018	0.795	0.731	0.138	0.535	0.224
Rec d1	165	192	162	176								
Acetate												
Baseline	117	125	118	133	7.6	0.741	0.010	0.885	0.921	0.136	0.427	0.266
Rec d1	115	137	113	125								
Propionate												
Baseline	27.1	30.1	26.6	29.6	1.95	0.244	0.088	0.562	0.012	0.057	0.442	0.181
Rec d1	26.4	30.0	25.2	25.8								
A:P ratio[2]												
Baseline	4.43	4.21	4.58	4.52	0.226	0.152	0.678	0.781	0.082	0.675	0.121	0.685
Rec d1	4.45	4.69	4.81	4.50								
Butyrate												
Baseline	17.6	18.3	17.4	18.2	1.13	0.967	0.363	0.763	0.174	0.630	0.950	0.378
Rec d1	17.6	18.8	18.3	18.5								
Iso-butyrate												
Baseline	1.04	1.03	1.00	1.06	0.079	0.782	0.244	0.660	0.547	0.604	0.012	0.571
Rec d1	1.00	1.11	0.97	1.10								
Valerate												
Baseline	2.26	2.39	2.22	2.37	0.191	0.967	0.231	0.957	0.257	0.575	0.557	0.947
Rec d1	2.15	2.34	2.16	2.37								
Iso-valerate												
Baseline	2.76	2.87	2.62	2.87	0.288	0.927	0.200	0.933	0.087	0.364	0.061	0.310
Rec d1	2.54	2.88	2.53	2.87								
Lactate												
Baseline	0.07	0.00	0.00	0.01	0.341	0.627	0.718	0.482	0.003	0.651	0.567	0.494
Rec d1	0.90	0.78	0.32	0.90								

FR= Feed restriction and re-feeding (in days), C= Concentrate allocation, Y= inclusion of yeast, x= interaction between treatments

[1] Baseline data were collected on d 30, the deprivation day was d 31, during which animals were offered 75% of their previously recorded PMR intake and spent 6 h of the day fasting. Recovery days 1 and 2 were the subsequent 24-h periods (d 32 and 33, respectively), on rec d 1 cows were offered the PMR at a rate of 125% of their previously recorded PMR intake.

[2] Acetate to propionate ratio

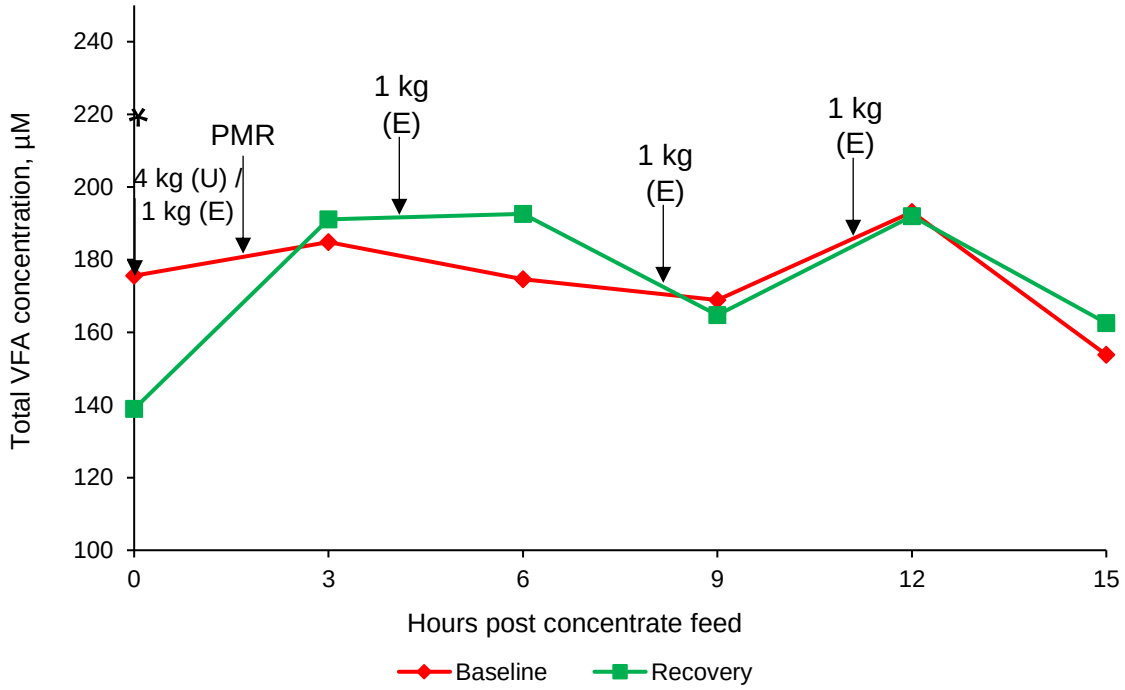


Figure 4.4: Mean rumen volatile fatty acid (VFA) concentration (µM) in dairy cows during baseline (♦), and recovery day one (■). Meal times for even (E) and uneven (U) diets and partial mixed ration (PMR) are shown with an arrow (↓; SED = 11.6; Time (h), P < 0.001; FR, P = 0.731; Time x FR, P = 0.013). * P < 0.05.

4.3.5 Microbial community analysis amplicon sequencing of 16S rRNA gene

Following sequencing there was a total of 27.4 million sequences, before quality control subsampling was conducted, and there were a total of 30,265 OTU identified. The libraries were normalised by subsampling to 40,000 reads per sample and there were 4257 OTU in total across all samples, those classified as low abundance (total number of reads per OTU <10) were removed from the dataset. Coverage was between 98.1% and 99.6% per library, measured by Good's statistic (Good, 1953). The total abundance taxonomic summary at phylum level were Bacteroidetes (40%), Firmicutes (31%), Proteobacteria (8%), Spirochaetes (7%), Euryarchaeota (6%), and Fibrobacteres (3%; Figure 4.5). The remaining 5% consisted of low abundance taxonomic groups and unclassified bacteria.

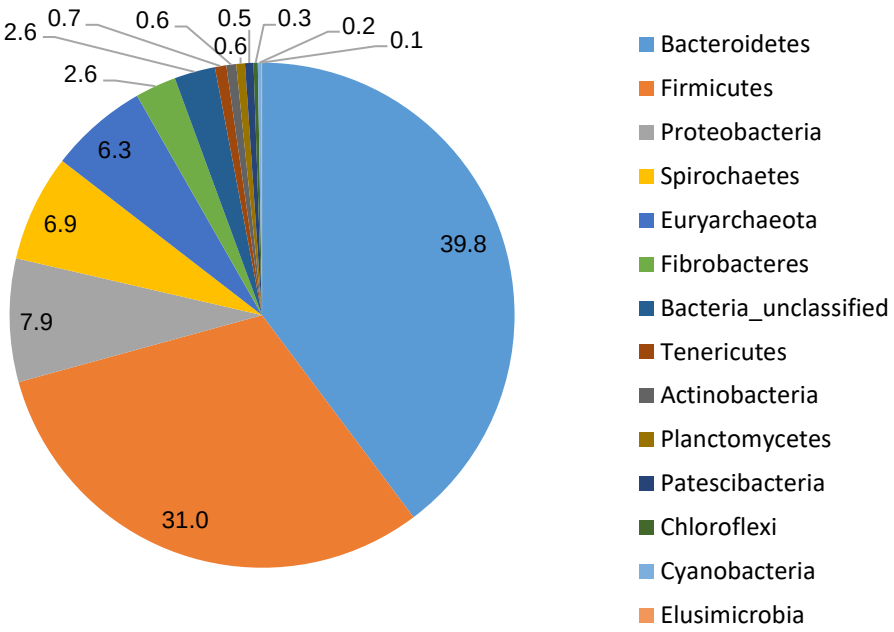


Figure 4.5: Relative abundance (%) of phyla of total dataset

4.3.5.1 Alpha Diversity

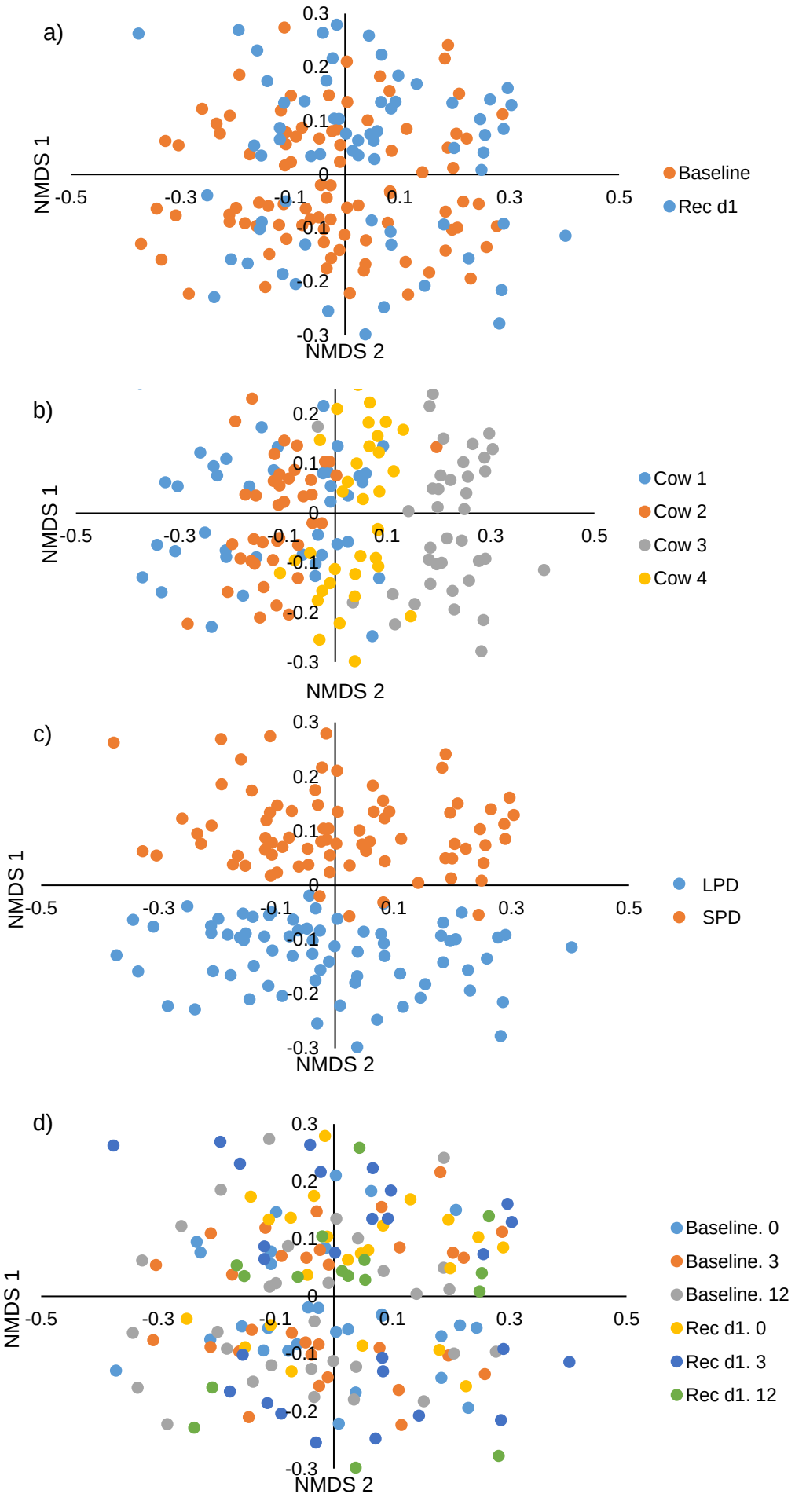
The number of observed OTU (OBS) decreased by 47 (P = 0.020) on rec d1 compared to the baseline, while the Chao index increased by 146 on rec d1 compared to the baseline (Table 4.6). There was a decrease in OTU evenness as the inverse Simpson index decreased by 8.9 (P < 0.001) on rec d1 compared to baseline. There was also a decrease in Shannon index decreased by 0.20 (P < 0.001) in the rec d1 compared to the baseline.

Table 4.6: Alpha diversity of rumen microbial community in dairy cows before (Baseline) and after (Rec d1) a short-term feed restriction period

	Treatment			
	Baseline	Rec d1	SED	P-value
OBS	1404	1356	20.6	0.020
Chao1 index	1717	1863	34.3	<0.001
Inverse Simpson index	50.0	41.1	2.16	<0.001
Shannon index	5.11	4.91	0.041	<0.001

4.3.5.2 Beta Diversity

Bray Curtis dissimilarity was affected (AMOVA $P < 0.05$) by FR, cow, phase, FR x time, FR x cow, FR x phase, and FR x yeast (Figure 4.6). There was dissimilarity (AMOVA $P < 0.0001$) between all cows in the pair wise comparison (Table 4.7), and there also was a combination effect of FR and individual cow, for three out of the four cows the microbial community was significantly dissimilar ($P < 0.001$) between the baseline and rec d1 (Table 4.8). Bray Curtis dissimilarity was significant ($P < 0.0001$) between the baseline and rec d1, within each of the respective solid and liquid digesta phases (Table 4.9). With the Bray Curtis metric there was a combined effect of FR and yeast ($P < 0.0001$), yet when comparing the pairwise comparisons the differences were only driven by a change in FR rather than with supplementation (Table 4.10).


a)
NMDS 1
NMDS 2
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.5
-0.3
-0.1
0.1
0.3
0.5
Baseline
Rec d1
b)
NMDS 1
NMDS 2
Cow 1
Cow 2
Cow 3
Cow 4
c)
NMDS 1
NMDS 2
LPD
SPD
d)
NMDS 1
NMDS 2
Baseline. 0
Baseline. 3
Baseline. 12
Rec d1. 0
Rec d1. 3
Rec d1. 12

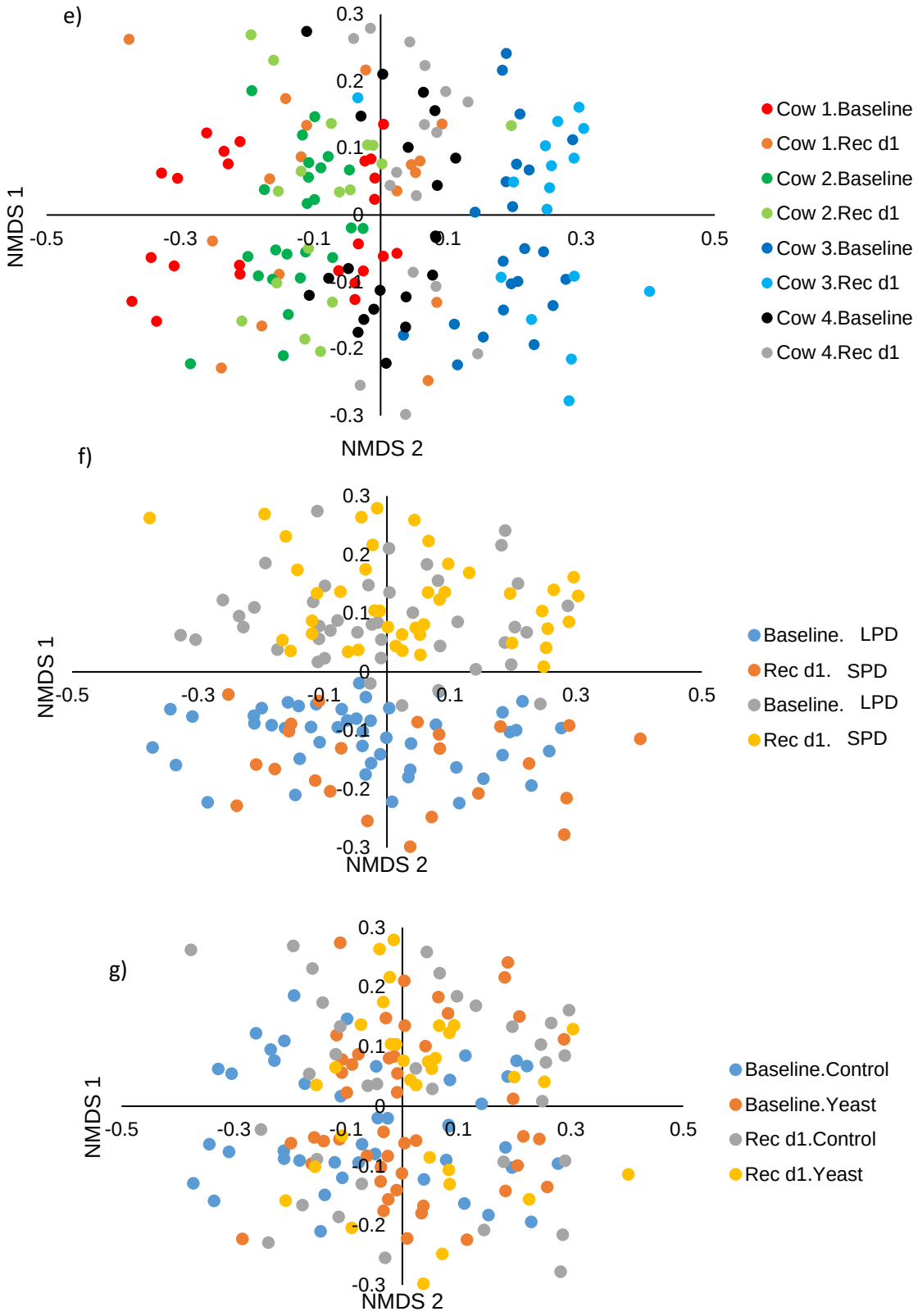


Figure 4.6: NMDS plot (Stress value 0.21556) based on Bray Curtis dissimilarity matrix, for a) feed restriction (FR; P < 0.001) b) Cow (P < 0.001) c) Digesta phase (P < 0.001) d) FR x Time (P < 0.001) e) FR x Cow (P <0.001) f) FR x Digesta phase g) FR x Yeast (P < 0.001)

Table 4.7: Pair wise comparisons of the effect of feed restriction (FR) on individual cows, using analysis of molecular variance (AMOVA)

1.Cow	1.FR	2.Cow	2.FR	P-value
Cow 1	Baseline	Cow 1	Rec d1	<0.0001*
Cow 2	Baseline	Cow 2	Rec d1	0.0063
Cow 3	Baseline	Cow 3	Rec d1	0.0009*
Cow 4	Baseline	Cow 4	Rec d1	0.001*

Table 4.8: Pairwise comparisons of cow and feed restriction (FR), using analysis of molecular variance (AMOVA)

		Cow 1		Cow 2		Cow 3	
		Baseline	Rec d1	Baseline	Rec d1	Baseline	Rec d1
Cow 1	Baseline	–	–	–	–	–	–
	Rec d1	–	–	–	–	–	–
Cow 2	Baseline	0.0044	–	–	–	–	–
	Rec d1	–	0.1596	–	–	–	–
Cow 3	Baseline	<0.0001*	–	<0.0001*	–	–	–
	Rec d1	–	<0.0001*	–	<0.0001*	–	–
Cow 4	Baseline	<0.0001*	–	<0.0001*	–	<0.0001*	–
	Rec d1	–	0.0271	–	0.0204	–	0.0002*

Table 4.9: Pair wise comparisons of digesta phase and feed restriction (FR), using analysis of molecular variance (AMOVA)

1.Phase	1.FR	2.Phase	2.FR	P-value
LPD	Baseline	LPD	Rec d1	<0.0001*
SPD	Baseline	SPD	Rec d1	<0.0001*
LPD	Baseline	SPD	Baseline	<0.0001*
LPD	Rec d1	SPD	Rec d1	<0.0001*

Table 4.10: Pair wise comparisons of feed restriction (FR) and yeast supplementation, using analysis of molecular variance (AMOVA)

1.FR	1.Yeast	2.FR	2.Yeast	P-value
Baseline	No yeast	Baseline	Yeast	0.0113
Recovery	No yeast	Rec d1	Yeast	0.1619
Baseline	No yeast	Rec d1	No yeast	<0.0001*
Baseline	Yeast	Rec d1	Yeast	<0.0001*

4.3.5.3 Discriminant analysis (Identification of Taxonomic Biomarkers; LEfSe)

On rec d1 at 0 h, immediately following the restriction period there was an increased relative abundance of OTU00005 classified to the type strain *Treponema bryantii* (100%) and OTU00004 and OTU00026 related to *Methanobrevibacter ruminantium* (98%) and *Methanosphaera cuniculi* (98%), respectively, compared to the baseline (Table 4.11). Three hours after re-feeding there was an increase in OTU00027, OTU00157, OTU00084 related to *Acinetobacter chinensis* (100%), *Stenotrophomonas maltophilia* (98%), and *Lactobacillus malefermentans* (98%), respectively, and at 12 h post feeding OTU related to *Bifidobacterium longum* (99%; Wong *et al*. 2019) and *Succiniclasticum ruminis* (95%; Van Gylswyk 1995) had increased, as well as *Prevotella bryantii* (99%; Fraga *et al*. 2018) and *Ruminococcaceae bromii* (95%; Ze *et al*., 2012).

There was an increased relative abundance ($P < 0.05$) of OTU00001, OTU00012, OTU00017 all related to *Prevotella* in LPD and an increase in *Methanobrevibacter ruminantium* and *Treponema bryantii* in SPD on rec d1 compared to the baseline (Table 4.12). Differences between FR period and yeast supplementation ($P < 0.001$) were mainly due to an increase in OTU00006, OTU00026 both assigned to *Methanobrevibacter* on rec d1 in cows that received the diet without yeast supplementation, while there was an increase in the *Treponema bryantii* (100%; Stanton and Canale-Parola, 1980) on rec d1 when cows were supplemented with yeast (Table 4.13).

Table 4.11: OTU level taxonomic biomarkers for dairy cows during a short-term feed restriction period at 0, 3, 12 hours post morning feed (Linear discriminant analysis (LDA) score > 2.0, P < 0.05)

	P-value	LDA score	% Seqs	Silva 132 SEED taxonomy (mixed rank)	BLASTn Type (% Ident) (Accessed 05/11/2021)	Reference
Baseline.0						
OTU00020	0.004515	2.12	0.787	*Fibrobacter* (Genus; 100%)	*Fibrobacter succinogens* (93%)	Suen *et al*. (2011)
OTU00010	0.000135	2.11	1.34	Unclassified *Ruminococcaceae* (Family; 85%)	*Intestinimonas gaboonensis* (92%)	Mourembou *et al*. (2017)
Baseline.3						
OTU00008	0.004223	2.58	1.70	*Lactobacillus* (Genus; 96%)	*Latilactobacillus sakei* (98%)	Won *et al*. (2020)
OTU00019	0.001392	2.11	0.802	Unclassified Bacteroidales (Class; 64%)	*Microbacter margulisiae* (86%)	Sanchez-Andrea *et al*. (2014)
Baseline.12						
OTU00003	3.99E-06	3.2	5.37	Unclassified *Gammaproteobacteria* (Genus; 86%)	*Frischella perrara* (88%)	Engel *et al*. (2015)
OTU00015	0.000429	2.36	0.942	Unclassified *Gammaproteobacteria* (Genus; 60%)	*Gilliamella intestini* (86%)	Praet *et al*. (2017)
Rec d1.0						
OTU00004	8.32E-10	2.92	3.33	*Methanobrevibacter* (Genus; 100%)	*Methanobrevibacter ruminantium* (98%)	Leahy *et al*. (2010)
OTU00005	1.15E-09	2.77	3.15	*Treponema* (Genus; 91%)	*Treponema bryantii* (100%)	Stanton and Canale-Parola (1980)
OTU00026	9.27E-08	2.21	0.636	*Methanobacteriaceae* (Family; 100%)	*Methanosphaera cuniculi* (98%)	Biavati *et al*. (1988)
Rec d1.3						
OTU00027	1.05E-08	2.83	0.620	*Acinetobacter* (Genus; 100%)	*Acinetobacter chinensis* (100%)	Hu *et al*. (2019)
OTU00157	6.19E-09	2.13	0.118	*Stenotrophonomas* (Genus; 100%)	*Stenotrophomonas maltophilia* (98%)	Dalia *et al*. (2017)
OTU00084	0.001164	2.06	0.215	*Lactobacillaceae* (Family; 99%)	*Lactobacillus malefermentans* (98%)	Russell and Walker (1953)
Rec d1.12						
OTU00006	9.01E-10	2.69	1.90	*Methanobrevibacter* (Genus; 100%)	*Methanobrevibacter millerae* (99%)	Rea *et al*. (2007)
OTU00007	0.000714	2.23	1.71	*Acidaminococcaceae* (Family; 100%)	*Succiniclasticum ruminis* (98%)	Van Gylswyk (1995)
OTU00050	6.50E-08	2.22	0.339	Unclassified *Bifidobacterium* (Genus; 100%)	*Bifidobacterium longum* (99%)	Wong *et al*. (2019)
OTU00018	0.000455	2.08	0.827	*Prevotella* (Genus; 100%)	*Prevotella bryantii* (99%)	Fraga *et al*. (2018)
OTU00085	6.75E-06	2.01	0.214	*Ruminococcaceae* (Family; 86%)	*Ruminococcaceae bromii* (96%)	Ze *et al*. (2012)

Table 4.12: OTU level taxonomic biomarkers for dairy cows during a short-term feed restriction period in the liquid (LPD) and solid (SPD) digesta phase (Linear discriminant analysis (LDA) score > 2.0, P < 0.05)

	P-value	LDA score	% Seqs	Silva 132 SEED taxonomy (mixed rank)	BLASTn Type (% Ident) (Accessed 05/11/2021)	Reference
Baseline.LPD						
OTU00003	1.08E-10	3.26	5.37	Unclassified *Gammaproteobacteria* (Genus; 86%)	*Frischella perrara* (88%)	Engel *et al*. (2015)
OTU00015	9.89E-13	2.52	0.942	Unclassified *Gammaproteobacteria* (Genus; 60%)	*Gilliamella intestini* (86%)	Praet *et al*. (2017)
OTU00018	2.12E-15	2.22	0.827	*Prevotella* (Genus; 100%)	*Prevotella bryantii* (99%)	Fraga *et al*. (2018)
OTU00039	6.25E-10	2.03	0.480	*Prevotella* (Genus; 100%)	*Prevotella ruminicola* (93%)	Purushe *et al*. (2010)
Rec d1.LPD						
OTU00002	7.75E-08	2.99	5.27	*Acidaminococcaceae* (Family; 96%)	*Succiniclasticum ruminis* (94%)	Van Gylswyk (1995)
OTU00001	5.20E-10	2.69	5.95	*Prevotella* (Genus; 100%)	*Prevotella ruminicola* (97%)	Purushe *et al*. (2010)
OTU00006	2.07E-10	2.66	1.90	*Methanobrevibacter* (Genus; 100%)	*Methanobrevibacter millerae* (100%)	Rea *et al*. (2007)
OTU00019	2.02E-15	2.35	0.802	Unclassified Bacteroidales (Class; 64%)	*Microbacter margulisiae* (86%)	Sanchez-Andrea *et al*. (2014)
OTU00012	2.55E-08	2.26	1.29	*Prevotella (Genus*; 100%*)*	*Prevotella ruminicola* (93%)	Purushe *et al*. (2010)
OTU00017	7.65E-18	2.2	0.835	*Prevotella* (Genus; 99%)	*Prevotella brevis* (91%)	Avguštin *et al*. (1997)
Baseline.SPD						
OTU00008	0.000334	2.62	1.70	*Lactobacillus* (Genus; 96%)	*Lactobacillus sakei* (98%)	Won *et al*. (2020)
OTU00013	4.09E-07	2.18	1.25	Unclassified *Clostridiales* (Class*;* 85%)	*Gracilibacter thermotolerans* (87%)	Lee *et al*. (2006)
OTU00016	1.12E-05	2.17	0.839	*Ruminococcaceae* (Family; 100%)	*Ruminococcus bromii* (96%)	Ze *et al*. (2012)
OTU00035	5.23E-05	2.01	0.508	*Fibrobacter* (Genus; 100%)	*Fibrobacter succinogens* (96%)	Suen *et al*. (2011)
Rec d1.SPD						
OTU00004	4.49E-23	3.03	3.33	*Methanobrevibacter* (Genus; 100%)	*Methanobrevibacter ruminantium* (98%)	Leahy *et al*. (2010)
OTU00005	1.07E-20	2.92	3.15	*Treponema* (Genus; 74%)	*Treponema bryantii* (100%)	Stanton and Canale-Parola (1980)
OTU00027	2.52E-10	2.64	0.620	*Acinetobacter* (Genus; 100%)	*Acinetobacter chinensis* (100%)	Hu *et al*. (2019)
OTU00009	1.10E-09	2.34	1.46	*Bacteroidales* (Class; 97%)	*Gallalistipes aquisgranensis* (86%)	Zenner *et al*. (2021)
OTU00026	6.18E-15	2.31	0.636	*Methanobacteriaceae* (Family; 100%)	*Methanosphaera cuniculi* (97%)	Biavati *et al*. (1988)
OTU00010	1.11E-05	2.07	1.34	Unclassified *Ruminococcaceae* (Family; 85%)	*Intestinimonas gaboonensis* (92%)	Mourembou *et al*. (2017)
OTU00036	1.63E-17	2.06	0.501	*Clostridiales* (Class; 82%)	*Saccharofermentans acetigenes* (94%)	Chen *et al*. (2010)

OTU00057	4.00E-21	2.00	0.319	Unclassified *Spirochaetaceae* (Family; 91%)	*Treponema bryantii* (95%)	Stanton and Canale-Parola (1980)

Table 4.13: OTU level taxonomic biomarkers for dairy cows during a short-term feed restriction period when supplemented with or without a live yeast (Linear discriminant analysis (LDA) score > 2.0, P < 0.05)

	P-value	LDA score	% Seqs	Silva 132 SEED taxonomy (mixed rank)	BLASTn Type (% Ident) (Accessed 05/11/2021)	Reference
Baseline.No yeast						
OTU00003	4.60E-06	3.05	5.37	Unclassified *Gammaproteobacteria* (Genus; 86%)	*Frischella perrara* (88%)	Engel *et al*. (2015)
OTU00021	0.010543	2.15	0.757	Unclassified *Prevotellaceae* (Family; 92%)	*Prevotella copri* (88%)	Iljazovic *et al*. (2021)
OTU00016	0.007705	2.00	0.839	*Ruminococcaceae* (Family; 100%)	*Ruminococcus bromii* (96%)	Ze *et al*. (2012)
Baseline.Yeast						
OTU00015	0.000198	2.27	0.943	Unclassified *Gammaproteobacteria* (Genus; 60%)	*Gilliamella intestini* (86%)	Praet *et al*. (2017)
Rec d1.No yeast						
OTU00027	8.50E-09	2.68	0.620	*Acinetobacter* (Genus; 100%)	*Acinetobacter chinensis* (100%)	Hu *et al*. (2019)
OTU00006	1.22E-09	2.57	1.90	*Methanobrevibacter* (Genus; 100%)	*Methanobrevibacter millerae* (99%)	Rea *et al*. (2007)
OTU00026	1.73E-08	2.17	0.636	*Methanobacteriaceae* (Family; 100%)	*Methanosphaera cuniculi* (98%)	Biavati *et al*. (1988)
OTU00157	8.53E-06	2.01	0.118	*Stenotrophonomas* (Genus; 100%)	*Stenotrophomonas maltophilia* (98%)	Dalia *et al*. (2017)
Rec d1.Yeast						
OTU00004	1.12E-08	2.82	3.33	*Methanobrevibacter* (Genus; 100%)	*Methanobrevibacter ruminantium* (98%)	Leahy *et al*. (2010)
OTU00005	8.19E-11	2.73	3.15	*Treponema* (Genus; 91%)	*Treponema bryantii* (100%)	Stanton and Canale-Parola (1980)

4.3.5.4 Protozoa

The FR period had no effect ($P < 0.05$) on the abundance of the protozoa *Isotrichidae* relative to the *Ophryoscolecidae* (Table 4.14). There was however a trend for an interaction ($P = 0.056$) between FR and pattern of concentrates fed; the relative abundance of *Isotrichidae* tended to increase on rec d1 in cows fed E while there was no change in U.

Table 4.14: Relative abundance (*Isotrichidae* cells per 1000 cells of *Ophryoscolecidae*) of rumen protozoa in dairy cows receiving an even (E) or uneven (U) pattern of concentrate allocation either with (+) or without (–) yeast supplementation, during a short-term feed restriction period (FR)

	Treatments					P-value						
	E+	E–	U+	U–	SED	C	Y	C x Y	FR	FR x C	FR x Y	FR x C x Y
Base	16.7	21.2	19.0	18.1	4.59	0.156	0.972	0.403	0.136	0.056	0.284	0.656
Rec d1	24.8	24.4	19.2	16.3								

FR= Feed restriction and re-feeding (in days), C= Concentrate allocation, Y= inclusion of yeast, x= interaction between treatments

[1] Baseline data were collected on d 30, the deprivation day was d 31, during which animals were offered 75% of their previously recorded PMR intake and spent 6 h of the day fasting. Recovery days 1 and 2 were the subsequent 24-h periods (d 32 and 33, respectively), on rec d 1 cows were offered the PMR at a rate of 125% of their previously recorded PMR intake.

4.4 Discussion

4.4.1 Forage and diet composition

The current study was conducted to determine the effect of short-term FR and re-feeding on the rumen microbiome, rumen metabolism, and performance of high yielding dairy cows fed different concentrate patterns and either with or without a live yeast. The basal PMR was the same for all treatments, and all cows received the same proportion of forage and concentrates. The DM content of the grass silage was lower than described by Sinclair *et al*. (2015) however the mean DM content of the PMR was consistent with results reported by Tayyab *et al*. (2019) for rations containing GS and MS in the UK. The NDF content of GS and MS were also comparable to those reported by Tayyab *et al*. (2019). Forage pH and VFA concentration and ratios (including lactate) were similar to those of Little *et al*. (2018) and Purcell *et al*. (2016).

4.4.2 Performance

Upon re-feeding following the FR period, cows can enter a period of overeating which can affect both rumen function and the microbiome (Thomson *et al*., 2018; Oetzel, 2007). In the current study, DMI decreased on the day of FR by 6.36 kg as only 75% of the PMR was provided, and upon reintroduction feed intake increased by 5.12 kg on rec d1 compared to the baseline. These findings are supported by Patterson *et al*. (1998) who reported an increase in DMI following a short-term (six hour) restriction period. In contrast, results by Chelikani *et al*. (2004) and Thomson *et al*. (2018) reported that a short-term FR had no effect on daily DMI. However, the timings of the FR period were different, as in the current study the restriction occurred overnight so the FR and recovery periods (rec d1 and rec d2) were measured independently on different days. In the study of Thomson *et al*. (2018) the feed was removed at 0830 h for six hours, and so the FR and initial recovery periods were on the same day, which may have balanced each other out resulting in no apparent effect on DMI.

Following FR, milk yield decreased and failed to return to the baseline level within two days of recovery, despite DMI doing so in the same timeframe. While FR did not affect mean total VFA concentration there was a significant decrease in concentration at 0600 h prior to re-feeding, and in mean propionate concentration, which most probably limited energy availability, causing milk yield to decline. Similarly, Abdelatty *et al*. (2017) and Thomson *et al*. (2018) concluded that a period of FR reduced milk yield. Velez and Donkin (2005) reported that milk yield returned to baseline within four days following FR, a finding in agreement with the current study. There are strong links between rumen microbial profile and milk production, and Weimer *et al*. (2017) reported that when the rumen experienced

severe change in microbial composition milk yield only returned back to baseline when the microbial communities returned to the original composition. In the current study the microbial community was recorded for one day following FR yet had not returned to baseline composition in this time, and it is not possible to determine whether the return of the rumen microbiome and milk yield to baseline levels occurred together. Milk yield was higher in cows fed E during the baseline period, a finding supported by Sova *et al*. (2013), although following FR there was no difference between treatments. Milk protein content (g/kg) increased on rec d1 by 1.36 g/kg, a change that may have been due to the lower milk yield during as milk protein yield (kg/d) remained unchanged on rec d1. Similarly, Abdelatty *et al*. (2017) reported that during the recovery period milk protein content increased by 0.1% while protein yield (kg/d) was unchanged. Likewise, milk fat followed a similar pattern, with a tendency for fat content (g/kg) to increase on rec d1 while yield (kg/d) remained similar.

4.4.3 Rumen pH, ammonia and volatile fatty acids

Rumen pH increased by pH 0.38 at 0600 h prior to re-feeding compared to the baseline. During FR the VFA produced by fermentation would continue to be absorbed, but the rate of synthesis was likely reduced due to the lack of substrate available for fermentation (Thomson *et al*., 2018). Furthermore, cows may have continued to ruminate and therefore saliva would have continued to be produced which would have a positive buffering effect, increasing rumen pH (Thomson *et al*., 2018). Krause and Oetzel (2005) concluded that following re-feeding pH dropped as VFA production increased due to increased DMI with the return of substrate availability for microbial fermentation. Likewise, in the current study rumen pH declined by 1.16 units in the first 6 h post feeding on rec d1. Mean rumen pH was consistent during the baseline and rec d1, however the pH range increased during rec d1. Mean pH on rec d2 was lower than the baseline, indicating rumen conditions, such as VFA absorption, required longer than 24 h to recover after feed reintroduction despite DMI returning back to normal within that time. Krause and Oetzel (2006) reported that following a 50% FR rumen pH took 72 h to recover to the baseline. During the baseline period yeast increased rumen pH, however following the FR period yeast had no effect. This result contrasts with those of Ferreira (2019) who suggested that the effects of yeast supplementation were greater when the rumen was compromised.

In the current study, FR had no effect on mean total VFA concentration compared to the baseline. However there was diurnal variation, with a nadir reached prior to re-feeding for total and individual VFA, and then increased above baseline level within 3 h post initial feed, before returning to the baseline for the remainder of the day. Volatile fatty acids help stimulate epithelial function and therefore with chronic FR, when VFA concentrations are low

the absorptive capacity of the epithelium can be temporarily reduced, resulting in an accumulation of VFA in the rumen immediately following re-feeding (Zhang *et al*., 2013). These findings align with the pH results in the current study; when VFA concentration was at its highest, pH was at its lowest suggesting accumulation in the rumen. Following re-feeding mean NH_3 concentration increased, and the diurnal variation also changed, with NH_3 concentration peaking at 3 h post concentrate feed, as it was 58.2 mg/L higher than on the baseline day. There is little literature that has investigated the effect of a short-term FR on rumen NH_3 concentration in dairy cows. However, NH_3 may have increased due to the increase in DMI and microbial activity, reflected by the increase in VFA at those time points.

4.4.4 Rumen microbiota

During rec d1 there was an apparent decrease in both OTU richness (OBS) and increase measured using Chao index, OBS is based on the actual data while the Chao index is calculated as an estimation based full depth of coverage (Thukral, 2017). During the period of FR there was less substrate available in the rumen from the digestion of rapidly degradable carbohydrates (Thomson *et al*., 2018) leading to a reduction in microbial activity, may have resulted in a decrease in microbial abundance and diversity, reflected by a drop in VFA concentration. Similar results were reported by McCann *et al*. (2016) who reported a reduction in species richness following a FR period. There was a difference in Bray Curtis dissimilarity between the baseline and rec d1 within respective LPD and SPD samples in the current study, suggesting that the FR period caused a shift in the entire rumen microbial community.

There is no ‘normal’ rumen microbiome, as core microbiota vary across species, diet and geographical location, although the baseline microbial communities were consistent with control data from other studies (Henderson *et al*., 2015; Clemmons *et al*., 2018; Plazier *et al*., 2017). During the FR period, rapidly degradable carbohydrates were likely absorbed from the rumen, with fibrous material remaining in the rumen, encouraging an increase in fibrolytic bacteria, reflected in an increased relative abundance of *Treponema bryantii* (OTU00005) which is associated with fibre degradation and acetogenesis, (Liu *et al*., 2014; Santos *et al*., 2011). There was also an increase in *Methanobrevibacter* which are associated with high fibre environments as they utilise hydrogen generated as a by-product of fibre degradation (Newbold *et al*., 2015; Morgavi *et al*., 2010). Previous research has identified *Methanobrevibacter* as the main genera of archaea in the rumen (Henderson *et al*., 2015). On rec d1 there was an increase in OTU assigned to *Methanobrevibacter ruminantium* and *Methanosphaera cuniculi* which have been reported to be low methane emitters (Misiukiewicz *et al*., 2021). On the other hand, there was also an increase in the

OTU assigned to *Methanobrevibacter millerae* on rec d1 compared to the baseline which has been linked to higher methane emissions (Danielsson *et al*., 2017), therefore a period of FR followed by re-feeding may lead to an increase in methane production.

With the reduction in OTU diversity following the FR period, the rumen microbial community did not return to baseline immediately following re-feeding, instead colonising bacteria such as lactate producers including *Lactobacillus* and *Bifidobacteria* increased which can proliferate before other niche groups of bacteria have the opportunity (Yang *et al*., 2018). This is supported by an increase in rumen lactate on rec d1 and a decrease in rumen pH in the current study. After 12 h post re-feeding the rumen microbiota profile had not recovered to baseline composition and further research is necessary to determine the length of time the rumen takes to recover and the impact of chronic FR on the rumen microbiome.

On rec d1, there was an increased abundance of methane producing archaea both with or without yeast supplementation, and additionally when cows were supplemented with yeast there was also in increase in *Treponema bryantii* suggesting that the rumen may be better able to recover from a FR period compared to when cows did not receive yeast due to the increased abundance of the fibrolytic bacteria. During the period of FR, cows continued to ruminate to aid fibre degradation which would have become the predominant nutrient in the rumen once the rapidly degradable carbohydrates were digested shortly into the period of FR. The potential increase in rumination may have increased the proportion of oxygen present in the rumen which may have in turn have compromised fibrolytic activity in cows fed the diet without yeast. When cows received yeast in the diet it may have improved conditions for fibrolytic bacteria by scavenging oxygen from the rumen (Chaucheyras-Durand *et al*., 2008). Ferreira *et al*. (2019) concluded that yeast is more beneficial to the rumen when conditions are compromised which supports the result that the effect of yeast supplementation on the microbial community was greater on rec d1 than without yeast.

4.5 Conclusions

At re-feeding, following a period of FR DMI increased, however milk yield decreased and was below the baseline level until recovery day 4. Rumen pH decreased in the recovery period (rec d1 and rec d2) and did not return to baseline within two days. Mean VFA (total, acetate, butyrate and valerate) concentrations were unaffected by the feed restriction however there was a change in diurnal variation on rec d1, with a lower concentration immediately following the restriction period prior to re-feeding and increasing three hours post feeding, compared to the baseline indicating a change in microbial activity. Propionate concentration decreased while NH_3 concentration increased on rec d1 compared to the baseline. Following FR, there was a reduction in alpha and beta diversity, with an increased

relative abundance of *Methanobrevibacter*, compared to the baseline potentially due to a reduction in substrate availability. With yeast supplementation there was increased abundance of fibrolytic *Treponema bryantii* compared to without on rec d1. The effect of repeated feed restriction on rumen metabolism and performance and possible acclimatisation requires further investigation.

Chapter 5: The effect of *Yucca schidigera* (De-Odorase® and live yeast (Yea-Sacc®) supplementation in diets high in rumen degradable protein on the performance, rumen metabolism, the microbiome and nitrogen balance in high yielding dairy cows

5.1 Introduction

Ammonia emissions contribute to the formation of fine particle matter with a diameter smaller than 2.5 µm (PM2.5) and associated adverse health conditions (Giannakis *et al*., 2019). The UK government launched the Clean Air Strategy to reduce the number of people living in locations above the WHO guideline level of 10 µg/m^3 by half by 2025 (GOV, 2019). The policy covers pollution produced by industry, transport, farming, and households. Agriculture accounts for 88% of UK NH_3 emissions and can occur during slurry application, cattle housing, slurry storage, and grazing, therefore reducing NH_3 losses will require a whole farm system approach (Guthrie *et al*., 2018; Bussink and Oenema, 1998).

In the UK, housing dairy cattle all year round has become increasingly common due to higher productivity compared to grazing at pasture, as grass quality can vary greatly throughout the year, the DM intake of grass is lower than total mixed rations, and access to pasture is limited on many farms (Fontineli *et al*., 2005; Bargo *et al*., 2002a). However outdoor grazing can be beneficial, with lower feed and labour costs and improved foot health compared to zero grazing systems (Haskell *et al*., 2006; Meul *et al*., 2012). Grass, particularly in the early grazing season is also high in rumen degradable protein (RDP; Totty *et al*., 2013). When dairy cow diets are oversupplied with RDP, NH_3 is absorbed across the rumen epithelium and converted to urea in the liver, which can then either be recycled back to the rumen across the rumen wall or via salivary secretions, or excreted in the urine (Sannes *et al*., 2002; Getahun *et al*., 2019). When urine and faeces are mixed the urea in urine comes into contact with the enzyme urease present in faecal material and NH_3 is released (Huntington and Archibeque, 1999). This along with other forms of N in cattle slurry such as nitrous oxide and nitrate have negative implications for the environment (Hynes *et al*., 2016).

Yucca schidigera extract is rich in saponins which are a group of high molecular weight glycosides, with the saccharide chain units (1-8 residues) linked to steroidal aglycone moiety (Patra and Saxena, 2009). The glycofraction has demonstrated NH_3 binding capabilities while the steroidal saponins have been recognised for their anti-protozoal and anti-bacterial properties in the rumen which may assist in the regulation of the release of NH_3 from protein degradation in the digestive tract (Wallace *et al*., 1994; Saeed *et al*., 2017). Whilst studies have been undertaken *in vitro* and *in vivo* there is little information on the effects of *Y. schidigera* on the microbial community and diversity. Furthermore *Y. schidigera* extract has

been reported to have an NH_3 binding capacity and may therefore reduce the rate and extent of NH_3 absorption from the rumen and subsequently the amount of urea excreted in the urine (Wallace *et al*., 1994).

The inclusion of live yeasts in cattle rations have been reported to increase the utilisation of trace amounts of dissolved oxygen, particularly at the interface of the cellulolytic bacteria and fibre, thereby stimulating rumen bacterial growth (Chaucheyras-Durand *et al*., 2008). An increase in microbial growth and the subsequent increase of NH_3 utilisation from microbial fermentation (Jouany, 2006) which may improve N capture and performance. However, few studies have been undertaken on the combined effects of Yucca extract and live yeast on rumen metabolism, the microbiome and nitrogen (N) excretion in dairy cows.

The objective was to determine the effect of *Yucca schidigera* extract and a live yeast supplementation in diets high in rumen degradable protein on rumen metabolism, the microbiome, nitrogen balance, and performance of high yielding dairy cows.

5.2 Materials and methods

The procedures for the animals used were conducted in accordance with the UK Animals Scientific Procedures Act (1986; amended 2012) and were approved by the local ethics committee at Harper Adams University.

5.2.1 Animals and experimental design

Six Holstein-Friesian dairy cows in their third lactation that had previously been fitted with permanent rumen cannulas (10 cm diameter; Bar Diamond, Idaho, USA) were used. The cows were 76 days (SE ± 24.8) post-calving, weighed 650 kg (± 26.2), and yielding 37 kg (±1.9) of milk per day at the start of the study. Each cow was randomly assigned to one of three dietary treatments in each of three periods of seven weeks duration, and remained on study for 21 weeks (147 days). For the first six weeks of each period the cows were group housed in a pen bedded with sawdust with a concreted area in front of the feed barrier which was manually scraped out twice a day. In the seventh week of each period the cows were housed in individual metabolism stalls fitted with mattresses for five days for rumen and digestibility sample collection. Cows had continuous access to water at all times.

The experimental design was a 3 x 3 Latin rectangle, with a Control total mixed ration (TMR) without supplementation (C), TMR plus De-Odorase® (D), TMR plus De-Odorase® and Yea-Sacc® (DY). Each treatment was fed for seven weeks, and during the final week of each period performance, rumen metabolism, microbiome and digestibility measurements were undertaken.

5.2.2 Forages and diets

All cows received the same basal TMR (Table 5.1) via individual Calan gates (American Calan, Northwood, NH, USA) at a rate of 105% of the previous recorded intake at approximately 0800 h, and refusals were collected three times a week (Monday, Wednesday and Friday). In addition, the diets were either unsupplemented or supplemented with De-Odorase®, or De-Odorase® and Yea-Sacc® (Alltech UK). The dose rate for De-Odorase® was 5 g/cow/day and 1 g/cow/day for Yea-Sacc®. The additives were mixed with 100 g of ground barley as a carrier and provided as a topdressing to the TMR, which was mixed into the top of the diet immediately after feeding, with cows fed the Control diet also receiving 100 g of ground barley without any supplementation. The diets were formulated according to Thomas (2004) to meet the metabolisable energy and metabolisable protein requirements of a dairy cows yielding 40 kg/d at 32 g/kg protein and 40 g/kg fat content.

Table 5.1: Dietary formulation (kg/kg DM) of the basal total mixed ration (TMR) fed to dairy cows fed the Control TMR (C), TMR with De-Odorase® (D) or TMR with De-Odorase® and Yea-Sacc® (DY)

Ingredient	TMR
Grass silage	0.550
Barley	0.212
Sugar beet	0.072
Soypass[1]	0.064
Wheat distillers dark grains	0.031
Rapeseed meal	0.031
Soya bean meal (Hipro)	0.021
Palm kernel meal	0.009
Minerals/vitamins[2]	0.004
Megalac[3]	0.004
Molasses	0.003
Predicted composition (g/kg DM)	
Forage:concentrate (DM basis)	55:45
Crude protein	178
NDF	329
Sugar	58
Starch	127
Rumen degradable starch	104
Oil	41
ME[4], MJ/kg DM	12.1
MPE[5], g/kg DM	106
MPN[6], g/kg DM	125
MPB[7], g/kg DM	25
MPE[5], % of requirements	100
MPN[6], % of requirements	121

[1] A rumen protected source of soybean (KW Alternative Feeds, Leeds, UK)
[2] Minerals/Vitamins premix (KW Alternative Feeds, Leeds, UK), major minerals g/kg: Ca 220, P 30, Mg 80 Na 80, trace minerals mg/kg: Cu 1000; I 400, Mn 4000; Se 160, Zn 3000; Vitamins (IU): A 1,000,000; D_3 300,000; E 4,000; B_{12} 135.
[3] A rumen-protected source of fat (Volac, Royston, UK).
[4] ME, Metabolisable energy
[5] MPE, Metabolisable protein- rumen energy limited
[6] MPN, Metabolisable protein- rumen nitrogen limited
[7] MPB, Metabolisable protein from bypass protein

5.2.3 Experimental routine

5.2.3.1 Intake and milk parameters

The experimental routine is shown in Table 5.2. During the sampling week of each period intake was recorded daily. On day three of each sampling period, intake was also measured at four hourly intervals from 0800 to 2000 h. Grass silage and TMR samples were collected daily during the sampling period and stored at -20°C for subsequent analysis. The cows were milked twice daily using a portable milking machine (Milkline, London, UK) at 0600 and 1600 h and the yield recorded. During week seven of each period milk samples were collected on four occasions (two AM and two PM) for subsequent composition analysis (fat, protein, lactose, and urea-N). Body weight and condition score (Ferguson *et al*., 1994) were recorded at the same time of day (after morning milking) at the start and end of each period.

Table 5.2: Experimental routine for sampling week of dairy cows fed a Control total mixed ration (TMR; C), TMR with De-Odorase® (D) or TMR with De-Odorase® and Yea-Sacc® (DY)

	Week Seven						
Sampling	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Milk yield (AM and PM)	X	X	X	X	X	X	X
Milk composition	PM	AM		PM	AM		
Intake (Weighed feed at 0, 4, 8, 12, 24 h)	X	X	0, 4, 8, 12 h	24 h	X	X	X
Blood sampling	0700, 0900, 1100, 1300						
Digestibility		Apparatus on at 1200	X	X	X	X	Apparatus off at 1200
Slurry sampling[1]							X
Rumen fluid sampling[2]				0, 3, 6, 9, 12, 17 h	24 h		

[1] Urine and faecal matter collected for slurry analysis after digestibility sampling completed at 1200 h
[2] Rumen sampling for pH, VFA, NH_3, microbiome analysis at 0, 3, 6, 9, 12, 17, 24 h post feeding

5.2.3.2 Blood sampling

Blood samples were collected at 0700, 0900, 1100 and 1300 h by venipuncture via the jugular vein using fluoride/oxalate (grey) for glucose and lithium heparin (green) for urea, β-hydroxybutyrate (BHB) and NH_3 vacutainers (BD Vacutainer, Plymouth, UK) on day one of each sampling period. The samples were centrifuged at 1500 g for 15 min and the plasma extracted. Plasma ammonia analysis was conducted within 30 minutes of sampling and the rest of the plasma was stored at -20°C for subsequent analysis.

5.2.3.3 Diet digestion

On days two to seven of each sampling period, total urine output was collected for five days using a modified catheter bag (Shop Optimum, West Yorkshire, UK) fitted with a pipe connected to a 25 L barrel (Figure 3.1 and 3.2). Modified catheter bags were secured over the vulva of the cow with Velcro® straps which were glued to the cows using EvoStick® compact adhesive. One litre of 20% sulphuric acid was added to each 25 L barrel to maintain urinary pH below pH 3.0. After each 24 h period a 1% subsample was taken and stored at -20°C for subsequent analysis. The barrels were then emptied and cleaned for the next day's collection. Faecal samples were also collected daily for the same five consecutive days by collecting all deposited material from the floor on three occasions over the 24 h. Faecal material was weighed, and a 1% subsample of the daily output stored at -20°C prior to bulking the five sampled days together for each cow per period and subsequent analysis.

5.2.3.4 Slurry ammonia

On day seven of each sampling period additional urine and faecal samples were collected to determine the pH, total N and NH_3 concentration of slurry. Spot samples of urine and faeces were taken and mixed in a 10 L bucket at a ratio of 2:1, respectively (i.e. 1.5 kg faeces with 750 g urine), and incubated in a 2.5 L plastic bottle for 24 h at room temperature (Dospatliev *et al*., 2015). For the first six hours pH was recorded at hourly intervals, and then at 24 h. Subsamples (approximately 100 g) were also taken at each time point and stored at -20°C for subsequent analysis.

5.2.3.5 Rumen pH, ammonia and volatile fatty acids

Rumen fluid samples were collected on day three of each sampling period, at 0800 (immediately before morning feeding), 1100, 1400, 1700, 2000, and 0100 h, using a method adapted from Martin *et al*. (1999). Four grab samples of digesta were collected from the ventral region of the rumen by inserting an arm directly down approximately 50 cm through the cannula and grabbing a large handful of digesta then placing into a bucket. Further fluid was collected by inserting a 250 ml glass bottle into the same area. The rumen fluid and digesta was then strained through four layers of muslin cloth to separate the solid digesta from the liquid. The pH of the strained rumen fluid was recorded immediately after samples were taken using a calibrated pH meter (Bibby Scientific Limited, Staffordshire, UK), and liquid samples were then stored in a 25% w/v HPO_3 solution at -20°C for subsequent analysis of VFA and NH_3.

5.2.3.6 Microbial community analysis

Rumen fluid and digesta samples collected at 1400, 1700 and 2000 h were also stored in 15% glycerol solution at -20°C for subsequent microbial community analysis of liquid phase digesta (LPD) and solid phase digesta (SPD). Strained rumen fluid was also stored in a 10% formalin/saline solution at room temperature for ciliate protozoa identification and counting.

5.2.4 Chemical analysis

Forage, TMR, and faecal samples were bulked between days for each period, and the sub-samples analysed for DM (943.01), CP (990.03; intra-assay CV of 2.48%) and ash (942.05) according to AOAC (2012), with NDF (intra-assay CV of 1.36%) determined according to Van Soest *et al*. (1991) and expressed exclusive of residual ash. Milk composition was analysed by National Milk Laboratories (NML; Wolverhampton, UK) for fat, protein, lactose and urea using near midinfrared (MIR; Foss, Denmark). Urine and slurry samples were analysed for total N using the Kjeldahl method (MAFF, 1986).

Blood plasma samples were analysed for glucose, β-hydroxybutyrate (BHB), urea and ammonia (with intra-assay CV of 2.97, 3.68, 3.61, and 2.64%, respectively) using a Cobas Miras Plus autoanalyser (ABX Diagnostics, Bedfordshire, UK). The kits used were: GLUC-HK, Ref GU611; RANBUT, Ref RB1008; and UREA, Ref UR221 and NH3, Ref AM1015, respectively (Randox Laboratories, County Antrim, UK).

The VFA concentration of grass silage and rumen fluid was analysed by GC using methods according to Erwin *et al*. (1961) using a column (DBFFAP, 30 m x 0.250 mm x 0.2 µm; Agilent J and W, GC columns, UK) and flame ionisation detector (Agilent 6890, Stockport, UK) as described in Section 2.13. Lactate analysis was conducted on rumen fluid samples by high performance liquid chromatography (Agilent 1100, Germany) as described in Section 2.14. Rumen and slurry NH_3 concentrations were measured from a method adapted from MAFF (1986) and using an auto-titrator (FOSS 1030 auto-titrator, FOSS, Warrington, UK; Buchi Labortechnik AG CH-9230, Flawil, Switzerland).

Microbial community analysis was undertaken using 16S rRNA gene amplicon sequencing (as described in Section 2.16). The DNA extraction was undertaken at Harper Adams University using the bead beating protocol based on Yu and Morrison (2004; Section 2.16.3). Amplicon libraries were prepared using dual index primers based on Kozich *et al*. (2013; Section 2.16.4), the order of the primers used are described in Appendix 8.2. The libraries were quality assessed using an Agilent 2100 Bioanalyzer System (Agilent Technologies. Santa Clara, CA, US) and sequenced by Edinburgh Genomics using the Illumina MiSeq v2

250 paired end reagent kit (Illumina UK, Cambridge, UK.). Ciliate protozoa identification and counts of relative abundance of *Isotrichidae* and *Ophryoscolecidae* was conducted using a light microscope (Olympus CX31, Olympus, Tokyo) with a 10X objective and 10X/20 eyepiece (Section 2.16.7).

5.2.5 Statistical analysis

Performance and rumen metabolism parameters were evaluated by repeated measures analysis of variance, and digestibility parameters were evaluated by ANOVA as a 3 x 3 factorial design as a Latin rectangle design using GenStat Release 18.1 (VSN International Ltd). The repeated measure was time of sampling during the day (h), with main effect of dietary treatment (Tr). The N balance equations are described in Section 2.10. Results are reported as treatment means with SED, with the level of significance set at $P < 0.05$, and a tendency stated at $P < 0.10$.

Microbial community data were analysed using mothur v1.44.0 (Schloss *et al*., 2011), assembling paired end sequences and removing low quality sequences. Sequence counts in each library were normalised by subsampling to 38,500 sequences per sample in the liquid phase digesta, and 10,500 sequences per samples in the solid phase digesta. An operational taxonomic unit (OTU) based approach was used to describe the microbial community diversity. Sequences were clustered into operational taxonomic units (OTU) at 97% identity, and taxonomic classification of the representative sequences was carried out using the SILVA 138.1 SEED reference database (Yilmaz *et al*., 2014).

Microbial OTU richness and diversity were summarised using Chao1, Shannon, inverse Simpson, and number of observed OTU (OBS) indices. Beta diversity was calculated using the Bray Curtis dissimilarity metric, with a significance level from analysis of molecular variance (AMOVA) set at $P < 0.001$. Taxonomic biomarkers associated with respective treatment groups were determined by comparing relative abundance of OTU using Linear Discriminant Analysis (LDA) Effect Size (LEfSe; Segata *et al*., 2011) with minimum LDA score >2.0 and $P < 0.05$.

5.3 Results

5.3.1 Forage and diets

The nutritional composition (DM, CP, OM, NDF) of the TMR was 394 g/kg, 168, 920, 420 g/kg DM, respectively, and for the grass silage (GS) 301 g/kg, 131, 909, 487 g/kg DM, respectively (Table 5.3). The pH of the GS was 3.89, and the NH_3-N content was 19.8 g/kg of total N. The VFA profile (acetate, propionate, butyrate) of the GS was 36.5, 0.780, 0.328 g/kg, respectively, and the lactate concentration was 19.1 g/kg.

Table 5.3: Nutritional composition (g/kg DM) and fermentation characteristics of grass silage (GS), and the total mixed ration (TMR)

	GS	TMR
DM	301	394
CP	131	168
Ash	90.9	79.9
OM	909	920
NDF	487	420
Fermentation characteristics, g/kg		
pH	3.89	
NH_3-N, g/kg of total N	19.8	
Acetate	36.5	
Propionate	0.780	
Iso-butyrate	0.481	
Butyrate	0.328	
Iso-valerate	0.127	
Lactate	19.1	

5.3.2 Intake, production and milk composition

Dry matter intake decreased (P = 0.015) by 0.8 kg/d in cows when fed DY compared to the Control diet (Table 5.4). Milk yield and milk composition yield (fat and protein) were not affected (P > 0.05) by dietary treatment, with mean values of 33.9 kg/d for milk yield, and 1.50 kg/d, 1.15 kg/d, for milk fat and protein yield, respectively. Milk protein content decreased by 2.07 g/kg when cows were fed D compared to the Control. The feed conversion ratio was not affected (P > 0.05) by dietary treatment with a mean value of 1.78. Body weight and body condition score (BCS) were also not affected by dietary treatment with mean values of 736 kg and 3.0, respectively.

Table 5.4: Performance of dairy cows fed a Control total mixed ration (TMR; C), TMR with De-Odorase® (D) or TMR with De-Odorase® and Yea-Sacc® (DY)

	Treatments			SED	P Value
	C	D	DY		
Dry matter intake, kg/d	21.9[a]	21.7[a]	21.1[b]	0.23	0.015
Milk yield, kg/d	34.0	33.8	33.8	0.68	0.949
4% ECM, kg/d	39.2	39.1	38.5	1.31	0.826
Milk fat, kg/d	1.51	1.52	1.48	0.080	0.904
Milk fat, g/kg	44.1	44.0	43.9	1.98	0.992
Milk protein, kg/d	1.19	1.13	1.14	0.033	0.194
Milk protein, g/kg	34.9[a]	32.8[b]	33.6[b]	0.52	0.020
Milk lactose, g/kg	46.4	46.8	46.1	0.34	0.191
Milk urea-N, g/kg	0.271	0.265	0.253	0.0126	0.373
Feed conversion ratio[1]	1.76	1.74	1.86	0.090	0.422
Live weight, kg	738	731	739	13.2	0.789
Live weight change, kg[2]	0.35	0.64	0.69	0.298	0.503
Body condition score	3.01	2.98	2.92	0.107	0.694
Body condition score change[2]	0.292	0.167	0.042	0.003	0.305

ECM= Energy corrected milk, C= Control, D= De-Odorase® supplementation, Y=Yea-Sacc® supplementation, means along row with different superscript are significantly ($P < 0.05$) different
[1] kg of milk/ kg of DMI
[2] = change/day

5.3.3 Blood metabolites

De-Odorase® or De-Odorase® and Yea-Sacc® supplementation had no effect on blood metabolites concentration with mean values for ammonia, glucose, BHB and urea of 66.0, 4.22, 0.835, 3.61 mmol/L, respectively (Table 5.5). There was no interaction between dietary treatment and time for any of the metabolites measured (Figure 5.1).

Table 5.5: Plasma metabolites (mmol/L) of dairy cows fed a Control total mixed ration (TMR; C), TMR with De-Odorase® (D) or TMR with De-Odorase® and Yea-Sacc® (DY)

	Treatments			SED	P value
	C	D	DY		
Ammonia	65.2	67.9	64.9	2.24	0.456
Glucose	4.29	4.25	4.29	0.108	0.910
BHB	0.806	0.785	0.829	0.0267	0.314
Urea	3.52	3.83	3.48	0.209	0.249

C = Control, D= De-Odorase® supplementation, Y=Yea-Sacc® supplementation

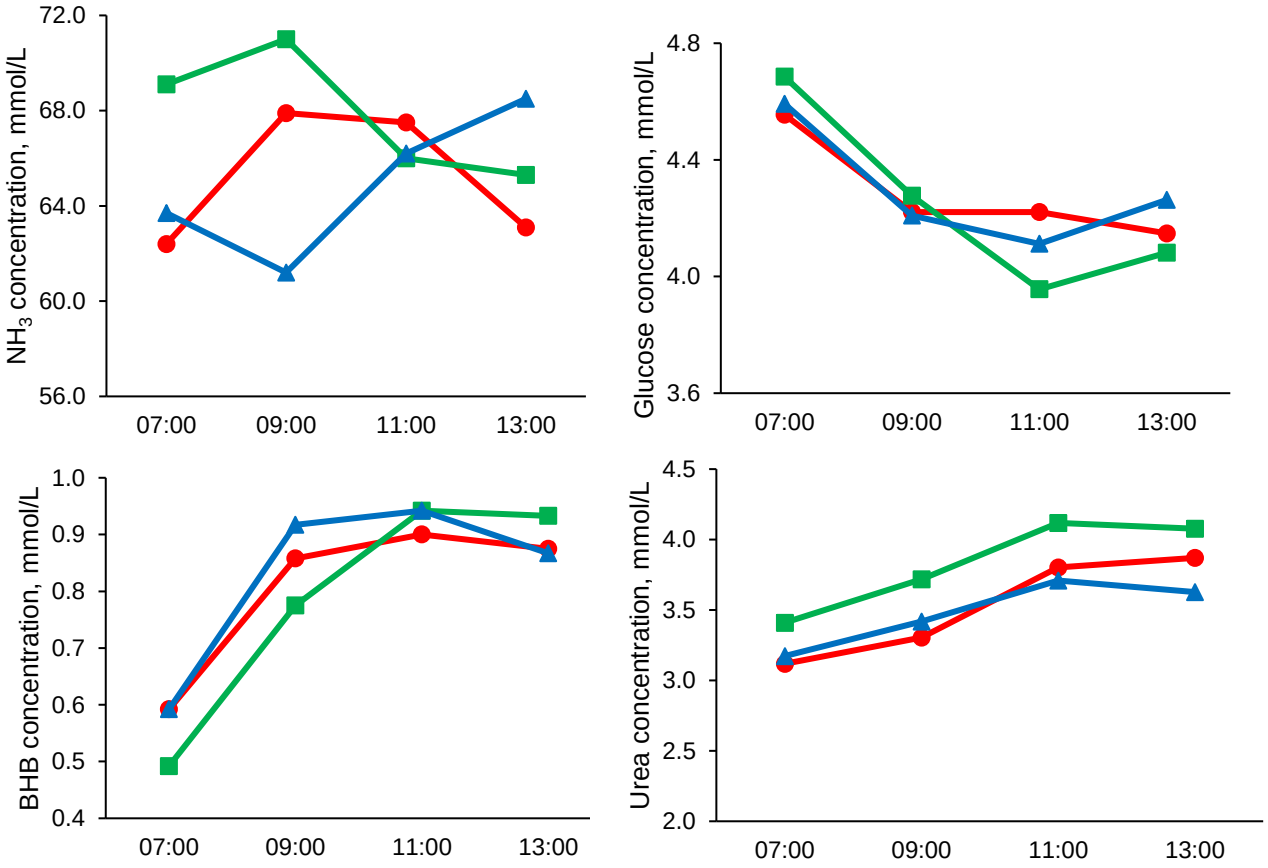


Figure 5.1: Plasma betahydroxybutyrate (BHB), plasma urea, plasma ammonia (NH_3), plasma glucose (mmol/L) of dairy cows fed a Control total mixed ration (TMR; ●), TMR with De-Odorase® (■) or TMR with De-Odorase® and Yea-Sacc® (▲). For plasma NH_3, SED= 4.88, Time, P = 0.932; Time x Treatment, P = 0.486, for plasma glucose, SED= 0.155, Time, P = <0.001; Time x Treatment, P = 0.317, for plasma BHB, SED= 0.082; Time, P = <0.001; Time x Treatment, P = 0.658, for plasma urea, SED= 0.267, Time, P < 0.001, Time x Treatment, P = 0.886.

5.3.4 Diet digestion and nitrogen balance

Nutrient intake (DM, OM, NDF) decreased (P < 0.05) when cows were fed DY by 0.8, 1.08, and 0.48 kg/d, respectively, compared to the Control (Table 5.6). Diet also had no effect on faecal output (DM, OM, NDF) with mean values of 5.70, 4.93, and 2.69 kg/d, respectively. Diet also had no effect on nutrient digestibility (DM, OM, NDF) with mean values of 0.734, 0.749, and 0.701 kg/kg, respectively.

Nitrogen intake was 32 g/d lower (P = 0.006) in cows fed DY than when fed the Control, but there was no effect of treatment on N faecal output, digested N or N digestibility, with mean values of 175 g, 352 g, 0.666 g/g, respectively (Table 5.7). Dietary treatment had no effect on urinary N output and the proportion of urinary-N of total faecal N, with mean values of 195 g and 522 g/kg, respectively. Dietary treatment also had no effect (P > 0.05) on milk N or milk N as a proportion of N intake, with mean values of 177 g and 342 g/kg, respectively.

Table 5.6: Diet digestibility of dairy cows fed a Control total mixed ration (TMR; C), TMR with De-Odorase® (D) or TMR with De-Odorase® and Yea-Sacc® (DY)

	Treatments				
	C	D	DY	SED	P-value
DM, kg/d					
Intake	21.9[a]	21.7[a]	21.1[b]	0.23	0.015
Output	5.78	5.76	5.56	0.143	0.277
Digestibility, kg/kg	0.736	0.734	0.731	0.0045	0.591
OM, kg/d					
Intake	20.2[a]	20.0[a]	19.1[b]	0.27	0.009
Output	4.99	4.98	4.82	0.123	0.321
Digestibility, kg/kg	0.752	0.750	0.747	0.0044	0.480
NDF, kg/d					
Intake	9.19[a]	9.12[a]	8.71[b]	0.133	0.013
Output	2.70	2.72	2.65	0.087	0.716
Digestibility, kg/kg	0.706	0.703	0.696	0.0072	0.388

C = Control, D= De-Odorase® supplementation, Y=Yea-Sacc® supplementation
Means along row with different superscript are significantly (P < 0.05) different

Table 5.7: Nitrogen balance of dairy cows fed a Control total mixed ration (TMR; C), TMR with De-Odorase® (D) or TMR with De-Odorase® and Yea-Sacc® (DY)

	Treatments				
N, g/d	C	D	DY	SED	P value
Intake	539[a]	532[a]	509[b]	6.9	0.006
Faecal output	174	176	174	8.7	0.986
Digested	365[a]	357[ab]	334[b]	10.0	0.040
Digestibility, g/g	0.673	0.669	0.656	0.0166	0.578
Urine	204	187	194	17.47	0.643
Urine N of total faecal N, g/kg	534	507	525	19.9	0.411
Milk N	187	177	178	5.2	0.194
N use efficiency, g/kg	345	330	350	8.8	0.130

C = Control, D= De-Odorase® supplementation, Y=Yea-Sacc® supplementation
Means along row with different superscript are significantly (P < 0.05) different

5.3.5 Slurry analysis

Dietary treatment had no effect on slurry pH, NH_3 concentration, total N or N loss in the first six hours of measurement with mean values of 8.41, 2.45 g/L, 5.679 g/L, 0.062 g/L (Table

5.8). At six hours of incubation, pH was 0.29 units lower (P =0.076) in slurry from cows fed D compared to when fed the Control diet (Figure 5.2).

Table 5.8: Slurry composition from dairy cows fed a Control total mixed ration (TMR; C), TMR with De-Odorase® (D) or TMR with De-Odorase® and Yea-Sacc® (DY)

	Treatments			SED	P value
	C	D	DY		
Slurry pH	8.52	8.34	8.37	0.098	0.201
Slurry ammonia, g/L	2.49	2.55	2.30	0.355	0.283
Slurry total N, g/L	5.72	5.63	5.68	0.276	0.949
Volatile N loss[1], g/L	-0.016	0.028	0.176	0.1155	0.351

C = Control, D= De-Odorase® supplementation, Y=Yea-Sacc® supplementation
[1] = N loss in first six hours

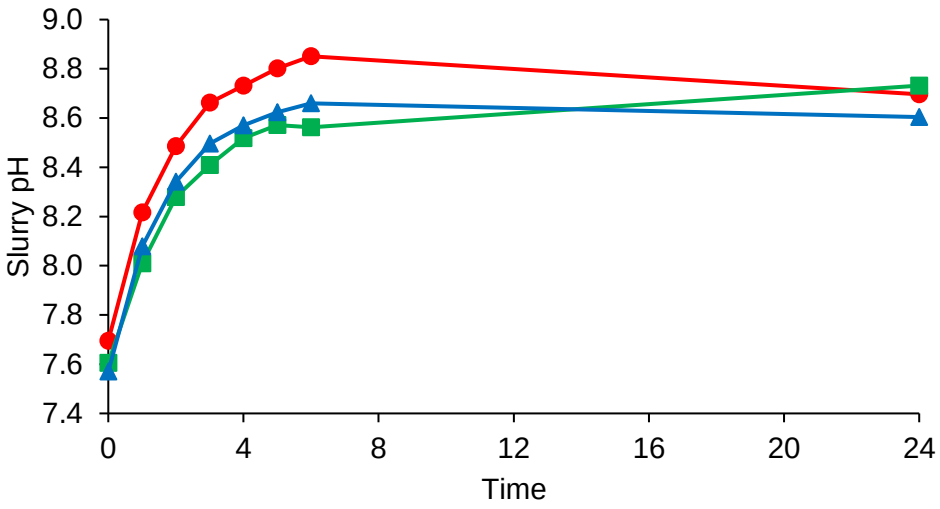


Figure 5.2: Slurry pH of dairy cows fed a Control total mixed ration (TMR; ●), TMR with De-Odorase® (■) or TMR with De-Odorase® and Yea-Sacc® (▲). (SED= 0.111; Time, P <0.001; Treatment, P = 0.201; Treatment x Time, P = 0.130).

5.3.6 Rumen pH, ammonia and volatile fatty acids

Mean rumen pH, pH range and mean NH_3 concentration were not affected (P > 0.05) by diet treatment, with mean values of pH 6.12, 0.728, 49.8 mg/L, respectively (Table 5.9). There were no interactions (P > 0.05) between total VFA or NH_3 concentration and time (Figure 5.3; Figure 5.4). Dietary treatment also had no effect (P > 0.05) on the total or individual (acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate) VFA concentration, with mean values of 82.2, 53.8, 15.5, 10.6, 0.40, 1.19, 0.84 µM, respectively (Table 5.10). At

1700 h however, rumen propionate concentration was 5.81 µM higher (P = 0.050) in cows when fed the Control diet than DY, and at 2000 h was 6.42 µM higher in cows fed the Control than either of the supplemented diets. Iso-butyrate concentration was 6.55 µM lower (P = 0.028) at 1400 h when cows were fed DY than D. Mean lactate concentration was not affected (P > 0.05) by dietary treatment with a mean value of 0.152 µM.

Table 5.9: Rumen metabolism of dairy cows fed a Control total mixed ration (TMR; C), TMR with De-Odorase® (D) or TMR with De-Odorase® and Yea-Sacc® (DY)

	Treatments				
	C	D	DY	SED	P-value
Mean rumen pH	6.10	6.10	6.16	0.057	0.496
Maximum – minimum rumen pH	0.71	0.77	0.71	0.337	0.907
Mean rumen ammonia, mg/L	50.7	51.8	44.6	5.22	0.374

C = Control, D= De-Odorase® supplementation, Y=Yea-Sacc® supplementation

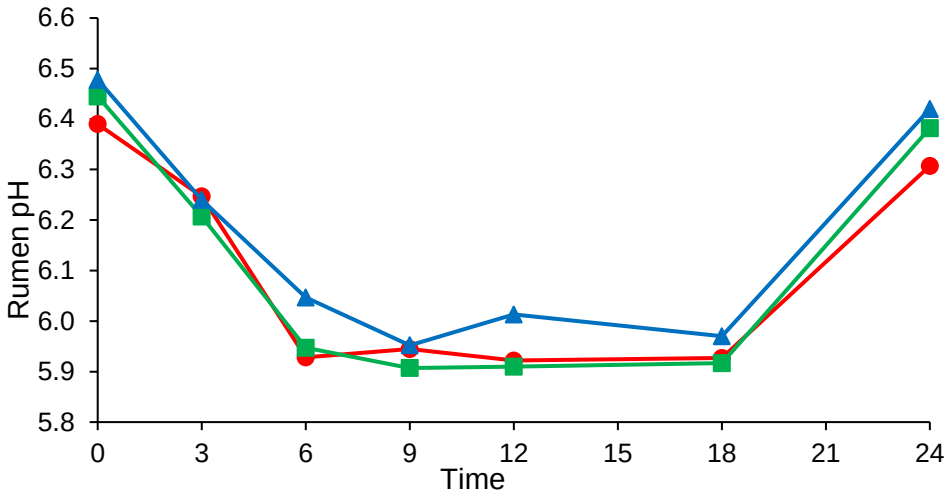


Figure 5.3: Rumen pH of dairy cows fed a Control total mixed ration (TMR; ●), TMR with De-Odorase® (■) or TMR with De-Odorase® and Yea-Sacc® (▲). (SED= 0.120; Time, P <0.001; Treatment, P = 0.496; Treatment x Time, P = 0.994).

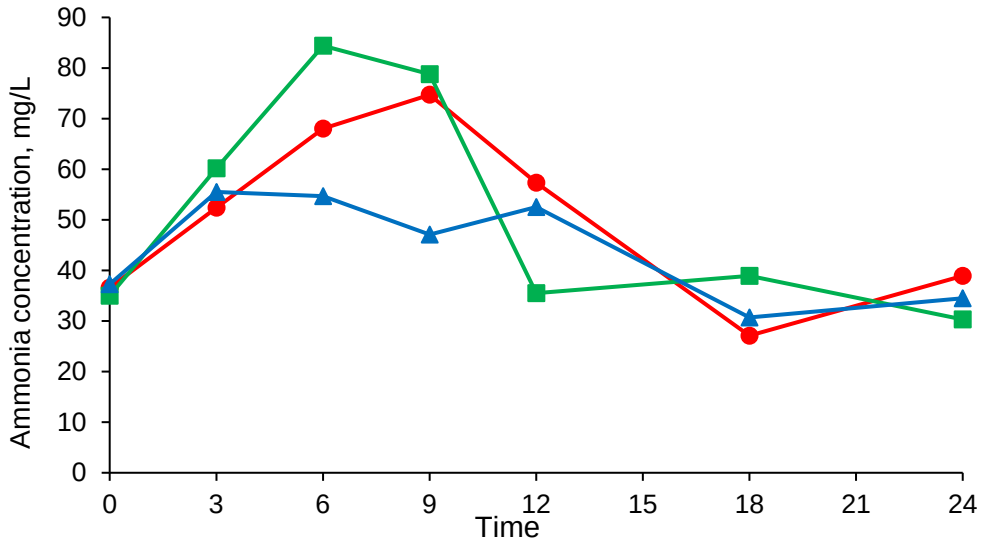


Figure 5.4: Rumen ammonia of dairy cows fed a Control total mixed ration (TMR; ●), TMR with De-Odorase® supplementation (■) or TMR with De-Odorase® and Yea-Sacc® supplementation (▲). (SED= 12.70; Time, P <0.001; Treatment, P = 0.374; Treatment x Time, P = 0.274).

Table 5.10: Rumen volatile fatty acid content (µM) of dairy cows fed a Control total mixed ration (TMR; C), TMR with De-Odorase® (D) or TMR with De-Odorase® and Yea-Sacc® (DY)

	Treatments			SED	P-value		
	C	D	DY		Tr	Ti	Tr x Ti
Total VFA							
0800 h	81.4	77.8	69.6	15.36	0.643	0.894	0.200
1100 h	67.5	85.9	91.6				
1400 h	82.9	97.3	69.4				
1700 h	95.0	76.6	65.4				
2000 h	103	76.3	73.6				
0200 h	81.8	88.1	88.8				
0800 h (24 h)	88.0	78.5	88.1				
Acetate							
0800 h	54.5	52.5	46.0	10.49	0.695	0.749	0.292
1100 h	43.8	57.1	61.2				
1400 h	52.8	60.8	44.6				
1700 h	60.4	46.4	41.5				
2000 h	65.3	49.6	47.5				
0200 h	55.1	57.5	59.5				
0800 h (24 h)	60.5	52.6	59.6				
Propionate							
0800 h	14.3	13.6	12.7	2.85	0.638	0.273	0.050
1100 h	12.7	15.4	16.6				
1400 h	16.4	20.4	13.8				

1700 h	18.8	16.5	13.0				
2000 h	20.7	14.4	14.1				
0200 h	14.5	16.9	16.3				
0800 h (24 h)	14.5	13.8	15.5				
Butyrate							
0800 h	10.5	9.75	8.89	1.825	0.304	0.831	0.155
1100 h	9.00	10.8	11.0				
1400 h	11.0	13.0	8.65				
1700 h	12.7	10.9	8.77				
2000 h	14.0	10.0	9.68				
0200 h	10.2	11.3	10.7				
0800 h (24 h)	10.8	9.91	10.7				
Iso-butyrate							
0800 h	0.39	0.41	0.38	0.080	0.490	0.171	0.028
1100 h	0.34	0.42	0.44				
1400 h	0.48	0.49	0.42				
1700 h	0.60	0.38	0.31				
2000 h	0.51	0.32	0.35				
0200 h	0.33	0.35	0.35				
0800 h (24 h)	0.40	0.38	0.42				
Valerate							
0800 h	0.98	0.91	0.89	0.244	0.699	0.059	0.221
1100 h	0.94	1.18	1.34				
1400 h	1.26	1.51	1.00				
1700 h	1.46	1.41	1.02				
2000 h	1.70	1.23	1.17				
0200 h	1.09	1.28	1.28				
0800 h (24 h)	1.10	1.06	1.12				
Iso-valerate							
0800 h	0.69	0.67	0.71	0.179	0.994	0.005	0.230
1100 h	0.76	0.98	0.98				
1400 h	0.94	1.12	0.88				
1700 h	1.00	0.96	0.81				
2000 h	1.06	0.72	0.83				
0200 h	0.68	0.77	0.81				
0800 h (24 h)	0.75	0.70	0.81				
Mean lactate	0.046	0.206	0.205	0.1825	0.618	-	-

C= Control, D= De-Odorase® supplementation, Y= Yea-Sacc® supplementation,
Tr= Treatment, Ti= Time

5.3.7 Microbial community analysis amplicon sequencing of 16S rRNA gene

Following sequencing the result was a total of 16.6 million sequences, before quality control subsampling was conducted, and a total of 11,780 OTU were identified in the LPD samples and 6,965 OTU in the SPD samples. To optimise the depth of coverage, LPD samples were

normalised by subsampling to 38,500 reads per sample, while SPD samples were normalised to 10,500 reads per sample. Low abundance OTU (number of reads per OTU below 10) were removed from the dataset, resulting in 2630 OTU in the LPD dataset and 1647 OTU in the SPD dataset. Coverage was measured using Good's statistic was between 98.2% and 99.0% per library of LPD samples and between 95.9% and 98.0% per library of SPD samples. For LPD samples the relative abundance at phylum level were Bacteroidetes (47%), Firmicutes (23%), Euryarcheota (8%), Spirochaetes (7%), unclassified bacteria (5%), Proteobacteria (5%); the remaining 5% consisted of low abundance taxonomic groups (Figure 5.5). For SPD samples the relative abundance at phylum level were Bacteroidetes (39%), Firmicutes (32%), Spirochaetes (15%), Euryarcheota (8%), Fibrobacteres (3%); the remaining 3% consisted of unclassified bacteria and low abundance groups.

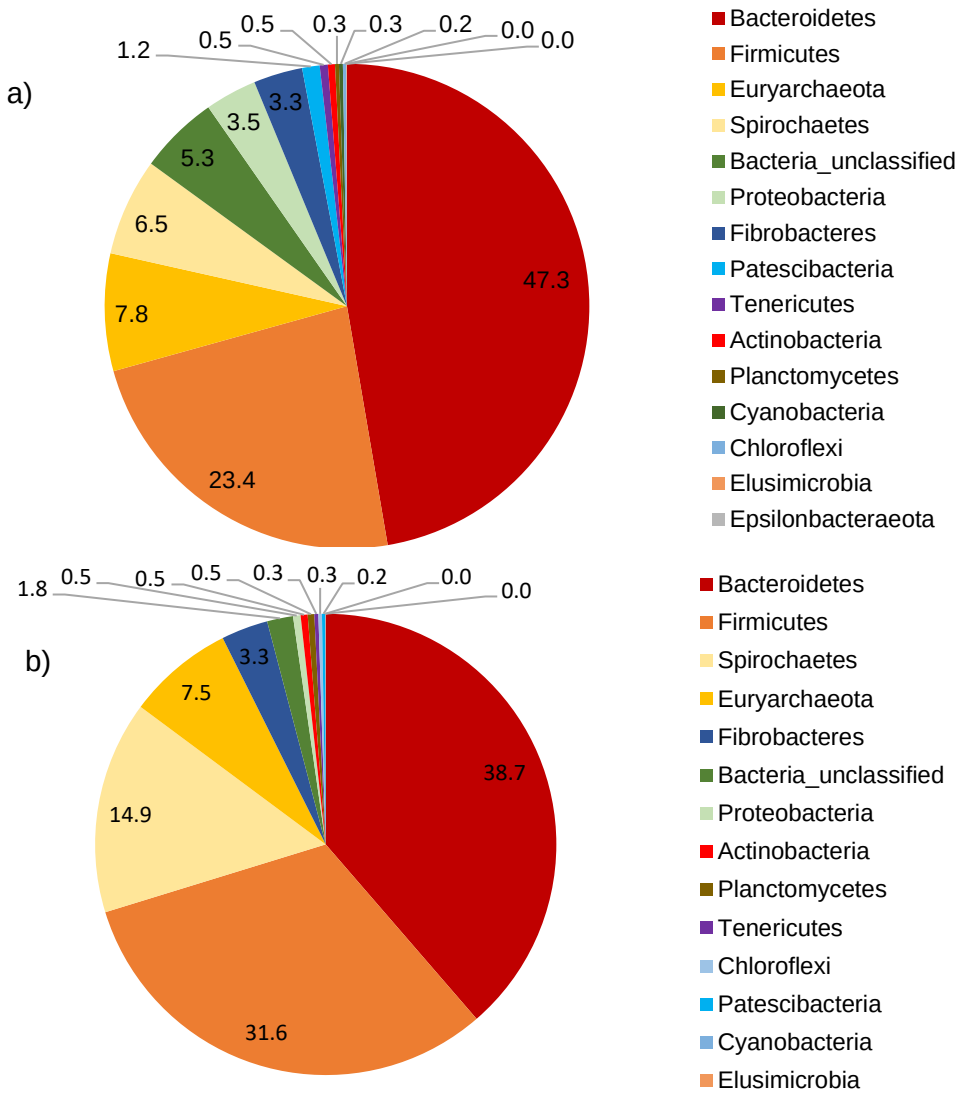


Figure 5.5: Overview of phyla of a) liquid phase digesta (LPD) and b) solid phase digesta (SPD) in the rumen of dairy cows fed a Control total mixed ration (TMR; C), TMR with De-Odorase® supplementation (D) or TMR with De-Odorase® and Yea-Sacc® supplementation (DY)

5.3.7.1 Alpha Diversity

Dietary treatment had no effect (P > 0.05) on observed OTU (OBS) and Chao in both LPD and SPD samples (Table 5.11). However, the inverse Simpson index tended to decrease by 6.6 (P = 0.053) in LPD samples, and decrease by 5.2 (P = 0.060) in SPD samples when cows were fed diets supplemented with D or DY compared to the Control diet. Treatment had no effect (P > 0.05) on the Shannon index for LPD samples, while Shannon index decreased by 0.21 (P = 0.040) when cows were supplemented with D compared to the Control.

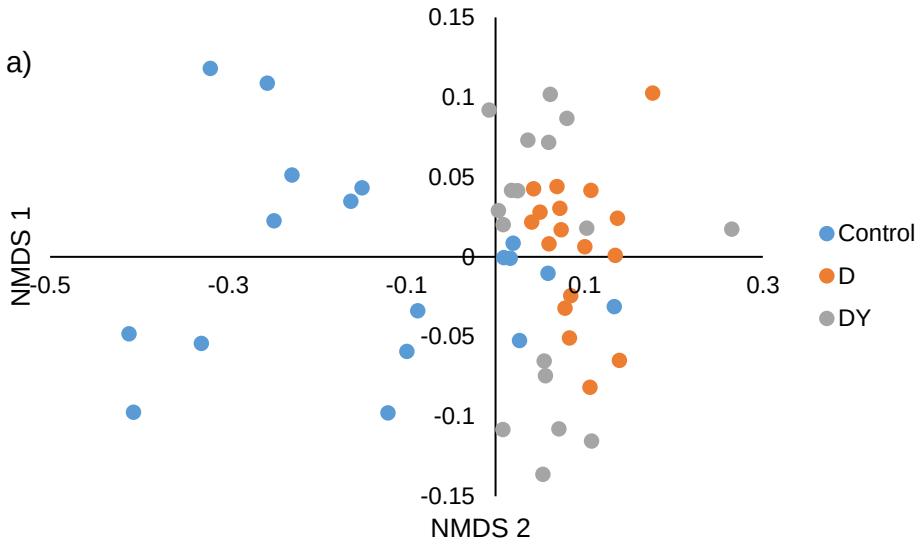
Table 5.11: Alpha diversity of rumen microbial community in liquid phase digesta (LPD) and solid phase digesta (SPD) in dairy cows fed a Control total mixed ration (TMR; C), TMR with De-Odorase® (D) or TMR with De-Odorase® and Yea-Sacc® (DY)

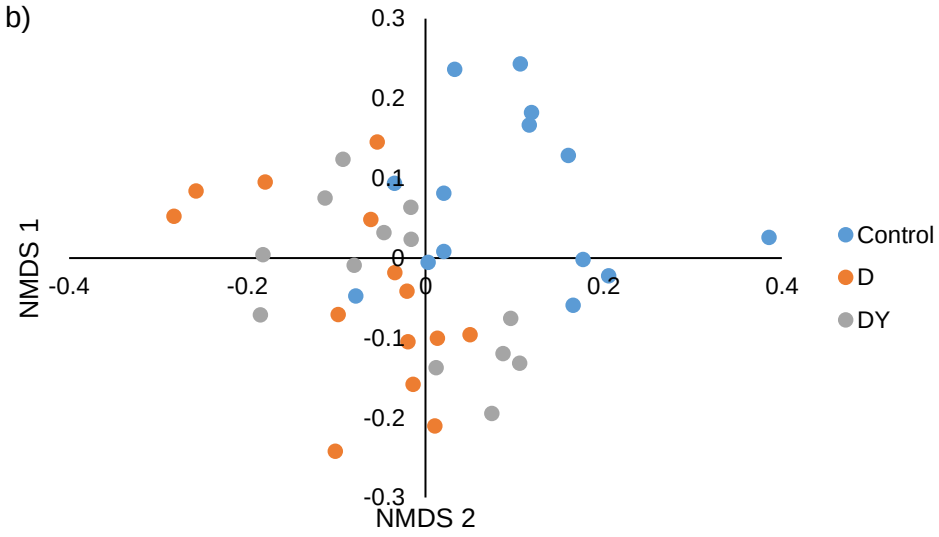
	Treatment			SED	P-value
	C	D	DY		
LPD					
OBS	1392	1418	1428	32.6	0.548
Chao1 Index	1961	2028	1937	73.1	0.468
Inv. Simpson Index	41.3	34.4	35.1	2.57	0.053
Shannon Index	4.90	4.79	4.83	0.056	0.187
SPD					
OBS	823	814	852	33.9	0.534
Chao1 Index	1246	1257	1337	88.4	0.559
Inv. Simpson Index	32.2	24.2	29.7	2.88	0.060
Shannon Index	4.71[a]	4.50[b]	4.69[a]	0.075	0.040

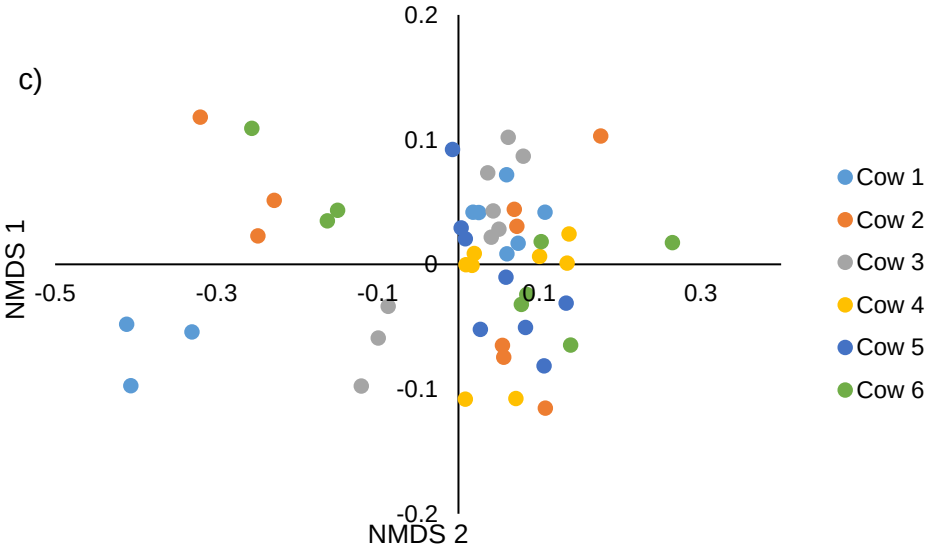
C= Control, D= De-Odorase® supplementation, Y= Yea-Sacc® supplementation
Means along row with different superscript are significantly (P < 0.05) different

5.3.7.2 Beta Diversity

Dietary treatment had an effect (AMOVA; P < 0.001) on Bray Curtis dissimilarity between microbial communities in either LPD or SPD samples (Figure 5.6). In LPD samples there was a difference (P < 0.001) between cows fed D and DY compared to the Control, although there was no difference (P = 0.0139) between D and DY (Table 5.12). In SPD samples but not LPD D and not DY was found to have significant Bray Curtis dissimilarity from the Control (P < 0.001). Bray Curtis dissimilarity differed (P < 0.001) between individual cows only in the liquid phase, while in the solid phase individual cow tended to have an effect (P = 0.086) on clustering. This was not supported by any individual pairwise comparisons (P > 0.001) between either digesta phase or cow (Table 5.13), although individual cow rumen microbiome dissimilarity was found at values of P < 0.05. Time of sampling had no effect (P > 0.05) on Bray Curtis clustering in either of the digesta phases.


a)
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.5
-0.3
-0.1
0.1
0.3
NMDS 1
NMDS 2
Control
D
DY


b)
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.2
0
0.2
0.4
NMDS 1
NMDS 2
Control
D
DY


c)
0.2
0.1
0
-0.1
-0.2
-0.5
-0.3
-0.1
0.1
0.3
NMDS 1
NMDS 2
Cow 1
Cow 2
Cow 3
Cow 4
Cow 5
Cow 6

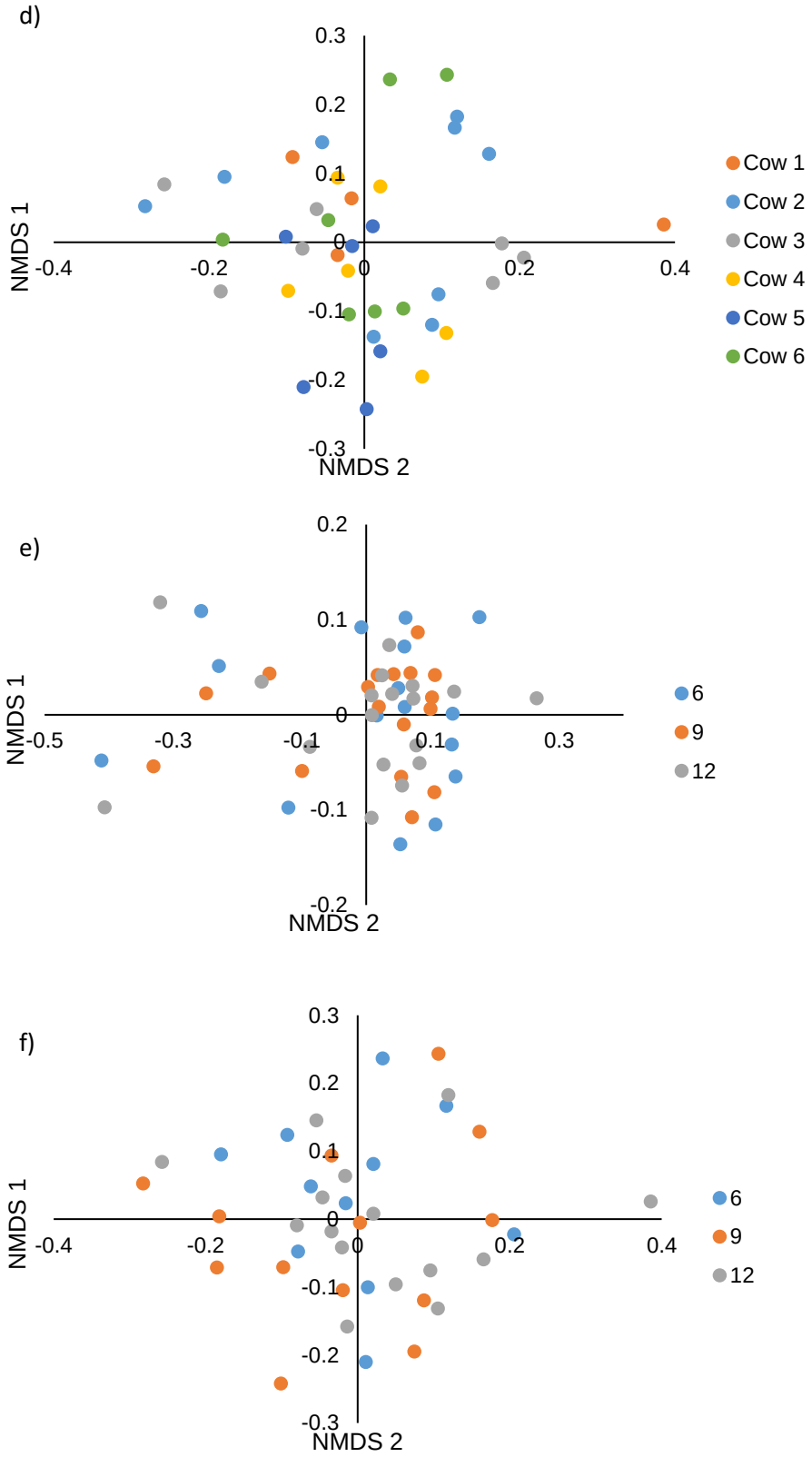


Figure 5.6: NMDS plot (Stress value 0.18732 for LPD samples, and 0.15694 for SPD samples), for a) Dietary treatment in LPD ($P < 0.001$) b) Dietary treatment in SPD ($P < 0.001$) c) Individual cow in LPD ($P < 0.001$) d) Individual cow in SPD ($P = 0.086$) e) Time in LPD ($P = 0.538$) f) Time in SPD ($P = 0.568$)

Table 5.12: Pair wise comparisons of digesta phase and diet supplementation, using analysis of molecular variance (AMOVA)

1.Phase	1.Diet	2.Phase	2.Diet	P-value
LPD	Control	LPD	DY	0.0001*
LPD	Control	LPD	D	<0.0001*
LPD	DY	LPD	D	0.0139
SPD	Control	SPD	DY	0.0246
SPD	Control	SPD	D	0.0001*
SPD	DY	SPD	D	0.1175

C = Control, D= De-Odorase® supplementation, Y=Yea-Sacc® supplementation

Table 5.13: Pair wise comparisons of Bray Curtis dissimilarity between digesta phase and individual cow, using analysis of molecular variance (AMOVA)

1.Phase	1.Cow	2.Phase	2.Cow	P-value
LPD	Cow 1	LPD	Cow 2	0.2607
LPD	Cow 1	LPD	Cow 3	0.0717
LPD	Cow 1	LPD	Cow 4	0.0501
LPD	Cow 1	LPD	Cow 5	0.0735
LPD	Cow 1	LPD	Cow 6	0.2643
LPD	Cow 2	LPD	Cow 3	0.0213
LPD	Cow 2	LPD	Cow 4	0.0129
LPD	Cow 2	LPD	Cow 5	0.0061
LPD	Cow 2	LPD	Cow 6	0.1785
LPD	Cow 3	LPD	Cow 4	0.0489
LPD	Cow 3	LPD	Cow 5	0.0357
LPD	Cow 3	LPD	Cow 6	0.0096
LPD	Cow 4	LPD	Cow 5	0.1577
LPD	Cow 4	LPD	Cow 6	0.0034
LPD	Cow 5	LPD	Cow 6	0.0099
SPD	Cow 1	SPD	Cow 2	0.5326
SPD	Cow 1	SPD	Cow 3	0.6665
SPD	Cow 1	SPD	Cow 4	0.1614
SPD	Cow 1	SPD	Cow 5	0.1362
SPD	Cow 1	SPD	Cow 6	0.5964
SPD	Cow 2	SPD	Cow 3	0.4006
SPD	Cow 2	SPD	Cow 4	0.1111
SPD	Cow 2	SPD	Cow 5	0.0517
SPD	Cow 2	SPD	Cow 6	0.4621
SPD	Cow 3	SPD	Cow 4	0.1678
SPD	Cow 3	SPD	Cow 5	0.1706
SPD	Cow 3	SPD	Cow 6	0.3532
SPD	Cow 4	SPD	Cow 5	0.1068
SPD	Cow 4	SPD	Cow 6	0.1023
SPD	Cow 5	SPD	Cow 6	0.1541

5.3.7.3 Discriminant analysis (Identification of Taxonomic Biomarkers; LEfSe)

In LPD samples there was a higher relative abundance in OTU00013 and OTU00030 both assigned to the genus *Prevotella*, and in both digesta phases there was an increase in OTU00014 assigned to *Methanobrevibacter olleyae* (98%; Table 5.14; Table 5.15) in cows fed the Control diet. When cows were fed D there was an increase in OTU00024 related to *Gimesia aquarii* (83%) and OTU00006 assigned to *Prevotella ruminicola* (100%). Individual cow affected ($P < 0.001$) Bray Curtis dissimilarity in the liquid phase, with an increase in relative abundance in OTU00044 (*Prevotella ruminicola*) in Cow 2 (Table 5.16).

Table 5.14: OTU level taxonomic biomarkers for dairy cows fed a Control total mixed ration (TMR), TMR with De-Odorase® (D) or TMR with De-Odorase® and Yea-Sacc® (DY) in the liquid phase digesta (Linear discriminant analysis (LDA) score > 2.0, P < 0.05)

	P-value	LDA score	% Seqs	Silva 132 SEED taxonomy (mixed rank)	BLASTn Type (% Ident) (Accessed 12/11/2021)	Reference
LPD.Control (C)						
OTU00013	2.08E-05	2.2	1.43	*Prevotella* (Genus; 99%)	*Prevotella brevis* (90%)	Avguštin *et al*. (1997)
OTU00030	0.0024064	2.02	0.586	*Prevotellaceae* (Family; 100%)	*Prevotella bryantii* (91%)	Fraga *et al*. (2018)
OTU00014	0.0034449	2.19	1.24	*Methanobrevibacter* (Genus; 100%)	*Methanobrevibacter olleyae* (98%)	Rea *et al*. (2007)
LPD.De-Odorase® (D)						
OTU00024	0.0023337	2.02	0.817	*Patescibacteria* (Phylum; 86%)	*Gimesia aquarii* (83%)	Wiegand *et al*. (2020)
OTU00006	0.013291	2.32	2.40	*Prevotella* (Genus; 100%)	*Prevotella ruminicola* (96%)	Purushe *et al*. (2010)
LPD.DY						
OTU00021	0.0016922	2.43	0.909	Unclassified *Gammaproteobacteria* (Class; 83%)	*Chelonobacter oris* (87%)	Kudirkiene *et al*. (2014)
OTU00011	0.018033	2.04	1.55	*Prevotella* (Genus; 100%)	*Prevotella ruminicola* (94%)	Purushe *et al*. (2010)

C = Control, D= De-Odorase® supplementation, Y=Yea-Sacc® supplementation

Table 5.15: OTU level taxonomic biomarkers for dairy cows fed a Control total mixed ration (TMR) in the solid phase digesta (Linear discriminant analysis (LDA) score > 2.0, P < 0.05)

	P-value	LDA score	% Seqs	Silva 132 SEED taxonomy (mixed rank)	BLASTn Type (% Ident) (Accessed 12/11/2021)	Reference
SPD.Control						
OTU00014	0.0023113	2.01	1.27	*Methanobrevibacter* (Genus; 100%)	*Methanobrevibacter olleyae* (99%)	Rea *et al*. (2007)

Table 5.16: OTU level taxonomic biomarkers for individual dairy cows fed a Control total mixed ration (TMR; C), TMR with De-Odorase® (D) or TMR with De-Odorase® and Yea-Sacc® (DY; Linear discriminant analysis (LDA) score > 2.0, P < 0.05)

	P-value	LDA score	% Seqs	Silva 132 SEED taxonomy (mixed rank)	BLASTn Type (% Ident) (Accessed 12/11/2021)	Reference
LPD. Cow 2						
OTU00044	2.08E-05	2.09	1.55	*Prevotella* (Genus)	*Prevotella ruminicola* (96%)	Purushe *et al*. (2010)

5.3.7.4 Protozoa

Relative abundance of *Isotrichidae* cells tended to decrease (P = 0.074) by 20 cells per 1000 cells of *Ophryoscolecidae* when cows were supplemented with De-Odorase® and by 41 when supplemented with DY compared to the baseline (Table 5.17). There was no interaction (P > 0.05) between dietary treatment and time on the relative abundance of protozoa.

Table 5.17: Relative abundance (*Isotrichidae* cells per 1000 cells of *Ophryoscolecidae*) of rumen protozoa in dairy cows fed a Control total mixed ration (TMR; C), TMR with De-Odorase® (D) or TMR with De-Odorase® and Yea-Sacc® (DY)

	Treatment				P-value		
	C	D	DY	SED	Tr	Ti	Tr x Ti
1400 h	166	129	134	25.8	0.074	0.180	0.615
1700 h	149	156	110				
2000 h	144	115	91				

C = Control, D= De-Odorase® supplementation, Y=Yea-Sacc® supplementation, Tr= Treatment, Ti= Time

5.4 Discussion

5.4.1 Diet and forages

The current study was conducted to determine the effect of supplementing the diet with *Y. schidigera* or *Y. schidigera* and a live yeast on the performance, rumen metabolism and the microbiome in high yielding dairy cows. All cows were fed the same basal TMR and were then provided with the appropriate supplement. The chemical analysis of the grass silage was consistent with other studies conducted in the UK, although the NH_3-N was lower than some other results (Sinclair *et al*., 2015; Tayyab *et al*., 2018). The De-Odorase® and Yea-Sacc® were supplemented at doses in accordance with manufacturers' recommendations.

5.4.2 Performance

There is much literature to suggest that *Y. schidigera* has no effect on performance in dairy cows (Wilson *et al*., 1998; Śliwiński *et al*., 2004; Singer *et al*., 2008). There was a decrease in DMI when cows were supplemented with DY, although dietary treatment had no effect on milk yield or milk fat content, similarly in Chapter 3 yeast had no effect on milk yield or composition. Lovett *et al*. (2006) reported similar results with the effect of *Y. schidigera*, and suggested that despite a decrease in dry matter intake (DMI) with *Y. schidigera*, performance was maintained by an increase in digestibility and improved microbial efficiency. In the current study however, whole tract digestibility was not affected by treatment. In contrast, there is evidence in previous research which suggests that yeast supplementation had no effect on DMI (Jiang *et al*., 2017; Dias *et al*., 2018), which does not comply with the results in the current study where DMI was lower in cows when yeast was included in the diet. Milk protein content (g/kg) in the current study decreased in cows fed D or DY compared to the Control, although milk protein yield was unchanged. Yeast has been reported to have no effect on milk protein content (Kumprechtová *et al.,* 2019; Ambriz-Vilchis *et al*., 2017), therefore the change in milk protein is more likely to be due to the supplementation with De-Odorase®. Wilson *et al*. (1998) reported a minor decrease in milk protein (g/kg) with *Y. schidigera* supplementation, and although this was not significant (P = 0.130), the reduction in milk protein may have been due to the antimicrobial properties of saponins found in *Y. schidigera.* This may have reduced the synthesis of microbial protein in the rumen (Singer *et al*., 2008), although microbial protein synthesis was not measured in the current study.

5.4.3 Diet digestibility and slurry analysis

In the current study, there was a decrease in nutrient intake with DY compared to the other treatments, which is principally due to the decrease in DMI as all cows were fed the same basal TMR. However, digestibility and N balance were not affected by D or DY supplementation. Śliwiński *et al*. (2004), Holtshausen *et al*. (2009) and Hristov *et al*. (1999) all concluded that *Y. Schidigera* had no effect on nutrient digestibility, whilst Patra and Saxena (2009) suggested that *Y. Schidigera* supplementation may have a negative effect on NDF digestibility in the rumen due to the reduction in protozoal activity, although total tract digestibility may be unaffected due to a change in site of digestion. Supplementation of yeast has also been reported to have no effect on total tract digestibility (Ferreira *et al*., 2019).

Ammonia is a basic compound with a pKa value of 9.3, and consequently at pH 9.3 the compound is in equilibrium with approximately 50% in the form of NH_3 and the remaining 50% in the form NH_4^+ (ammonium; Sigurdarson *et al*., 2018). At a lower pH there remains a higher portion of the compound in the aqueous solution (NH_4^+), therefore reducing the amount of volatile NH_3 which could be lost to the environment (Sigurdarson *et al*., 2018). When the urea present in urine and the urease in faeces react, NH_3 is produced in slurry (Huntington and Archibeque, 1999). The glycofraction of *Y. schidigera* has been reported to have NH_3 binding properties and therefore can bind to the NH_3 and keep the compound in the form of NH_4^+ rather than emitting the NH_3 as a gas, which would in turn be reflected as a lower slurry pH which is more stable (Wallace *et al*., 1994; Kavanagh *et al*.; 2019). Dietary treatment had no significant effect on total slurry N or NH_3-N concentration, however, there was a tendency that after six hours slurry pH was lower ($P = 0.076$) with D at pH 8.55 than the Control at pH 8.85. At the pH levels at six hours, the potential loss of volatile NH_3 in slurry was 0.553 mg/L in cows fed the Control diet compared to a loss of 0.312 mg/L of volatile NH_3 in cows fed D, which is a difference of nearly 44% between the dietary treatments. When applied to a farm situation the supplementation of D may beneficially reduce volatile NH_3 loss from slurry. During this experiment there was no recorded gas production, therefore any gas (e.g. NH_3) production from the slurry may occur after 24 h, so the implementation of slurry management measures may be more important once the slurry is stored in a lagoon rather than in the cattle shed.

5.4.4 Rumen metabolism and the microbiota

Dietary treatment had no effect on rumen metabolism (pH and VFA concentration), which is similar to that reported by Hristov *et al*. (1999) and Benchaar *et al*. (2008), who suggested that the effects of *Y. schidigera* on the rumen can vary with diet and dose rate. Rumen NH_3

concentration was not affected in the current study, a finding supported by Benchaar *et al*. (2008) and Wilson *et al*. (1998). However, in *in vitro* studies *Y. Schidigera* have reported a decrease in NH_3 concentration, which was suggested to be due to reduced proteolysis in the rumen caused by the antiprotozoal activity of the saponins (Wallace *et al*., 1994; Singer *et al*., 2008). Yeast has been reported to increase rumen pH and promote the growth of fibrolytic and lactate utilising bacteria due to its capacity to scavenge excess oxygen from the rumen (Chaucheyras-Durand *et al*., 2008). However, in the current study these effects were not apparent, findings which contrast with those reported in Chapter 3, which concluded that yeast supplementation tended to increase rumen pH and increase the relative abundance of an unclassified bacteria from the Clostridiales class compared to without yeast. However, direct conclusions on the individual effects of yeast are not possible as it was fed alongside *Y. Schidigera* which may have overshadowed any effect the yeast may have had.

Before conducting the bioinformatics, the sequencing data was normalised so each library contained the same number of sequences, keeping the number as high as possible without removing too many low abundance libraries. The number of sequences in the libraries of the SPD samples were considerably lower than the LPD, therefore the decision was made to subsample the LPD samples to 38,500 sequences and the SPD samples to 10,500 to maximise the most sequences in each digesta phase. Similar to the previous chapters, the microbial phyla identified in the current study were consistent with the expected composition of rumen microbiota characterised in previous studies (e.g. Henderson *et al*., 2015). Although some (Planctomycetes) have been reported in the rumen, they are more usually associated with free living aquatic and terrestrial environments and may therefore have been ingested and be transient to the rumen.

In the current study there was a reduction in OTU diversity in both the LPD and SPD samples in cows fed D, which may be a result of the antimicrobial properties of *Y. schidigera* (Wang *et al*., 2012). Cellulolytic bacteria have been reported to be more susceptible to lysis from *Y. schidigera* (Wang *et al*., 2000), they have a higher species richness than amylolytic bacteria, which are more diverse in function than cellulolytic bacteria (Bowen *et al*., 2018), therefore a decrease in the abundance of cellulolytic bacteria may have reduced diversity in the microbial community. In the current study there was no effect of *Y. schidigera* supplementation on relative the abundance of Firmicutes, but there was a decrease in *Methanobrevibacter* compared to cows when fed the Control diet suggesting that there may be a lower production of methane in cows fed diets supplemented with *Y. schidigera* although methane production was not measured in the current study. The effects of *Y. schidigera* on methane production are inconclusive (Holtshausen *et al*., 2009; Wang *et al*.,

2011). Protozoa are also associated with methane production in the rumen as they are involved in fibre degradation which contributes to the production of H_2 as a by-product, which archaea then utilise to produce methane (Wallace *et al*., 1994; Holtshausen *et al*., 2009). The trend for a reduction in protozoal numbers in cows fed a live yeast and *Y. schidigera* in the current study could therefore provide an explanation for the reduction in *Methanobrevibacter*.

5.5 Conclusion

Supplementing the diet with DY reduced DMI compared to the other treatments but did not affect milk yield, although there was a decrease in milk protein concentration in cows when fed either D or DY compared to the Control. Dietary treatments had no effect on plasma metabolites, whole-tract apparent digestibility, N balance, or rumen metabolism. Slurry pH tended to decrease compared to the Control after six hours, indicating that supplementation with D may reduce the loss of volatile NH_3, although there was no effect on slurry NH_3 concentration, total N or loss of volatile N. Alpha and beta diversity decreased in rumen fluid from cows fed D compared to the Control, indicating a reduction in the variety of OTU in the rumen. Supplementing with DY tended to reduce protozoal numbers, and in both the solid and liquid phase digesta there was a lower relative abundance of *Methanobrevibacter* in cows when fed D or DY compared to the Control which may reduce methane production. For future research the study should be repeated with different doses of De-Odorase® to better understand it effects on performance and rumen function.

Chapter 6: General discussion

6.1 Introduction

This thesis has characterised the effect of feeding issues which can commonly occur on farm such as diet selection (Chapter 3), short-term feed restriction (Chapter 4), and reducing the excretion of N (Chapter 5) on the performance, digestibility, rumen metabolism and microbiome of high yielding dairy cows, and the effect of supplementation a live yeast or *Y. schidigera*. The main priorities when managing dairy cattle are health, production and fertility, and a significant factor in controlling these are by diet (Bowen *et al*., 2018). It is common for dairy cattle to be housed throughout the year and receive nutrients in the form of a TMR to better monitor and control the balance of nutrients, ensuring maximum production while maintaining optimal health (March *et al*., 2014). However, studies have shown that cattle are able to sort through the TMR and tend to favour shorter particles such as concentrates over longer forage particles, a problem that can be exacerbated by poor mixing by the mixer wagon (Tayyab *et al*., 2018). When cows express sorting behaviour they change both the level and pattern of concentrate intake (Leonardi and Armentano, 2003), which can alter rumen fermentation and performance (DeVries *et al*., 2008). Furthermore, due to errors such as insufficient supply of feed or pasture, or feed equipment failure, cattle can experience periods of short-term feed restriction. For example, Tayyab *et al*. (2018) reported that 34% of the farms surveyed in the UK had no feed left in the morning prior to feeding. These practices can increase the risk of cows developing SARA especially when the feed is returned, as cows can enter a period of overeating to compensate, consuming a higher intake of concentrates (Thomson *et al*., 2018).

Active dry yeasts are well accepted to have beneficial effects on livestock performance, including increasing performance (Barrera *et al*., 2019; Chaucheyras-Durand *et al*., 2008). Yeast has been reported to have three main effects on the rumen microbiota: improvement of rumen maturity by favouring microbial establishment, stabilisation of ruminal pH and interactions with lactate metabolising bacteria, and increasing fibre degradation and interactions with plant cell wall degrading microbes (Chaucheyras-Durand *et al*., 2008). Yeast also improves fibre degradation by scavenging oxygen due to its high respiratory activity, therefore shifting rumen conditions to become more anaerobic and so more favourable for fibre degrading bacteria (Newbold *et al*., 1996).

Ammonia emissions contribute to the formation of fine particle matter with a diameter smaller than 2.5 µm (PM2.5) and are associated to many adverse health conditions (Giannakis *et al*., 2019). The UK government launched the Clean Air Strategy to reduce the number of people living in locations above the WHO guideline level of 10 µg/m^3 by half by 2025 (GOV,

2019). The policy covers pollution produced by industry, transport, farming, and households. Often cows receive diets which contain high levels of RDP which can lead to increased levels of N being excreted (Chowdhury, 2022). Nitrogen is excreted in urine mainly in the form of urea which reacts with the urease enzyme in faeces to produce NH_3 in slurry, which as a volatile compound NH_3 can contribute to air pollution and have negative environmental effects (Huntington and Archibeque, 1999). Agriculture accounts for 88% of UK ammonia emissions and can occur during slurry application, cattle housing, slurry storage, and grazing, and reducing ammonia losses would require a whole farm system approach (Bussink and Oenema, 1998). *Yucca schidigera*, more commonly used in monogastric nutrition, contain saponins which have a glycofraction with the capacity to bind to NH_3 potentially both in the rumen and in slurry, which may reduce the emissions of volatile ammonia, along with other N compounds such as nitrous oxide into the environment (Wallace *et al*., 1994; Saeed *et al*., 2018). Furthermore, *Y. schidigera* contains steroidal saponins which act as a natural detergent have antiprotozoal and antibacterial properties, which may reduce methane production as protozoa are closely linked to methanogens and methane production (Wallace *et al*., 1994).

The objectives of the series of studies were: To determine the effect of pattern of concentrate allocation when fed with or without supplementation of a live yeast on rumen metabolism, the microbiome, animal performance, and whole tract digestibility in high yielding dairy cows; determine the effect of short-term feed restriction and re-feeding on rumen metabolism, the microbiome and performance of high yielding dairy cows when fed different concentrate patterns and either with or without a live yeast, and to determine the effect of *Yucca schidigera* extract and a live yeast supplementation in diets high in rumen degradable protein on rumen metabolism, the microbiome, nitrogen balance, and performance of high yielding dairy cows .

6.2 Optimising methodology

There were issues with the original PCR protocol (Section 2.16.4.1), and therefore the method was optimised, as described in Section 2.16.4.2. When following the original PCR protocol, initially the results appeared acceptable, then the bands of DNA in the gel became weak and unclear, often showing no bands at all despite accurate pipetting and using fresh reagents. There were two possible reasons why the process was not working: lack of apparent amplification or poor quality gel. The amplification may not have worked due to the degradation of primers or the template DNA, incorrect annealing temperature/ time or insufficient amplification/ cycles. During the analysis the primers were repeatedly thawed and refrozen which may have caused them to degrade (Pollock *et al*., 2018). The template

DNA may have degraded due to the introduction of DNases through contamination (Gohl *et al*., 2016). Therefore all surfaces and equipment were wiped down with DNase wipes, and tips and PCR tubes were sterilised under UV light for 30 minutes. Running 20 cycles during the PCR schedule did result in sufficient amplification, however there was reluctance to increase the number of cycles much further as this can result in amplification bias, and the relative abundances of bacteria have reported to change with increased cycles, skewing results (Kanagawa, 2003). Using TBE buffer instead of TAE in the agarose gel has been reported to improve the quality and clarity of the bands (Sanderson *et al*., 2014). Furthermore, running the gel with a higher voltage can reduce the clarity of the DNA bands and the ladder (Pollock *et al*., 2018). The original method was therefore adapted by changing the PCR program to exactly follow Kozich *et al*. (2013) increasing the number of cycles to 30, using freshly prepared primers, using 1% TBE buffer instead of 1% TAE, and with lower voltage (60 v/cm). These changes improved the quality of the results (Figure 6.1).

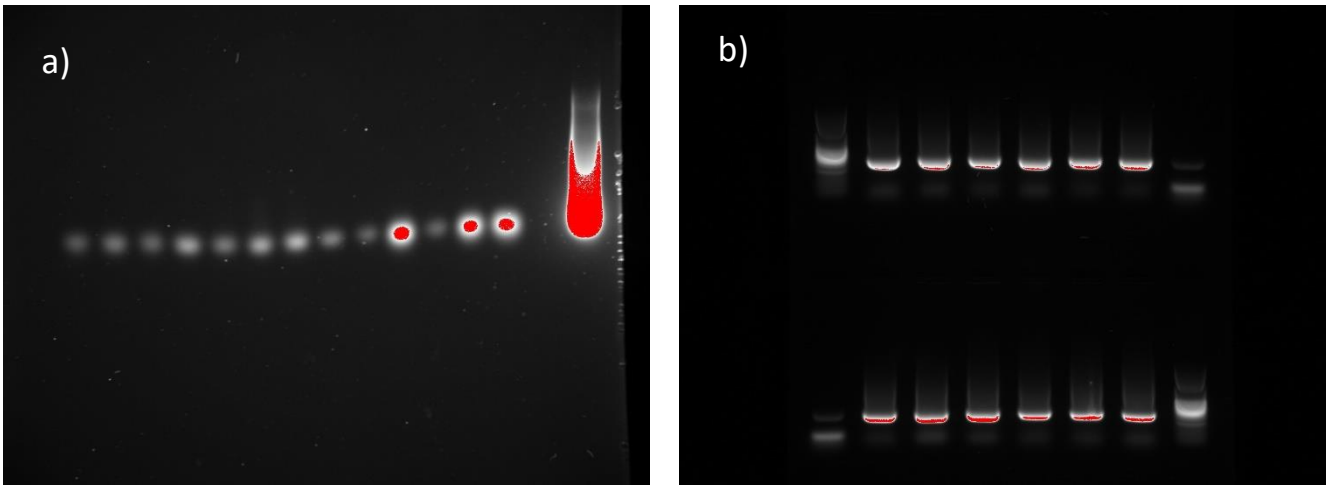


Figure 6.1: Comparison of a) original PCR protocol with b) optimised PCR protocol

6.3 Effect of supplementation of a live yeast on performance and rumen function

Across all studies a live yeast (Yea-Sacc®, Alltech UK) was supplemented in the diets under a range of situations. In Chapter 3 yeast was supplemented while cows were fed different patterns of concentrates to simulate sorting behaviour (even or uneven eating pattern). In Chapter 4 cows received yeast supplementation while experiencing a period of short-term feed restriction. In Chapter 5 cows were fed a diet high in RDP while supplemented both with a live yeast and *Yucca schidigera* extract (De-Odorase®) to determine if N use efficiency could be increased and the loss of N as NH_3 in slurry decreased.

In Chapter 3, supplementing the diet with a live yeast and altering pattern of concentration allocation had no significant effect on the performance or whole tract digestibility in dairy

cows. Changes to rumen metabolism included an increase in rumen pH with yeast supplementation. The inclusion of live yeast also affected the Bray Curtis dissimilarity, although the effects were not strong with an increase in the abundance of unclassified *Clostridiales* in cows fed yeast and an increase of unclassified species of *Prevotella* and *Gammaproteobacteria* when cows were not fed yeast. Ferreira *et al*. (2019) reported that the effects of supplementing with a live yeast were greater when the rumen conditions were challenged (i.e. by a high concentrate diet). In Chapter 3 with altered pattern of concentrates there were only small effects of the yeast on the rumen microbial community which were not reflected at animal level with minimal effects on performance or rumen metabolism, suggesting that the rumen was not sufficiently challenged. Prior to the study, preliminary work was conducted using 6 kg of concentrates for the even/uneven pattern of allocation, although the level of concentrates used in Chapter 3 appeared to cause a sufficiently large decrease in rumen pH, and therefore the decision was made to decrease the amount of concentrates fed to 4 kg to avoid the potential of compromising the health of the cows. However, the extent that the concentrates were decreased may have been too large, and therefore did not elicit an effect on the cows. It should be borne in mind that under commercial feeding practices, cows that select for concentrates change both their pattern and amount of concentrates that they consume. By only changing the pattern of concentrates in the current study it was not possible to determine the combined effect of pattern and level of concentrate intake, and therefore may have under-estimated the effects seen on farm

When subjected to a period of short-term feed restriction (Chapter 4), there was no effect of yeast supplementation on performance or rumen metabolism, although there was evidence that the rumen microbial community recovered more rapidly with yeast supplementation, principally by increasing the relative abundance of *Treponema bryantii*. This suggests that when there was additional challenge to the rumen (i.e. from feed restriction), yeast provided some benefit to cellulolytic bacteria growth, although similar to Chapter 3, this was not reflected in performance or rumen function parameters. In Chapter 5, cows received yeast supplementation in addition to *Y. schidigera*, *Y. schidigera* benefited the rumen microbial community by decreasing the relative abundance of *Methanobrevibacter* compared to the Control which may reduce methane production (Holtshausen *et al*., 2009; Danielsson *et al*., 2017). By decreasing the initial pH in slurry *Y. schidigera* may also reduce the loss of volatile ammonia into the environment. In contrast, there appeared to be little additional benefit of supplementing the diet with yeast in addition to *Y. schidigera,* with no effect on performance, digestibility or rumen function. It could be theorised that when using supplements in combination the effects of *Y. schidigera* outweigh the effects of yeast in the rumen. It may be

considered that the supplementation of yeast could benefit the rumen when fed with *Y. schidigera* due to its ability to stabilise rumen pH and encourage fibrolytic activity, however Wang *et al*. (2000) reported that *Y. schidigera* can have antimicrobial properties, particularly the lysis of fibrolytic bacteria. Therefore the beneficial properties of yeast supplementation may be negated if *Y. schidigera* reduced the size of the fibrolytic microbial population.

It is clear that the effects of yeast supplementation are not consistent when fed under different feeding conditions, and the current results suggest that there is much variation of the efficacy of yeast between individual cows. In Chapter 3, yeast had an effect on the microbial community of every cow, however the microbial communities of each cow were substantially different from each other ($P < 0.001$) both with or without yeast supplementation (Table 3.13) suggesting that the effect of yeast was not consistent between all cows and the effect of individual cow may have overridden the effect of yeast (Table 6.1). Whereas in Chapter 5, there was an effect of individual cow on the Bray Curtis dissimilarity in the LPD samples ($P < 0.001$) and a tendency in the SPD samples ($P = 0.086$), however when assessing the individual pairwise comparisons there were no differences in the microbial communities between individual cows ($P > 0.001$) when fed the dietary treatments in either digesta phase. There were no common taxa across the studies when the cows were fed yeast (Table 6.2), and furthermore there were no common taxa in the individual cows when diets were supplemented with yeast across the studies, suggesting that the effect of yeast supplementation on the rumen microbiota is not specific to a small/specific group between cows/rumen, and may affect many taxa at a smaller level and therefore not be detected with the LDA.

Table 6.1: Bray Curtis dissimilarity metric of individual cows in Chapters 3, 4 and 5

	Stress value	P-value
Chapter 3	0.21556	<0.001
Chapter 4	0.21556	<0.001
Chapter 5	0.18732	LPD: <0.001 SPD: 0.086

Table 6.2: OTU level taxonomic biomarkers for dairy cows fed diets with and without yeast in Chapters 3, 4 and 5 (Linear discriminant analysis (LDA) score > 2.0, P < 0.05)

	Without yeast	Yeast
Chapter 3	*Chelonobacter oris* (87%)	*Gracilibacter thermotolerans* (87%)
	Prevotella copri (89%)	
Chapter 4	–	–
Chapter 5 LPD	*Prevotella brevis* (90%)	*Chelonobacter oris* (87%)
	Prevotella bryantii (91%)	*Prevotella ruminicola* (94%)
	Methanobrevibacter olleyae (98%)	
Chapter 5 SPD	*Methanobrevibacter olleyae* (99%)	

Further work is therefore required to identify if there are any biomarkers present in the rumen contents or faecal matter which can be assessed to identify whether the supplementation of yeast is likely to be more effective in individual cows in terms of performance and rumen function, allowing the farmer to have a targeted approach to supplementing the herd to optimise output.

6.4 Effect of individual cow variation on performance and rumen function

An important question is highlighted from the current thesis regarding why the rumen microbiome of individual cows responded differently when fed the same diets and additives? Through omics-based analysis it had been established that the rumen microbiota plays an important role in many aspects of production and health in dairy cows including feed efficiency, methane production, milk yield and rumen acidosis (Li *et al*., 2019). Previous work has identified that the variation of the individual microbiome can influence performance and rumen function parameters, however it has been reported to contribute to a lesser extent compared to the effect of the dietary treatments. Which contrast with the results in the current studies, particularly in Chapter 3 where there is more variation between individual cows (Shaani *et al*., 2018; Henderson *et al*., 2015).

In Chapter 3 the differences between cows was most notable, with clear differences between the Bray Curtis dissimilarity in both the SPD and LPD samples and many discriminant taxa identified between cows (Table 6.1), while in Chapter 5 the differences were less pronounced with only an effect in the LPD and only one discriminant taxa reported in one cow. There are a range of factors which may have led to a change in the degree of variation between the studies including: diet, climate, and parity (Li *et al*., 2019). The cows used in the current studies had been at Harper Adams University for 6 months prior to the commencement of Chapter 3, while in Chapter 5 they had been on the premises for over 2 years. Furthermore, in Chapter 5 the cows were 18 months older and in their third lactation compared to Chapter 3. By the time of the study reported in Chapter 5 the cows had both

had longer in their housing environment and so the rumens of the cows were more likely to have adapted to environment and therefore have less inter-cow variation compared to Chapter 3, although further work is necessary to determine the length of time required for the rumen to adjust to a new environment and factors which may influence this. In addition, Xue *et al*. (2018) concluded that once animals reach adulthood (> 2 years old) they have established a 'stable' microbiome which does not vary significantly with parity, suggesting that in the current study the age of cows alone did not greatly affect the differences in individual cow variation between the studies. In Chapter 3 the diet was 1 MJ/kg DM of energy and 144 g/kg DM of starch higher than the TMR provided in Chapter 5 and could therefore be considered to be more challenging to the rumen due to an increased risk of SARA, supported by Figure 6.2 which highlights that rumen pH was lower than pH 5.8 for approximately 12 h, indicating SARA, while rumen pH observed in the study reported in Chapter 5 did not drop below pH 5.9. If there was greater challenge on the rumen from the diet in Chapter 3 this may have exacerbated any changes in the individual microbiome. These effects emphasise the benefit of using a Latin square study design, as this accounts for animal variation in the statistical analysis of the results.

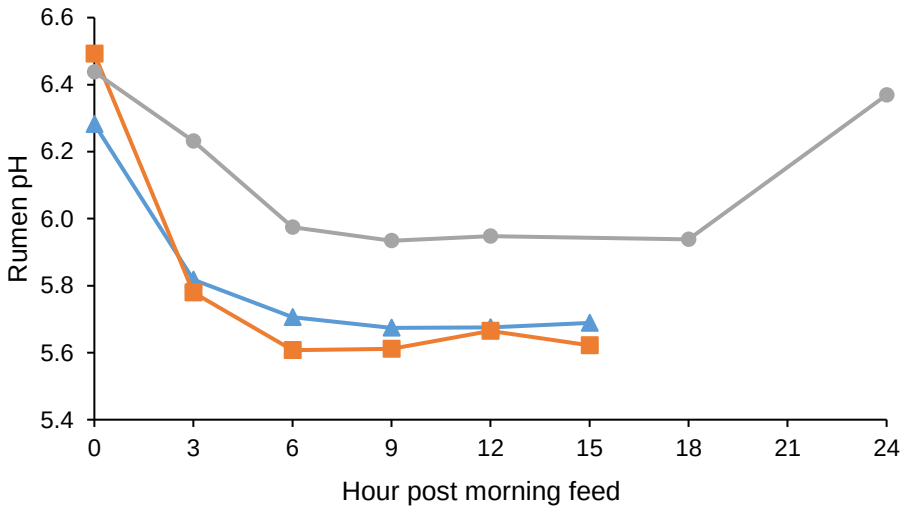


Figure 6.2: Rumen pH in Chapters 3 (▲), 4 (■) and 5 (●)

Zhu *et al.* (2021) hypothesised that the rumen microbiota were composed of two key components, the 'core microbiota' consisting of taxa crucial for functional stability, and the 'dynamic microbiota' with a plasticity allowing for adaption to swift changes in the environment, such as diet. Furthermore, Li *et al*., (2019) concluded that different taxa of the rumen microbiota have different levels of heritability. Taxa from the Bacteroidetes phylum are very versatile in function and have been reported to have a low heritability, and are therefore influenced heavily by environmental factors such as diet (Li *et al*., 2019). On the

other hand, taxa from the Firmicutes phylum, with fibrolytic properties, have been shown to have a higher estimated heritability than Bacteroidetes, and therefore are less affected by diet (Li *et al*., 2019). Heritable taxa such as *Succinivibrionaceae* and *Clostridiales* have also been reported to interact with other taxa and therefore may be concluded to be keystone members of the microbiota, as their presence may be necessary for microbial activity to occur despite not being directly involved in the process (Li *et al*., 2019). If keystone taxa have high heritability then a cow with low levels of these keystone taxa may have lower rates of feed degradation and therefore lower feed efficiency than those with higher levels, regardless of diet. At birth the rumen of a calf is not established or functional, the initial microbial community is acquired from the surrounding metacommunity, including skin during suckling and grooming, colostrum and milk, and the external environment (Curtis and Sloan, 2004; Rey *et al*., 2014). There are a range of factors which can affect the degree of microbial establishment including snatch calving, the cleanliness of the environment, colostrum intake/quality, pre weaning diet (Rey *et al*., 2014). As a result, even cows born and raised on the same farm may have different microbiomes, and furthermore when cows are moved to different environments they may take different lengths of time for the microbiome to adapt despite being fed the same diet and being kept in the same pens.

The rumen is very resilient to change, and when subjected to 95% exchange with the rumen contents of another cow the microbiota returned to the original composition within 10 days in the majority of the cows (Weimer *et al*., 2017). There are a range of physical and physiological factors which may influence the rumen microbiota including: rumination rate, rumen outflow rate, N recycling and abundance of protozoa (Clemmons *et al*., 2019). Cows with increased rumination rate would contribute to an increased proportion of smaller feed particles in the rumen which would lead to a faster rate of degradation due to a larger feed particle surface area and therefore increase outflow, encouraging the growth and activity of the liquid phase rumen microbiota, rate, potentially increasing DMI and therefore milk yield (Zebeli *et al*., 2012). If cows have a higher degree of N recycling a larger proportion of N would be returned to the rumen in the form of urea, potentially reducing the amount of N excreted in urine (Huntington and Archibeque, 1999). The rumen epithelium has been reported to have a distinctly different relative microbial community compared to the solid and liquid digesta phase communities (de Mulder *et al*., 2017). Epimural bacteria been linked to the hydrolysis of urea as part of nitrogen recycling (Wetzels *et al*., 2017), and therefore with an increase in dietary RDP there may be changes to the abundance of epimural bacteria, although this was not measured in the current study.

6.5 Effect of digesta phase on the rumen microbiome

There are multiple methods available to sample the rumen microbiota including oesophageal tubing, bolus or rumenocentesis (Ji *et al*., 2017). Research has shown that there can be significant variation in relative microbial communities between different rumen environments (solid associated, liquid associated and epimural) therefore attention needs to be paid to the bias of distributed bacteria for each digesta phase especially for studies using liquid as the representative sample (de Mulder *et al*., 2017; Ji *et al*., 2017).

The solid digesta phase is strongly associated with cellulolytic bacteria and secondary colonisers such as *Lachnospiraceae* or *Christensenellaceae* (de Mulder *et al*., 2017). The bacteria adhere to the fibrous material with the aid of biofilms to degrade the structural polysaccharides (Bowen *et al*., 2018). Bacteroidetes is often the most predominant phyla in the liquid phase, which is supported by the results in the current studies (Table 6.3). Rumen fluid contains dissolved sugars and readily degradable carbohydrates, and bacteria from the Bacteroidetes phyla are very diverse in their function, showing amylolytic, cellulolytic and proteolytic properties (Bowen *et al*., 2018). As a result, there tends to be a higher diversity within the solid fraction than the liquid as cellulolytic bacteria are more specific in their role than amylolytic bacteria, and there can be a greater number of methanogens in the solid phase, as the liquid phase tends to be predominated by *Prevotella* (Bowen *et al*,. 2018). Previous research has observed the *Prevotella* genera to be highly abundant in the microbial community, accounting for up to 50% of all sequence reads, regardless of diet (Henderson *et al*., 2015; Jami and Mizrahi, 2012). McCann *et al*. (2014) suggest that there is much diversity of function between species and strains of *Prevotella*, with the abilities to degrade amylose and amylopectin. *Prevotella* does not have cellulolytic properties though *Prevotella ruminicola* has been found to improve cellulose digestion when cocultured with *Fibrobacter succinogenes* and *Ruminicola flavefaciens* (Fondevila and Dehority, 1996).

Table 6.3: Relative abundance (%) of the top three most abundant phyla in the liquid (LPD) and solid (SPD) samples in studies 3, 4 and 5

Chapter 3		Chapter 4		Chapter 5	
LPD	SPD	LPD	SPD	LPD	SPD
Bacteroidetes (45.2%)	Bacteroidetes (38.9%)	Bacteroidetes (44.0%)	Bacteroidetes (36.2%)	Bacteroidetes (47.31%)	Bacteroidetes (38.65%)
Firmicutes (28.6%)	Firmicutes (36.0%)	Firmicutes (27.6%)	Firmicutes (33.7%)	Firmicutes (23.37%)	Firmicutes (31.6%)
Proteobacteria (12.6%)	Spirochaetes (6.8%)	Proteobacteria (11.9%)	Spirochaetes (8.88%)	Euryarchaeota (7.83%)	Spirochaetes (14.9%)

In Chapter 3, OTU richness increased in cows fed an uneven diet compared to even, in the SPD samples but not the LPD. An uneven diet promotes both fibrolytic and amylolytic activity more distinctly than an even diet, possibly due to the large intake of concentrates in the morning feeding followed by the forage based PMR for the remainder of the day, while with the even diet the nutrient intake is more consistent during the day encouraging a more stable microbial community. In the liquid phase the microbiota are versatile in function, while in the solid phase an uneven diet would encourage the growth and activity of a larger range of taxa to accomplish nutrient degradation hence increasing the diversity (Tapio, *et al*., 2017; Bowen *et al*., 2018). In contrast, in Chapter 5 there was a decrease in diversity in the solid phase with D supplementation as *Y. schidigera* has been reported to have antimicrobial properties. Additionally, Wang *et al*. (2000) reported that cellulolytic bacteria were more susceptible to lysis, and as they are more abundant in the solid phase this would therefore reduce diversity.

6.6 Effect of time on performance and rumen function

Measurements of rumen function (pH, VFA, lactate, NH_3) were sampled every 3 h from the morning feed to assess the effect of treatment on the diurnal variation of the variables across all three studies. In Chapter 5, there was minimal effect of time on rumen metabolism as cows received a TMR which was consistent throughout the day, whereas sampling time was a more important factor in Chapter 3 as the difference in pattern of concentrates caused more diurnal changes to rumen pH and ammonia concentration. In Chapter 4 when cows received feed following the FR period there were also considerable diurnal changes to DMI, rumen pH and VFA concentration.

In Chapter 3, samples for the rumen microbiome were collected at the same time as samples for rumen fluid, and the decision was then made to analyse the microbiome in three of the time points based on changes in the diurnal variation in rumen metabolism (pH and VFA concentration), resulting in 0, 3, 12 h post morning feed being used. The same time points were used in Chapter 4 as the results from Chapter 3 served as the baseline prior to the FR period, so the baseline and rec d1 data were parallel. When comparing the most prevalent OTU at each time point in Chapters 3 and 4 there are none which are the same between studies, indicating substantial changes in the composition of the microbiome within the day and highlighting the importance of sampling multiple times points (Table 6.4). In contrast, in Chapter 5 the rumen microbiota samples were also taken at the same time points as the rumen metabolism samples (as with the other studies), but the decision was made to choose three time points where the other rumen parameters were more consistent,

so any changes to the microbiome would be due to the effect of the treatment and not overshadowed by the effect of time.

Table 6.4: OTU level taxonomic biomarkers found in cows in Chapters 3 and 4 at timepoints 0, 3 and 12 h post feeding for dairy cows (Linear discriminant analysis (LDA) score > 2.0, P < 0.05)

	Chapter 3	BLASTn Type (% Ident) (Accessed 12/11/2021)	Chapter 4	BLASTn Type (% Ident) (Accessed 12/11/2021)
O h				
	OTU00010	*Marseillibacter massiliensis* (92%)	OTU00004	*Methanobrevibacter ruminantium* (98%)
	OTU00009	*Gracilibacter thermotolerans* (87%)	OTU00010	*Intestinimonas butyriciproducens* (92%)
3 h				
	OTU00015	*Lactobacillus nangangensis* (99%)	OTU00011	*Loigolactobacillus coryniformis* (98%)
	OTU00006	*Lactobacillus fuchuensis* (100%)	OTU00008	*Latilactobacillus sakei* (98%)
			OTU00027	*Acinetobacter chinensis* (100%)
12 h				
	OTU00001	*Chelonobacter oris* (87%)	OTU00003	*Frischella perrara* (88%)
			OTU00050	*Bifidobacterium longum* (99%)
			OTU00007	*Succiniclasticum ruminis* (98%)

6.7 General conclusions

Altering the pattern of concentrate allocation, to simulate sorting behaviour in dairy cows, had no effect on performance, digestibility, rumen metabolism or the relative microbial community but changed the rumen pH diurnal pattern and resulted in the cows fed the uneven pattern of concentrates being at a higher risk of SARA. Supplementing the diet with a live yeast had no effect on performance or digestibility but tended to increase rumen pH. Additionally, there was an increase in diversity of the relative microbial community in cows that received yeast supplementation, indicating that supplementation results in some benefit to the rumen which is not always reflected in performance. However, an effect of pattern of concentrates may be seen if the treatments were more extreme or conducted in conjunction with changing level of concentrates. Implementing a short-term FR period followed by re-feeding, had a negative effect on performance and rumen metabolism, with a decrease in milk yield, rumen pH, and microbial diversity, compared to the baseline. Milk yield, rumen pH and the relative microbial community took longer than the allocated recovery period to return to baseline levels. On rec d1 there was an increased relative abundance of *Methanobrevibacter*, suggesting that with a FR period there may be an increased production of methane. As a result, short term feed restriction periods should be avoided on farm, though the effect of repeated feed restriction on rumen metabolism and performance and possible acclimatisation requires further investigation. Supplementing the diet with De-

Odorase® tended to decrease the pH of slurry after 6 h which may reduce the loss of volatile NH_3 to the environment, although in the current study there were no changes in slurry NH_3 or total N concentration. There was a decrease in the relative abundance of *Methanobrevibacter* when cows were fed De-Odorase® or De-Odorase® and Yea-Sacc® compared to the Control, suggesting that supplementing the diet with De-Odorase® may reduce methane production. There was also a decrease in DMI with De-Odorase® and Yea-Sacc® but dietary treatment had no effect on performance, N balance or rumen metabolism. For future research the study should be repeated with different doses of De-Odorase® to better understand the effects on performance and rumen function. Across the three studies, the effects of Yea-Sacc® on performance and rumen function were inconclusive, effects on the rumen microbial community were seen more in some cows than others while on the same treatments.

6.8 Limitations and future research

The aim of the first study was to determine the effect of changing pattern of concentrate allocation, to simulate sorting behaviour, on the performance, digestibility, rumen metabolism and microbiome in high yielding dairy cows. When cattle display diet selection they can alter both the pattern and level of concentrates, and the results from Chapter 3 showed that changing the pattern of concentrates alone did not elicit a significant effect on high yielding dairy cows, and therefore may not be a major concern on farm without changing level of concentrate intake as well. In a future study a higher proportion of concentrates should be used. To more accurately determine the overall effect of diet selection the study should be repeated as a 2 x 2 factorial Latin square with both pattern and level of concentrates as treatments. In the current study it was not feasible to evaluate this in addition to the supplementation of yeast, as there were not sufficient cows for 2 x 2 x 2 factorial Latin square study.

The chapters in the thesis have identified that there can be considerable variation in the microbiome between individual dairy cows, although the degree of variation was not consistent across the three studies, with a lower effect of individual cows on the relative microbial community in Chapter 5 compared to Chapter 3. More work is required to better understand factors which may drive these changes, and the time taken for cows to adjust to a new environment when under the same conditions and provided with the same diet. Additionally, further work is required to identify any biomarkers in the rumen microbiome to distinguish between cows, as this may lead to the possibility of a targeted approach to feed supplementation.

In the FR study (Chapter 4), the inclusion of a control group which does not undergo a period of FR would have made the results more robust. The current study was a before and after design, which can have limitations from external factors such as climate temperature and lactation stage. It was concluded in Chapter 4, that on the first recovery day following a short-term FR DMI increased by approximately 23%. Should a short-term feed restriction period occur on farm it is paramount that sufficient additional feed is provided on recovery day one to accommodate the cows to compensate otherwise the time taken for rumen parameters and performance to return to baseline levels may be extended further than the four days observed here. The effect of chronic FR requires further investigation was cows may not receive adequate feed to compensate for the period of overeating during the recovery days, extending the time taken for performance and rumen function to return to baseline levels.

At the dose rate used (5 g/cow/day) in Chapter 5, *Y. schidigera* had an effect on only a few variables, and there appeared to be little additive effect of supplementing the diets with yeast. Further work is required to determine if the is a dose effect of *Y. schidigera* in dairy cows, by repeating with higher levels of De-Odorase®. The methods used to analyse the slurry require further development, including incubating the slurry for a longer period to monitor gas production, as in the current study no measurable gas was collected in the first 24 h.

7.0 References

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8.0 Appendices

8.1 Buffers and reagents

NDF reagent

The NDF reagent was prepared by dissolving 93 g of di-sodium ethylene diamine tetra-acetic acid dehydrate (EDTA) and 34 g of sodium tetraborate in 3 L of hot distilled water, then 150 g of sodium dodecyl sulphate (SDS) and 50 ml of tri-ethylene glycol were added. In a separate beaker, 22.8 g of anhydrous disodium hydrogen phosphate was dissolved in 500 ml of hot distilled water, added to the first solution and mixed. The volume was made up to 5 L and pH was adjusted to lie between 6.9 and 7.1 using 0.1 M NaOH or 0.1 M HCl.

0.9% Saline solution (1 L)

9 g NaCl

Make up to 1 L with dH_2O and stir until dissolved

Add NaCl to dH_2O and stir until dissolved

EDTA (500 ml 0.5M pH 8.0)

93.5 g EDTA

Make up to 500 ml with dH_2O and stir until dissolved

Adjust pH up to pH 8.0 using NaOH

Sterilise in autoclave at 120°C for 20 minutes

Tris-HCl (500 ml 1M pH 8.0)

60.57 g Tris base

Make up to 500 ml with dH_2O and stir until dissolved

Adjust pH down to pH 8.0 using HCl (5M)

5M NaCl (500 ml 5M pH 8.0)

146 g NaCl

Make up to 500 ml with dH_2O and stir until dissolved

Adjust pH to pH 8.0

Sodium dodecyl sulfate (SDS; 50 ml 10% w/v)

5 g SDS

Make up to 50 ml with dH_2O and stir until dissolved

Filter sterilise (0.2 nm filter)

Ammonium acetate (250 ml 10M)

192.7 g ammonium acetate

Make up to 250 ml with dH_2O and stir until dissolved

Lysis buffer (40 ml)

EDTA 0.5M (4 ml)

Tris-HCl 1M (2 ml)

NaCl 5M (4 ml)

SDS 10% w/v (16 ml)

dH_2O (14 ml)

TE buffer

Tris-HCl 1M pH 8.0 (1 ml)

EDTA 0. dH_2O (14 ml)

5M pH 8.0 (200 µl)

dH_2O (100 ml)

Tris-acetate-EDTA (TAE) buffer (1 L)

100 ml TAE buffer (10x)

900ml dH_2O

Tris-borate-EDTA (TBE) buffer (1 L)

100 ml TBE buffer (10x)

900ml dH_2O

8.2 Primers

Table 8.1: Dual index primer sequences for study one

Period	Phase	Cow	Diet	Yeast	Time	i5 Forward Primer	i7 Reverse Primer
1	LAB	Cow 1	Even	Control	0	SB501	SA701
1	LAB	Cow 2	Uneven	Control	0	SB501	SA702
1	LAB	Cow 3	Uneven	Yeast	0	SB501	SA703
1	LAB	Cow 4	Even	Yeast	0	SB501	SA704
1	LAB	Cow 1	Even	Control	3	SB501	SA705
1	LAB	Cow 2	Uneven	Control	3	SB501	SA706
1	LAB	Cow 3	Uneven	Yeast	3	SB501	SA707
1	LAB	Cow 4	Even	Yeast	3	SB501	SA708
1	LAB	Cow 1	Even	Control	12	SB501	SA709
1	LAB	Cow 2	Uneven	Control	12	SB501	SA710
1	LAB	Cow 3	Uneven	Yeast	12	SB501	SA711
1	LAB	Cow 4	Even	Yeast	12	SB501	SA712
2	LAB	Cow 1	Uneven	Control	0	SB502	SA701
2	LAB	Cow 2	Even	Yeast	0	SB502	SA702
2	LAB	Cow 3	Even	Control	0	SB502	SA703
2	LAB	Cow 4	Uneven	Yeast	0	SB502	SA704
2	LAB	Cow 1	Uneven	Control	3	SB502	SA705
2	LAB	Cow 2	Even	Yeast	3	SB502	SA706
2	LAB	Cow 3	Even	Control	3	SB502	SA707
2	LAB	Cow 4	Uneven	Yeast	3	SB502	SA708
2	LAB	Cow 1	Uneven	Control	12	SB502	SA709
2	LAB	Cow 2	Even	Yeast	12	SB502	SA710
2	LAB	Cow 3	Even	Control	12	SB502	SA711
2	LAB	Cow 4	Uneven	Yeast	12	SB502	SA712
3	LAB	Cow 1	Uneven	Yeast	0	SB503	SA701
3	LAB	Cow 2	Even	Control	0	SB503	SA702
3	LAB	Cow 3	Even	Yeast	0	SB503	SA703
3	LAB	Cow 4	Uneven	Control	0	SB503	SA704

3	LAB	Cow 1	Uneven	Yeast	3	SB503	SA705
3	LAB	Cow 2	Even	Control	3	SB503	SA706
3	LAB	Cow 3	Even	Yeast	3	SB503	SA707
3	LAB	Cow 4	Uneven	Control	3	SB503	SA708
3	LAB	Cow 1	Uneven	Yeast	12	SB503	SA709
3	LAB	Cow 2	Even	Control	12	SB503	SA710
3	LAB	Cow 3	Even	Yeast	12	SB503	SA711
3	LAB	Cow 4	Uneven	Control	12	SB503	SA712
4	LAB	Cow 1	Even	Yeast	0	SB504	SA701
4	LAB	Cow 2	Uneven	Yeast	0	SB504	SA702
4	LAB	Cow 3	Uneven	Control	0	SB504	SA703
4	LAB	Cow 4	Even	Control	0	SB504	SA704
4	LAB	Cow 1	Even	Yeast	3	SB504	SA705
4	LAB	Cow 2	Uneven	Yeast	3	SB504	SA706
4	LAB	Cow 3	Uneven	Control	3	SB504	SA707
4	LAB	Cow 4	Even	Control	3	SB504	SA708
4	LAB	Cow 1	Even	Yeast	12	SB504	SA709
4	LAB	Cow 2	Uneven	Yeast	12	SB504	SA710
4	LAB	Cow 3	Uneven	Control	12	SB504	SA711
4	LAB	Cow 4	Even	Control	12	SB504	SA712
1	SAB	Cow 1	Even	Control	0	SA505	SA701
1	SAB	Cow 2	Uneven	Control	0	SA505	SA702
1	SAB	Cow 3	Uneven	Yeast	0	SA505	SA703
1	SAB	Cow 4	Even	Yeast	0	SA505	SA704
1	SAB	Cow 1	Even	Control	3	SA505	SA705
1	SAB	Cow 2	Uneven	Control	3	SA505	SA706
1	SAB	Cow 3	Uneven	Yeast	3	SA505	SA707
1	SAB	Cow 4	Even	Yeast	3	SA505	SA708
1	SAB	Cow 1	Even	Control	12	SA505	SA709
1	SAB	Cow 2	Uneven	Control	12	SA505	SA710
1	SAB	Cow 3	Uneven	Yeast	12	SA505	SA711
1	SAB	Cow 4	Even	Yeast	12	SA505	SA712
2	SAB	Cow 1	Uneven	Control	0	SA506	SA701
2	SAB	Cow 2	Even	Yeast	0	SA506	SA702
2	SAB	Cow 3	Even	Control	0	SA506	SA703
2	SAB	Cow 4	Uneven	Yeast	0	SA506	SA704
2	SAB	Cow 1	Uneven	Control	3	SA506	SA705
2	SAB	Cow 2	Even	Yeast	3	SA506	SA706
2	SAB	Cow 3	Even	Control	3	SA506	SA707
2	SAB	Cow 4	Uneven	Yeast	3	SA506	SA708
2	SAB	Cow 1	Uneven	Control	12	SA506	SA709
2	SAB	Cow 2	Even	Yeast	12	SA506	SA710
2	SAB	Cow 3	Even	Control	12	SA506	SA711
2	SAB	Cow 4	Uneven	Yeast	12	SA506	SA712
3	SAB	Cow 1	Uneven	Yeast	0	SA507	SA701
3	SAB	Cow 2	Even	Control	0	SA507	SA702

3	SAB	Cow 3	Even	Yeast	0	SA507	SA703
3	SAB	Cow 4	Uneven	Control	0	SA507	SA704
3	SAB	Cow 1	Uneven	Yeast	3	SA507	SA705
3	SAB	Cow 2	Even	Control	3	SA507	SA706
3	SAB	Cow 3	Even	Yeast	3	SA507	SA707
3	SAB	Cow 4	Uneven	Control	3	SA507	SA708
3	SAB	Cow 1	Uneven	Yeast	12	SA507	SA709
3	SAB	Cow 2	Even	Control	12	SA507	SA710
3	SAB	Cow 3	Even	Yeast	12	SA507	SA711
3	SAB	Cow 4	Uneven	Control	12	SA507	SA712
4	SAB	Cow 1	Even	Yeast	0	SA508	SA701
4	SAB	Cow 2	Uneven	Yeast	0	SA508	SA702
4	SAB	Cow 3	Uneven	Control	0	SA508	SA703
4	SAB	Cow 4	Even	Control	0	SA508	SA704
4	SAB	Cow 1	Even	Yeast	3	SA508	SA705
4	SAB	Cow 2	Uneven	Yeast	3	SA508	SA706
4	SAB	Cow 3	Uneven	Control	3	SA508	SA707
4	SAB	Cow 4	Even	Control	3	SA508	SA708
4	SAB	Cow 1	Even	Yeast	12	SA508	SA709
4	SAB	Cow 2	Uneven	Yeast	12	SA508	SA710
4	SAB	Cow 3	Uneven	Control	12	SA508	SA711
4	SAB	Cow 4	Even	Control	12	SA508	SA712

Table 8.2: Dual primer sequences for feed restriction study

Period	Phase	Cow	Diet	Yeast	Time	i5 Forward Primer	i7 Reverse Primer
1	LAB	Cow 1	Even	Control	0	SB501	SA701
1	LAB	Cow 2	Uneven	Control	0	SB501	SA702
1	LAB	Cow 3	Uneven	Yeast	0	SB501	SA703
1	LAB	Cow 4	Even	Yeast	0	SB501	SA704
1	LAB	Cow 1	Even	Control	3	SB501	SA705
1	LAB	Cow 2	Uneven	Control	3	SB501	SA706
1	LAB	Cow 3	Uneven	Yeast	3	SB501	SA707
1	LAB	Cow 4	Even	Yeast	3	SB501	SA708
1	LAB	Cow 1	Even	Control	12	SB501	SA709
1	LAB	Cow 2	Uneven	Control	12	SB501	SA710
1	LAB	Cow 3	Uneven	Yeast	12	SB501	SA711
1	LAB	Cow 4	Even	Yeast	12	SB501	SA712
2	LAB	Cow 1	Uneven	Control	0	SB502	SA701
2	LAB	Cow 2	Even	Yeast	0	SB502	SA702
2	LAB	Cow 3	Even	Control	0	SB502	SA703
2	LAB	Cow 4	Uneven	Yeast	0	SB502	SA704
2	LAB	Cow 1	Uneven	Control	3	SB502	SA705
2	LAB	Cow 2	Even	Yeast	3	SB502	SA706
2	LAB	Cow 3	Even	Control	3	SB502	SA707
2	LAB	Cow 4	Uneven	Yeast	3	SB502	SA708

2	LAB	Cow 1	Uneven	Control	12	SB502	SA709
2	LAB	Cow 2	Even	Yeast	12	SB502	SA710
2	LAB	Cow 3	Even	Control	12	SB502	SA711
2	LAB	Cow 4	Uneven	Yeast	12	SB502	SA712
3	LAB	Cow 1	Uneven	Yeast	0	SB503	SA701
3	LAB	Cow 2	Even	Control	0	SB503	SA702
3	LAB	Cow 3	Even	Yeast	0	SB503	SA703
3	LAB	Cow 4	Uneven	Control	0	SB503	SA704
3	LAB	Cow 1	Uneven	Yeast	3	SB503	SA705
3	LAB	Cow 2	Even	Control	3	SB503	SA706
3	LAB	Cow 3	Even	Yeast	3	SB503	SA707
3	LAB	Cow 4	Uneven	Control	3	SB503	SA708
3	LAB	Cow 1	Uneven	Yeast	12	SB503	SA709
3	LAB	Cow 2	Even	Control	12	SB503	SA710
3	LAB	Cow 3	Even	Yeast	12	SB503	SA711
3	LAB	Cow 4	Uneven	Control	12	SB503	SA712
4	LAB	Cow 1	Even	Yeast	0	SB504	SA701
4	LAB	Cow 2	Uneven	Yeast	0	SB504	SA702
4	LAB	Cow 3	Uneven	Control	0	SB504	SA703
4	LAB	Cow 4	Even	Control	0	SB504	SA704
4	LAB	Cow 1	Even	Yeast	3	SB504	SA705
4	LAB	Cow 2	Uneven	Yeast	3	SB504	SA706
4	LAB	Cow 3	Uneven	Control	3	SB504	SA707
4	LAB	Cow 4	Even	Control	3	SB504	SA708
4	LAB	Cow 1	Even	Yeast	12	SB504	SA709
4	LAB	Cow 2	Uneven	Yeast	12	SB504	SA710
4	LAB	Cow 3	Uneven	Control	12	SB504	SA711
4	LAB	Cow 4	Even	Control	12	SB504	SA712
1	SAB	Cow 1	Even	Control	0	SA505	SA701
1	SAB	Cow 2	Uneven	Control	0	SA505	SA702
1	SAB	Cow 3	Uneven	Yeast	0	SA505	SA703
1	SAB	Cow 4	Even	Yeast	0	SA505	SA704
1	SAB	Cow 1	Even	Control	3	SA505	SA705
1	SAB	Cow 2	Uneven	Control	3	SA505	SA706
1	SAB	Cow 3	Uneven	Yeast	3	SA505	SA707
1	SAB	Cow 4	Even	Yeast	3	SA505	SA708
1	SAB	Cow 1	Even	Control	12	SA505	SA709
1	SAB	Cow 2	Uneven	Control	12	SA505	SA710
1	SAB	Cow 3	Uneven	Yeast	12	SA505	SA711
1	SAB	Cow 4	Even	Yeast	12	SA505	SA712
2	SAB	Cow 1	Uneven	Control	0	SA506	SA701
2	SAB	Cow 2	Even	Yeast	0	SA506	SA702
2	SAB	Cow 3	Even	Control	0	SA506	SA703
2	SAB	Cow 4	Uneven	Yeast	0	SA506	SA704
2	SAB	Cow 1	Uneven	Control	3	SA506	SA705
2	SAB	Cow 2	Even	Yeast	3	SA506	SA706

2	SAB	Cow 3	Even	Control	3	SA506	SA707
2	SAB	Cow 4	Uneven	Yeast	3	SA506	SA708
2	SAB	Cow 1	Uneven	Control	12	SA506	SA709
2	SAB	Cow 2	Even	Yeast	12	SA506	SA710
2	SAB	Cow 3	Even	Control	12	SA506	SA711
2	SAB	Cow 4	Uneven	Yeast	12	SA506	SA712
3	SAB	Cow 1	Uneven	Yeast	0	SA507	SA701
3	SAB	Cow 2	Even	Control	0	SA507	SA702
3	SAB	Cow 3	Even	Yeast	0	SA507	SA703
3	SAB	Cow 4	Uneven	Control	0	SA507	SA704
3	SAB	Cow 1	Uneven	Yeast	3	SA507	SA705
3	SAB	Cow 2	Even	Control	3	SA507	SA706
3	SAB	Cow 3	Even	Yeast	3	SA507	SA707
3	SAB	Cow 4	Uneven	Control	3	SA507	SA708
3	SAB	Cow 1	Uneven	Yeast	12	SA507	SA709
3	SAB	Cow 2	Even	Control	12	SA507	SA710
3	SAB	Cow 3	Even	Yeast	12	SA507	SA711
3	SAB	Cow 4	Uneven	Control	12	SA507	SA712
4	SAB	Cow 1	Even	Yeast	0	SA508	SA701
4	SAB	Cow 2	Uneven	Yeast	0	SA508	SA702
4	SAB	Cow 3	Uneven	Control	0	SA508	SA703
4	SAB	Cow 4	Even	Control	0	SA508	SA704
4	SAB	Cow 1	Even	Yeast	3	SA508	SA705
4	SAB	Cow 2	Uneven	Yeast	3	SA508	SA706
4	SAB	Cow 3	Uneven	Control	3	SA508	SA707
4	SAB	Cow 4	Even	Control	3	SA508	SA708
4	SAB	Cow 1	Even	Yeast	12	SA508	SA709
4	SAB	Cow 2	Uneven	Yeast	12	SA508	SA710
4	SAB	Cow 3	Uneven	Control	12	SA508	SA711
4	SAB	Cow 4	Even	Control	12	SA508	SA712

Table 8.3: Dual primer sequences for De-Odorase® and Yea-Sacc® study

Phase	Period	Cow	Diet	Time	i5 Forward Barcode	i7 Reverse Barcode
LAB	1	Cow 1	Control	6	SA501	SA701
LAB	1	Cow 3	Control	6	SA501	SA702
LAB	1	Cow 6	D	6	SA501	SA703
LAB	1	Cow 5	D	6	SA501	SA704
LAB	1	Cow 2	DY	6	SA501	SA705
LAB	1	Cow 4	DY	6	SA501	SA706
LAB	1	Cow 1	Control	9	SA501	SA707
LAB	1	Cow 3	Control	9	SA501	SA708
LAB	1	Cow 6	D	9	SA501	SA709
LAB	1	Cow 5	D	9	SA501	SA710

LAB	1	Cow 2	DY	9	SA501	SA711
LAB	1	Cow 4	DY	9	SA501	SA712
LAB	1	Cow 1	Control	12	SA502	SA701
LAB	1	Cow 3	Control	12	SA502	SA702
LAB	1	Cow 6	D	12	SA502	SA703
LAB	1	Cow 5	D	12	SA502	SA704
LAB	1	Cow 2	DY	12	SA502	SA705
LAB	1	Cow 4	DY	12	SA502	SA706
LAB	2	Cow 2	Control	6	SA502	SA707
LAB	2	Cow 5	Control	6	SA502	SA708
LAB	2	Cow 1	D	6	SA502	SA709
LAB	2	Cow 4	D	6	SA502	SA710
LAB	2	Cow 6	DY	6	SA502	SA711
LAB	2	Cow 3	DY	6	SA502	SA712
LAB	2	Cow 2	Control	9	SA503	SA701
LAB	2	Cow 5	Control	9	SA503	SA702
LAB	2	Cow 1	D	9	SA503	SA703
LAB	2	Cow 4	D	9	SA503	SA704
LAB	2	Cow 6	DY	9	SA503	SA705
LAB	2	Cow 3	DY	9	SA503	SA706
LAB	2	Cow 2	Control	12	SA503	SA707
LAB	2	Cow 5	Control	12	SA503	SA708
LAB	2	Cow 1	D	12	SA503	SA709
LAB	2	Cow 4	D	12	SA503	SA710
LAB	2	Cow 6	DY	12	SA503	SA711
LAB	2	Cow 3	DY	12	SA503	SA712
LAB	3	Cow 6	Control	6	SA504	SA701
LAB	3	Cow 4	Control	6	SA504	SA702
LAB	3	Cow 2	D	6	SA504	SA703
LAB	3	Cow 3	D	6	SA504	SA704
LAB	3	Cow 1	DY	6	SA504	SA705
LAB	3	Cow 5	DY	6	SA504	SA706
LAB	3	Cow 6	Control	9	SA504	SA707
LAB	3	Cow 4	Control	9	SA504	SA708
LAB	3	Cow 2	D	9	SA504	SA709
LAB	3	Cow 3	D	9	SA504	SA710
LAB	3	Cow 1	DY	9	SA504	SA711
LAB	3	Cow 5	DY	9	SA504	SA712
LAB	3	Cow 6	Control	12	SA505	SA701
LAB	3	Cow 4	Control	12	SA505	SA702
LAB	3	Cow 2	D	12	SA505	SA703
LAB	3	Cow 3	D	12	SA505	SA704
LAB	3	Cow 1	DY	12	SA505	SA705
LAB	3	Cow 5	DY	12	SA505	SA706
SAB	1	Cow 1	Control	6	SA505	SA707
SAB	1	Cow 3	Control	6	SA505	SA708

SAB	1	Cow 6	D	6	SA505	SA709
SAB	1	Cow 5	D	6	SA505	SA710
SAB	1	Cow 2	DY	6	SA505	SA711
SAB	1	Cow 4	DY	6	SA505	SA712
SAB	1	Cow 1	Control	9	SA506	SA701
SAB	1	Cow 3	Control	9	SA506	SA702
SAB	1	Cow 6	D	9	SA506	SA703
SAB	1	Cow 5	D	9	SA506	SA704
SAB	1	Cow 2	DY	9	SA506	SA705
SAB	1	Cow 4	DY	9	SA506	SA706
SAB	1	Cow 1	Control	12	SA506	SA707
SAB	1	Cow 3	Control	12	SA506	SA708
SAB	1	Cow 6	D	12	SA506	SA709
SAB	1	Cow 5	D	12	SA506	SA710
SAB	1	Cow 2	DY	12	SA506	SA711
SAB	1	Cow 4	DY	12	SA506	SA712
SAB	2	Cow 2	Control	6	SA507	SA701
SAB	2	Cow 5	Control	6	SA507	SA702
SAB	2	Cow 1	D	6	SA507	SA703
SAB	2	Cow 4	D	6	SA507	SA704
SAB	2	Cow 6	DY	6	SA507	SA705
SAB	2	Cow 3	DY	6	SA507	SA706
SAB	2	Cow 2	Control	9	SA507	SA707
SAB	2	Cow 5	Control	9	SA507	SA708
SAB	2	Cow 1	D	9	SA507	SA709
SAB	2	Cow 4	D	9	SA507	SA710
SAB	2	Cow 6	DY	9	SA507	SA711
SAB	2	Cow 3	DY	9	SA507	SA712
SAB	2	Cow 2	Control	12	SA508	SA701
SAB	2	Cow 5	Control	12	SA508	SA702
SAB	2	Cow 1	D	12	SA508	SA703
SAB	2	Cow 4	D	12	SA508	SA704
SAB	2	Cow 6	DY	12	SA508	SA705
SAB	2	Cow 3	DY	12	SA508	SA706
SAB	3	Cow 6	Control	6	SA508	SA707
SAB	3	Cow 4	Control	6	SA508	SA708
SAB	3	Cow 2	D	6	SA508	SA709
SAB	3	Cow 3	D	6	SA508	SA710
SAB	3	Cow 1	DY	6	SA508	SA711
SAB	3	Cow 5	DY	6	SA508	SA712
SAB	3	Cow 6	Control	9	SB501	SA701
SAB	3	Cow 4	Control	9	SB501	SA702
SAB	3	Cow 2	D	9	SB501	SA703
SAB	3	Cow 3	D	9	SB501	SA704
SAB	3	Cow 1	DY	9	SB501	SA705
SAB	3	Cow 5	DY	9	SB501	SA706

SAB	3	Cow 6	Control	12	SB501	SA707
SAB	3	Cow 4	Control	12	SB501	SA708
SAB	3	Cow 2	D	12	SB501	SA709
SAB	3	Cow 3	D	12	SB501	SA710
SAB	3	Cow 1	DY	12	SB501	SA711
SAB	3	Cow 5	DY	12	SB501	SA712