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Title	Effect of cow feeding system on the quality and constituents of raw milk, dairy products and the rumen
Authors	O'Callaghan, Tom F.
Publication date	2018
Original Citation	O'Callaghan, T. F. 2018. Effect of cow feeding system on the quality and constituents of raw milk, dairy products and the rumen. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
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Download date	2026-11-17 12:00:44
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Effect of Cow Feeding System on the Quality and Constituents of Raw Milk, Dairy Products and the Rumen

Thesis submitted to the National University of Ireland for Degree of Doctor of Philosophy by

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Submitted: December 2017
Viva voce: February 9th, 2018

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism. All the work described herein is my own, with the following exceptions:

Chapter 1 – Mr Ivan Sugrue contributed to section 1.1.11

Chapter 3 – Miss Hope Faulkner contributed to volatile analysis of butter samples.

Chapter 4 – Cheese manufacturing was co-ordinated by Dr Diarmuid Sheehan. Mr David Mannion contributed to volatile analysis of Cheddar cheeses.

Chapter 6 – Dr. Rosa Vazquez Fresno contributed to the profiling of NMR spectra. Dr Arnau Serra-Cayuela contributed to statistical analysis of data.

_____ _____

Tom F. O'Callaghan Date

Abstract

Effect of Cow Feeding System on the Quality and Constituents of Raw Milk, Dairy Products and the Rumen

The overall aim of this thesis was to investigate the effects of pasture versus conventional indoor cow feeding systems on the composition, quality and characteristics of milk, dairy products and rumen microbiota. Initially the effects of perennial ryegrass (GRS), perennial ryegrass and white clover pasture (CLV) and total mixed ration (TMR) feeding of cows on the composition and nutritional quality of raw milk throughout an entire lactation was examined (Chapter 2). Cow diet was shown to have a significant effect ($P < 0.05$) on both the macro-composition and fatty acid content of milk throughout lactation. Milk from pasture fed cows had significantly higher ($P < 0.05$) concentrations of fat, protein, true protein and casein. Pasture derived milk was shown to have significantly higher ($P < 0.05$) content of conjugated linoleic acid and omega 3 fatty acids while TMR derived milk had significantly higher ($P < 0.05$) palmitic acid and omega 6 fatty acids. The aim of Chapter 3 was to examine the effects of different feeding systems on the composition, quality and sensory properties of mid lactation sweet cream butter. The nutritional composition of butters was improved by pasture feeding. Alterations in the fatty acid composition of butter between feeding systems contributed to significant differences in textural and thermal properties of the butters. Volatile analysis of butter by GC-MS identified 25 compounds present in each of the butters, five of which differed significantly ($P < 0.05$) based on feeding system. Chapter 4 examined the effects of diet on the composition, quality and sensory properties of Cheddar cheese throughout nine months of ripening. This study demonstrated the benefits of pasture derived feeding systems for production of Cheddar cheeses with enhanced nutritional and rheological quality compared with TMR feeding system. Pasture derived feeding systems were shown to produce Cheddar cheeses more yellow in colour than that of TMR, which was positively correlated with cheese β-carotene content. Feeding system had a significant effect on the fatty acid composition of the cheeses. Differences in the cheese fatty acid content were correlated with alterations to the Cheddar cheese rheological properties. Feeding system and ripening time had a significant effect on the volatile profile of the Cheddar cheeses. Principal component analysis of average

fatty acid profiles in milk, butter and Cheddar cheese showed clear separation of the products from the grazed pasture-based diets to that of a TMR system throughout lactation, offering insight into the ability to verify pasture derived milk and products by fatty acid profiling. In Chapter 5, 16s RNA MiSeq sequencing was applied to both solid and liquid fractions of cow rumen to examine the effect of cows feeding system on the rumen microbiota. There was a clear separation between the liquid and solid fractions. No major differences in the rumen microbiota composition between cows exposed to different diets was found, which is likely as a result of a shortened adaptation period. It is also clear that the majority of the rumen microbiota is still undiscovered. As such, further work is required to fully understand the effects of these diets on the rumen microbiota and its functionality. Untargeted ^{1}H-NMR was used to examine the effects of cows feeding system on the rumen and milk metabolome (Chapter 6). Our results show that feeding system impacted significantly on both the rumen and milk metabolome. This study has highlighted that ^{1}H-NMR metabolomics coupled with multivariate analysis is capable of distinguishing both rumen-fluids and milk samples derived from cows on different feeding systems. Finally, Chapter 7 discusses the major findings and general conclusions arising from the studies presented in this thesis.

Publications

Literature Reviews

- **O'Callaghan T**, Ross R, Stanton C, Clarke G: **The gut microbiome as a virtual endocrine organ with implications for farm and domestic animal endocrinology.** *Domestic Animal Endocrinology* 2016, **56:**S44-S55. (Chapter 1 [Part 2])

Experimental Papers

- **O'Callaghan TF**, Hennessy D, McAuliffe S, Kilcawley KN, O'Donovan M, Dillon P, Ross RP, Stanton C: **Effect of pasture versus indoor feeding systems on raw milk composition and quality over an entire lactation.** *Journal of Dairy Science* 2016, **99:**9424-9440. (Chapter 2)

- **O'Callaghan TF**, Faulkner H, McAuliffe S, O'Sullivan MG, Hennessy D, Dillon P, Kilcawley KN, Stanton C, Ross RP: **Quality characteristics, chemical composition, and sensory properties of butter from cows on pasture versus indoor feeding systems.** *Journal of Dairy Science* 2016, **99:**9441-9460. (Chapter 3)

- **O'Callaghan, T. F.,** Mannion, D. T., Hennessy, D., Mcauliffe, S., O'sullivan, M. G., Leeuwendaal, N., Beresford, T. P., Dillon, P., Kilcawley, K. N., Sheehan, J. J., Ross, R. P. & Stanton, C. 2017**. Effect Of Pasture Versus Indoor Feeding Systems On Quality Characteristics, Nutritional Composition, And Sensory And Volatile Properties Of Full-Fat Cheddar Cheese**. Journal Of Dairy Science, 100, 6053-6073. (Chapter 4)

Appendix

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- Faulkner, H.; **O'Callaghan, T.F.;** McAuliffe, S.; Hennessy, D.; Stanton, C.; O'Sullivan, M.G.; Kerry, J.P.; Kilcawley, K.N. **Effect of different forage types on the volatile and sensory properties of bovine milk**. *Journal of Dairy Science.* Article in Press.

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Abbreviations

RDI = Recommended daily intake

LDL = Low-density lipoprotein

HDL = High-density lipoprotein

CLA = Conjugated linoleic acid

n3 = Omega 3 fatty acids

n6 = Omega 6 fatty acids

MFGM = Milk fat globule membrane

Ig = Immunoglobulin

ACE = Angiostensin-I-converting enzymes

α = Alpha

β = Beta

miRNA = MicroRNA

TMR = Total mixed ration

GRS = Cows maintained outdoors on perennial ryegrass only pasture

CLV = Cows maintained outdoors on perennial ryegrass/white clover pasture

NMR = Nuclear magnetic resonance spectroscopy

GIT = Gastrointestinal tract

ENS = Enteric nervous system

CNS = Central nervous system

GABA = γ-amino butyric acid

HPA = Hypothalmic pituitary adrenal axis

GALT = Gastrointestinal associated lymphoid

SPF = Specific pathogen free

ConA = ConcanavalinA

BDNF = Brain derived neurotrophic factor

ETEC = Enterotoxigenic *E.coli*

DMI = Dry matter intake

FA = Fatty acid

AWB = Animal welfare body

HPRA = The health products regulatory authority

TN = Total nitrogen

CrP = Crude protein

NPN = Non protein nitrogen

NCN = Non casein nitrogen

Tp = True protein

Cp = Casein protein

Wp = Whey protein

UFL = unité fourragère lait

PDIA = sum of the feed protein ruminally undegraded and truly digested in the small intestine

PDIE = sum of PDIA and the microbial true protein that is truly digested in the small intestine

PDIN = sum of PDIA and PDIM when nitrogen is limiting

N = Nitrogen

FAME = Fatty acid methyl esters

EPA = Eicosapentaenoic acid

LA = Linoleic acid

ALA = α-linolenic acid

DHA = Docohexanoic acid

AI = Atherogenicity index

TI = Thrombogenecity index

SD = Standard deviation

NCGD = Neutral cellulose plus gammanase digestibility

SFA = Saturated fatty acid

MUFA = Monounsatturated fatty acids

PUFA = Polyunsatturated fatty acids

DM = Dry matter

FFA = Free fatty acids

GC-MS = Gas chromatography mass spectrometry

GC-FID = Gas chromatography flame ionisation detection

UV/DAD = Ultraviolet diode array detection

DSC = Differential scanning calorimetry

SPME = Solid phase micro extraction

RDA = Ranking descriptive analysis

PLSR = Partial least square regression analysis

M = Month

d = Day

h = Hour

TFAA = Total free amino acids

FAA = Free amino acids

FDR = False discovery rate

HSD = Honestly significant difference

HCA = Hierarcheal clustering analysis

VIP = Variable importance plot

GLM = General linear model

VFA = Volatile fatty acids

CHD = Coronary heart disease

CVD = Cardiovascular disease

Acknowledgements

*"**Success is peace of mind, attained only through self-satisfaction in knowing, you made the effort to do your best to become the best at which you are capable**"*

- John Wooden

This PhD was a fantastic opportunity for me to pursue my passion for dairy research, has provided me with many great opportunities to travel the world, and to meet and work with, many great people.

I wouldn't have reached this point, or for that matter, even pursued a career in science without the encouragement of my supervisors Prof. Catherine Stanton and Prof. Paul Ross. Following your advice, I pursued a degree in Food Science and have not looked back since. Thank you for your constant support, encouragement, advice and supervision throughout my Ph.D and career. I am very proud of what we have achieved together in this work.

I am very grateful to Dr David Wishart and everyone in Wishart Group and The Metabolomics Innovation Centre at the University of Alberta, who took me on board, were always offering to help and work with me during my time there, especially Rosa, Arnau and Ben; I am very grateful for all your help.

Back home, I was extremely lucky to be a part of a fantastic group in Teagasc, to my lab colleagues, I very much enjoyed my time and experiences with you all, to especially mention Paul, Elaine, Kiera, Jen, David, Claire, Rob, Calum, Sian, Ruairi, Stephen, Deirdre, Beatriz, Dani and Fergus, I will very much miss the craic we had and I am very grateful for all your help and support throughout this endeavour. It is essential to have people like this around you to successfully accomplish and deal with the challenges, and ups and downs of a PhD.

I very much enjoyed working with Sean Hogan, Kieran Kilcawley, John Tobin, Eoin Murphy, Mark Fenelon and Noel McCarthy in the Food Chemistry Department, and Kieran Downey in MTL, I have learned a lot from you all. Special thanks also to Pat Dillon, whom I very much enjoyed working with, and the advice and time given to me throughout this process was very valuable. Dr Seamus O'Mahony and Prof Fergus Shanahan of UCC have always been a major influence on me and I appreciate all the time and advice you have given me. I had

terrific support from my friends outside of Moorepark, too many to mention everyone here but particularly, Patrick, Davi, Tomás, Jack, Shane, Gavin, Paudie, Kevin, Conor, Paul, Nikki, Mary Claire, Mark and Brendan. I am very lucky to have such great friends. Especially to mention Fiona, who has been with me through this whole endeavour, always been very patient, a constant support, full of encouragement and kept me grounded and on track throughout this time.

I can't thank my brother and sister, Paddy and Kate, enough for always being there for me and for their constant support and encouragement; I have and always will appreciate it. Mr Jim Loomis, has been a great influence on me and a great friend, whose support I am very grateful to have. I have always received tremendous encouragement from my aunts, uncles and cousins. In particular to my uncles Denis O'Brien and Liam Molan; for the summers on the farm, providing me my first introduction to the world of agriculture and dairy science, and a foundation on which to build a career, I am always grateful for your patience, humour, and constant support.

This thesis is dedicated to my parents, Pat and Mary. For your never-ending care, love and support in all my endeavours, academic or otherwise; I am extremely grateful. Throughout my life, through your own example, you have instilled in me a passion for hard work, respect, and decency which I hope I have displayed to my peers and others throughout my Ph.D.

This Ph.D would not have been possible without the financial support received from Teagasc and the APC Microbiome Institute through Science Foundation Ireland.

Chapter 1
(Part 1)

Nutritional Aspects of Raw Milk: A Beneficial or Hazardous Food Choice

Submitted Book Chapter 9 in *Benefits and Hazards of Raw Milk* (Elsevier)

In-review

1.1 Abstract

Liquid milk is a highly nutritious beverage which has seen a decline in consumption in recent years. Milk is an excellent source of dietary fats, proteins, and minerals such as calcium and magnesium, particularly for growing children. Physiological and environmental factors can affect the composition and quality of milk and cows feeding system has been identified as an important factor which can alter milks nutritional status. Pasture based feeding systems have been shown to produce milks with significantly higher conjugated linoleic acid and omega 3 fatty acid contents over more conventional indoor total mixed ration feeding systems. There is an on-going debate on the potential benefits associated with the consumption of raw milk over its processed counterpart. However, the consumption of raw milk poses a very real and serious health risk, through potential ingestion of pathogenic bacteria. The argument against treated milk has focused on the depletion of milks nutritional quality as a result of heat during processing, but these claims have been found to have no scientific basis. Nevertheless, it is widely agreed upon by experts that the risk of exposure to pathogenic bacteria in raw milk far outweighs any such potential benefits.

1.2 Introduction

Milk is a unique biological fluid which has evolved to provide optimal nourishment to young mammals from the early stages of life. It supports nutritional, immunological and developmental aspects of the newborn and is an excellent source of dietary fat and protein. Bovine milk is primarily composed of water (~87%), macronutrients; including protein (~3.2%), fat (~3.5%) and lactose (~4.8%), and micronutrients consisting of salts and minerals. The composition of bovine milk is dictated by several factors which include breed, age, diet, health status and stage of lactation. In Western societies, the consumption of liquid milk has declined in recent years, partly as a result of claimed negative health effects associated with consumption of saturated fatty acids [1]. In Ireland, as an example, liquid milk sales for human consumption have fallen from 530.1 million litres in the year 2000 to 507.8 million litres in 2015 [2]. Indeed, milk has a high content of saturated fatty acids, whose consumption has been linked to weight gain, heart disease, high cholesterol and obesity [3]. But recent critical reviews and meta-analyses of the topic have concluded that there is at worst a neutral effect of liquid milk intake on multiple health outcomes. In fact, human ingestion of bovine milk may actually be beneficial in combating osteoporosis, cardiovascular disease, stroke, type-2-diabetes and some cancers [4, 5].

Raw unpasteurised milk was ingested by man for thousands of years prior to the introduction of pasteurisation in the last century. The debate between the benefits and drawbacks associated with raw milk consumption over pasteurised or heated milk has been on-going for several decades, and claims for raw milk include improved nutrition, reduced incidence of lactose intolerance and provision of good bacteria. However, these claims have no scientific basis [6]. There is some evidence that heat treatment of raw milk does significantly alter its immunomodulatory effect, albeit based on *in-vitro* studies [7]. Moreover, extensive epidemiological evidence suggests certain health benefits may be associated with raw milk consumption. "GABRIELA" is a European wide epidemiological study which reported an

inverse relationship between the consumption of raw milk and incidence of asthma [8], while another European wide study by Waser*, et al.* [9] also reported that the consumption of farm/raw milk may offer protection against asthma and allergy development. There is, however, a very real health risk associated with consumption of raw milk if contaminated with human pathogens resulting in serious illness. Pathogenic bacteria associated with raw milk include *Escherichia coli* O157, *Salmonella, Campylobacter, Staphylococcus aureus*, *Mycobacterium avium* subsp. *paratuberculosis* and *Listeria monocytogenes* [10].

Pasteurisation, first implemented by Louis Pasteur in the 1860s for use in wine, is centred on the realisation that heating of liquids improves their keeping quality during storage. Pasteurisation is now defined as the process of heating every particle of milk or milk product in properly designed and operated equipment to any one of the specified pasteurisation time-temperature combinations [11], most commonly 72 °C for 15 s, and is effective in destroying human pathogens or reducing their presence to a safe level. Pasteurisation was made a legal requirement of dairy processors and creameries for the sale of milk in Ireland in 1958 by the Irish Department of Agriculture [12] and in 1987 in an effort to reduce dairy related food borne illness, the FDA prohibited the interstate sales of unpasteurised milk and dairy products [13]. This practice has no doubt contributed to the decline in incidence of milk borne illness in the United States, which has fallen from 25% of all food and contaminated water outbreaks in 1938 to less than one per cent in 2011 [11].

In recent times, there has been an increased demand among consumers for more natural and organic foodstuffs [14]. Despite the commonly accepted food safety concerns, the consumption of raw milk has become more popular, stimulated at least in part by discussions and debates often held on the internet where information of questionable scientific value is frequently circulated [15]. With this in mind, focusing on bovine liquid milk, this chapter

presents an overview of the nutritional aspects of milk, the factors that affect its composition, and the benefits and hazards of raw milk consumption.

1.3 The Nutritional Argument for Milk

The nutritional quality of any food is derived both from its composition, and potentially more importantly, the bioavailability and contribution of these nutrients to their recommended daily intakes (RDI) for consumers [15]. Milk, in both raw or processed form, is a highly nutritious food product. Several arguments have been put forward in the past by advocates for raw milk consumption, a major one being that raw milk is better from a nutritional perspective than its heat processed counterpart as a result of the denaturation of beneficial heat labile components during heat processing. The consumption of milk (either raw or processed) has also been criticized in the past, as mentioned before, because of its high content of saturated and trans fatty acids and their links to dietary related diseases. As mounting research has shown, generalisations about milk and milk fatty acid content can be misleading, given the beneficial attributes of saturated and unsaturated fatty acids collectively present in milk fat [6, 16]. In fact, reviews of epidemiological studies have shown that there is no consistent relationship between high intake of dairy products and cardiovascular disease [17]. Lamarche, *et al.* [5] recently reported that milk intake has a neutral effect on multiple health outcomes including coronary heart disease, stroke and type 2 diabetes based on meta-analysis of epidemiological data. Furthermore, Armas, *et al.* [4] also concluded that consumption of milk is associated with several health benefits; including reduced risk of osteoporosis, cardiovascular disease, type 2 diabetes, hypertension, some cancers, stroke and better weight management.

1.4 Milk Fat

Dietary guidelines in the past have recommended limited intakes of saturated fatty acids (<7-10% of daily energy) due to their ability to increase both total and LDL-cholesterol levels in blood, which are risk factors for coronary heart disease [18]. However, increasing evidence and

meta-analysis is now available which show an inverse association [19, 20] and no significant relationship or association of saturated fatty acids with increased risk of cardiovascular disease [21-23].

Saturated fatty acids account for more than half of the milk fatty acid content, several of which have both positive and negative health effects [1]. This suggests that the saturated fatty acid content of food alone is not necessarily a useful criterion on which to base food choices [23]. For example, butyric acid (C4:0) has been reported as a modulator of gene function [24] and may play a role in cancer prevention [25]. Caprylic acid (C8:0) and capric acid (C10:0) may have antiviral activities and caprylic acid has been reported to delay tumor growth. Lauric acid (C12:0) has been suggested to possess antiviral and antibacterial functions, and may act as an anti-caries and anti-plaque agent [1]. In addition, stearic acid (C18:0) does not seem to increase serum cholesterol levels relative to other long chain fatty acids, and has shown no deleterious effect on cardiovascular disease risk [26, 27].

Milk is also a source of monounsaturated and polyunsaturated, omega-3 and omega-6 fatty acids and conjugated linoleic acid (CLA), the latter being the generic name for a group of isomers of linoleic acid ($C_{18:2\ n6}$) with a conjugated double bond. CLA has been the topic of much research because of multiple health benefiting attributes and interesting biological functions which include an impact on immune function, protective affects against cancer, obesity, diabetes and atherosclerosis in animal studies and human cell lines; see review by Yang, *et al.* [28]. The most prevalent form of CLA in bovine milk and ruminant derived products is the c9t11 isomer, aptly named rumenic acid, produced as an intermediate during the biohydrogenation of dietary linoleic acid to stearic acid by the ruminal microorganism *Butrivibrio fibrisolvens* [29]. Rumenic acid is also produced by the action of the delta9-desaturase enzyme on vaccenic acid (C18:1, t-11) in the mammary gland [30]. The content of CLA in bovine milk can vary considerably, but it is widely acknowledged that the dietary regimen can

have a significant effect on CLA concentration (see later). Generally, polyunsaturated and monounsaturated fatty acids are regarded as beneficial to human health. Oleic acid ($C_{18:1\ n9}$) is the most prominent monounsaturated fatty acid present in milk while linoleic acid and α-linolenic acid ($C_{18:3\ n3}$) are the main polyunsaturated fatty acids in milk fat [31]. Oleic acid has been reported as favourable for health with reports that high concentrations of monounsaturated fatty acids in the diet will lower both plasma cholesterol, LDL-cholesterol and triacylglycerol concentrations [32]. Industrially produced trans fatty acids have been viewed in the past as having negative health effects, with links to increased risk of cardiovascular disease through a negative influence on the ratio of LDL to HDL cholesterol [33]. However, the question has been raised whether ruminally derived vaccenic acid shares these negative properties. Vaccenic acid present in rumen derived products is a known dietary precursor to CLA_{c9t11} and data suggests that consumption of this trans fatty acid may impart health benefits beyond those associated with CLA [34]. Vaccenic acid is the major trans C18:1 fatty acid present in milk and its concentration is heavily dependent on the cows feeding regimen, where it has been shown that fresh pasture feeding results in greater concentration of this trans fatty acid. Vaccenic acid is produced through the incomplete biohydrogenation of linoleic and linolenic acids by bacteria in the rumen [16]. Turpeinen*, et al.* [35] have also demonstrated that consumed vaccenic acid can be desaturated by the Δ^9-desaturase enzyme to CLA_{c9t11} in the liver of humans.

The polyunsaturated fatty acids can be further classified to two major families, the omega 3 (n3) and omega 6 (n6) fatty acids, based on the location of the final double bond relative to the terminal methyl end of the molecule [36]. Linoleic acid and α-linolenic acid are termed essential fatty acids as they cannot be synthesized by the human body and these fatty acids are also precursors to the *n*6 and *n*3 series of fatty acids [37]. Both the *n*6 and *n*3 fatty acid families possess cardioprotective properties [38]. Both are also precursors to eicosanoids,

which are potent lipid mediator signalling molecules which play important roles in inflammation, in general *n*3 derived eicosanoids possess anti-inflammatory properties while *n*6 derived eicosanoids possess pro-inflammatory properties [37]. The ideal ratio of *n*3 to *n*6 fatty acids in the diet has been reported to be 1: 1-4, but changes in the Western diet in recent decades with increased consumption of fat and vegetable oils rich in *n*6 fatty acids have resulted in a ratio between 1:10 and 1:20 [39]. Coinciding with this increase in *n*6 fatty acids consumption are increases in chronic inflammatory diseases such as non-alcoholic fatty liver disease, cardiovascular disease, obesity, inflammatory bowel disease, rheumatoid arthritis and Alzheimer's disease [37]. Many studies have concluded that reducing the ratio of *n*6:*n*3 fatty acids could lower risks of cardiovascular disease, metabolic syndrome, diabetes and obesity [40]. In milk, the ratio of *n*6 and *n*3 fatty acids is favourable compared to most other non-marine products, particularly organic and fresh pasture derived milks [1, 40], which could aid in the ultimate goal to reduce the current *n*6 to *n*3 fatty acids ratio in the human diet to more desirable levels.

1.5 Milk Fat Globule Membrane (MFGM)

The fat globules in milk are composed of a non-polar lipid core, surrounded by a stabilising membrane composed of phospholipids and proteins including cholesterol, phosphatidylcholine and sphingomelin, glycolipids, gangliosides, membrane glycoproteins and proteins, referred to as the milk fat globule membrane (MFGM) [41]. Significant quantities of MFGM are present in dairy products and are particularly concentrated in cream after the separation of milk for butter manufacturing. Buttermilk is a subsequent by-product of the butter making process and is a commercially available rich source of MFGM. Many potential benefits of MFGM have been demonstrated through both *in vitro* and *in vivo* studies, particularly involving the inhibition of pathogenic bacterial colonisation. Human MFGM derived mucins have demonstrated prevention of *E. coli* adhesion to buccal epithelial cells *in vitro*, while bovine

derived mucin inhibited neuraminidase-sensitive rotavirus infection in MA104 cells [42, 43]. Muc1 is a highly glycosylated mucin found in MFGM and bovine derived Muc1 has been demonstrated to inhibit the binding of Gram negative bacteria to human intestinal cells *in vitro* [44]. Other *in vitro* studies have shown the ability of bovine milk oligosaccharides to prevent cellular invasion of *Campylobacter jejuni* [45]. A recent study demonstrated the effects of defatted MFGM in inhibiting the association of *Escherichia coli* O157:H7 with human HT-29 cells [46]. Therefore, the addition of MFGM as a functional food may present an alternative approach to reduce particular infections in humans.

1.6 Milk Proteins and Bioactive Peptides

Bovine milk is regarded as an important source of protein in the human diet. Milk typically contains ~3.2% protein, which can be subdivided into two major families; the caseins (insoluble) which account for ~80% of total protein and whey proteins (soluble) which account for ~20% of total protein. Generally, milk protein composition is highly heritable and dependent on genetic factors, and therefore, can vary between breeds and individual cows, unlike other milk components where the dietary regimen can significantly alter their composition (i.e. milk fat). Several external factors can, however, affect the milk protein content, including stage of lactation, milk yield, age and health status of the cow, energy intake and lipid supplementation. Adequate protein intake in humans is fundamental to a healthy diet with daily recommended intakes of 52-56 g/day for adult men and ~46 g/day for adult women, while these requirements can increase during pregnancy and in the elderly [47]. The importance of milk proteins for health status of the elderly has received much attention in the past, particularly for preventing the loss of body and muscle mass, termed sarcopenia [48]. Milk proteins have a high biological value and milk is therefore a good source of essential amino acids in the diet. In this respect, processing methods for the fractionation of milk proteins has formed the basis for the production of several high value functional foods. Milk

proteins possess a wide array of biological activities which include anti-microbial effects, improved absorption of nutrients, growth factors, enzymes, anti-bodies and immune stimulants [1]. The casein protein family is composed of (in descending order) α_{s1}-casein, α_{s2}-casein, β-casein and ϰ-casein, while the major whey proteins include β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulin's (IgG1, IgG2, IgA, IgM) and lactoferrin [49]. Whey proteins in particular have an excellent biological value which is a measure of how well and quickly the body can utilise the consumed protein. Whey protein is a rich source of both essential amino acids and branched chain amino acids [50]. Branched chain amino acids (valine, leucine and isoleucine) are thought to play an important role in tissue growth and repair, and protein metabolism in the translation-initiation pathway [51]. As a result of this, whey proteins have received much attention for the nutrition of athletes [52].

Milk casein and whey proteins have also been shown to be precursors of many beneficial bioactive peptides. Bioactive peptides are short amino acid chains that are inactive/dormant within the sequence of the parent protein, however during the gastrointestinal digestion process or through *in vitro* hydrolysis processing using proteases, the peptide sequences are released. These peptides are capable of exerting several health benefits on the host including antihypertensive, antioxidant and anti-inflammatory activity [53]. Mills*, et al.* [54] have comprehensively reviewed milk derived bioactive peptides associated with human health through numerous physiological responses including antihypertensive peptides, antithrombotic peptides, opioid peptides, casein phosphopeptides, immunomodulatory peptides and antimicrobial peptides. Angiotensin-I-converting enzymes (ACE) is a key enzyme involved in the regulation of blood pressure. As an example, it has been shown that lactic acid bacteria derived proteinases can act on milk proteins particularly caseins, releasing antihypertensive peptides which inhibit ACE activity to produce angiotensin-II [55-57]. Opioid peptides are those which have pharmacological similarities to opium. Casein and whey

proteins have been highlighted as potential sources of opioid derived peptide sequences. However, hydrolysis of the β-casein in particular has been shown to produce potent opioid peptides termed β-casomorphins [54], while the hydrolysis of κ-casein has been demonstrated to produce opioid antagonist peptide sequences known as casoxins [58]. Opioid peptide sequences have also been identified within the primary sequences of the whey protein fractions β-lactoglobulin, lactoferrin and bovine serum albumin [54]. Several milk derived peptides known as immunomodulatory peptides have also been shown to display immunostimulatory activity, of particular interest is the peptide isracidin produced by the action of chymosin on αs1-casein, which has demonstrated a protective effect against mastitis when injected into the udder of sheep and cows and antibacterial activity against *Staphylococcus aureus* and *Candida albicans* [59]. Milk is also a rich source of potent antimicrobial proteins and peptides; an example being lactoferricin derived from the whey protein lactoferrin, which has displayed an anti-microbial effect against Gram positive and Gram negative bacteria, including *Listeria monocytogenes* [60]. Hayes*, et al.* [57] demonstrated that fermentation of casein by *Lactobacillus acidophilus* DPC6026 produced antimicrobial peptides active against pathogenic strains of *Escherichia coli* and *Cronobacter sakazakii.*

1.7 Lactose

Lactose is a disaccharide consisting of a galactose monomer bound to glucose. In aqueous solution, it is present in equal amounts in two isomeric forms, alfa (α) and beta (β). It is a key source of calories in the milk of all mammals other than those of suborder Pinnipedia (seals, sea lions and walruses), where it is present either in trace amounts or not at all [61]. Lactose consumption has often been discouraged in the past as a result of a majority of the global population (estimated between 70 and 75%) suffering from lactose intolerance, where the adult individual lacks the necessary enzymes to break down the carbohydrate during digestion (i.e. β-galactosidase) and several symptoms occur as a result including gas, cramps, bloating and

diarrhea [62]. However, as a result of intense research in the past 20 years in the area of gut microbiota and commensal microorganisms; a plethora of benefits have been associated with consumption of lactose and lactose derivatives, (i.e lactulose) highlighting a prebiotic effect on the gut microbiome have come to light. Lactose has also been recognised to act as a dietary fibre, which are food components that are not digested in the small intestine of humans and that according to their chemical characteristics can be described as carbohydrates, carbohydrate analogues, lignin and lignin-like compounds [63]. As a result, review of the subject has resulted in calls for lactose to be redefined as a conditional prebiotic [62]. A prebiotic as originally defined by Gibson and Roberfroid [64] is "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health". Lactose has relatively low sweetening power, calorific value and glycemic index and promotes calcium and magnesium absorption [63]. Lactulose, a derivative of lactose (formed through the heat processing of milk), has been widely attributed to have several prebiotic properties which include stimulating the growth and/or activity of probiotic bacteria including *Bifidobacterium* and *Lactobacillus* as well as anti-inflammatory effects in inflammatory bowel disease, Crohn's disease and ulcerative colitis and has been shown to have a laxative effect [65].

1.8 Effect of Heat Treatment on Milk Nutritional Status

A number of health benefits derived from consumption of raw milk have been suggested revolving around hypothetical assumptions of superior nutritional quality of milk not subjected to heat processing. However, evidence has shown that the heat processing of milk by pasteurisation temperatures for the removal of pathogenic bacteria has no significant effect on its nutritional status [6, 66]. While pasteurisation does result in minor denaturation of the milk whey proteins (<7%) this denaturation has no impact on the nutritional quality of milk proteins [6] and Lacroix*, et al.* [67], through the use of animal studies, reported no significant difference

in protein digestibility between raw milk proteins and those exposed to pasteurisation heat treatment. Lacroix, *et al.* [68] investigated the effects of heat treatment on protein quality in human subjects, by evaluating nitrogen metabolism following a meal. The same metabolic utilization of milk protein nitrogen was observed for both raw and pasteurized milk. The commercial heat treatment of milk does not affect milk lipids and has no significant effect on the content of minerals and trace elements, including the bioavailability of calcium as reviewed by Claeys, *et al.* [15]. Certain vitamin fractions of milk have been shown to be affected by heat treatment. MacDonald, *et al.* [69] conducted meta-analysis of 40 studies investigating the effects of pasteurisation on vitamin levels in milks. This study reported a decrease in concentrations of vitamins B1, B2, C, and folate. However, these decreases do not affect the overall nutritional value of the milk as they are naturally found at relatively low levels and milk is not considered an important dietary source of these vitamins [69].

1.9 Improving Nutritional Composition through Dietary Regimen

As mentioned, there are several factors that can affect the composition and nutritional status of bovine milk. Dietary regimen is the single most important factor that can be targeted in an effort to manipulate the nutritional status of cow's milk for human consumption, a topic which has been reviewed by Dewhurst, *et al.* [31]. Several factors can influence feeding systems which include land availability, climate and dairy cow requirements. It is widely understood that the feeding system of dairy cows can have a direct impact on the composition of milk, particularly the fatty acid composition of milk fat [70, 71]. Indeed, the level of saturated and unsaturated fatty acids in milk fat is closely dependent on the nature of the diet [72]. Fresh grass feeding systems produce a milk fat with higher proportions of unsaturated fatty acids compared to those derived from conventional indoor housed total mixed ration (TMR) systems [73]. The feeding system can also have an effect on the natural colour of products, where silage and TMR based diets have been shown to produce dairy products that are much whiter in colour

than those of pasture feeding systems, which have a characteristic yellow colour that has been attributed to increased concentrations of β-carotene in milks from pasture diets [74, 75]. Fresh grass feeding of dairy cows has been significantly correlated with increased concentrations of milk CLA_{c9t11}. Indeed Couvreur*, et al.* [73] demonstrated increases in CLA and vaccenic acid of cow's milk with increasing concentrations of fresh grass in the diet from 0 to 100% of total feed. The supplementation of TMR feeding systems with unsaturated fatty acids has also been shown to beneficially alter the fatty acid composition of milk [76]. O'Callaghan*, et al.* [71] also demonstrated that a perennial rye grass only and perennial rye grass with 20% white clover sward produced milks with significantly higher concentrations of fat, protein and true protein throughout lactation than those consuming a TMR diet of maize silage, grass silage and concentrates. O'Callaghan*, et al.* [77], also demonstrated the benefits of pasture feeding on the nutritional and rheological properties of Cheddar cheese, with increased CLA, vacennic acid and omega 3 fatty acids. Table 1.1.1 provides some examples of recent studies investigating the effects of different feeding systems on milk composition.

Table 1.1.1. Examples of studies investigating the effects of different feeding systems on the composition of milk.

Experimental Design/Treatments (1-4)	Results	Ref
1: corn silage + alfalfa hay TMR containing calcium salts and lignosulfonate treated soybean meal. **2, 3 and 4: TMR + soybeans roasted at 115, 130 or 145 °C**	Supplementation with roasted soybeans: ↑ Long chain fatty acids ↑ Polyunsatturated fatty acids ↑ Conjugated Linoleic acid ↓ C16:0	[78]
1. Whole flaxseed **2. Whole linola** **3. Calcium salts of palm oil**	Supplementation with calcium salts of palm oil: ↑ milk yield ↑ C16:0 Supplementation with whole linola: ↑ Conjugated Linoleic acid + TVA	[79]
1. Perennial ryegrass **2. Perennial rye grass + 20% white clover** **3. TMR (maize silage + grass silage + concentrates)**	Perennial rye, Perennial rye + white clover: ↑ % Protein + % Fat ↑ % True Protein ↑ Conjugated linoleic acid ↑ Omega 3 fatty acids TMR: ↑ C16:0 ↑ Omega 6 fatty acids	[71]
1. TMR diet (60% grass silage and 40% concentrate) no fat source **2. TMR diet + megalac** **3. TMR diet + formaldehyde-treated whole linseed** **4. TMR diet + fish oil and formaldehyde-treated whole linseed**	Feeding Fish oil: ↓ Fat & protein content Feeding linseed oil: ↑ C18:3n3 ↓ Omega 6 / Omega 3	[80]
TMR + varying concentrations of whole flaxseed **1. 0 g/kg DM whole flaxseed (WF)** **2. 50 g/kg DM WF** **3. 100 g/kg DM WF** **4. 150 g/kg DM WF**	↑ Whole flaxseed = ↓ Yields of fat, protein and total solids and proportions of short- and medium-chain FA ↑ Whole flaxseed = ↑18:0, cis9-18:1, trans9-18:1, cis9,trans11-18:2, cis9,12,15-18:3 19:0 and 20:0 in milk fat ↑ Whole flaxseed = ↓cis9,12-18:2, trans9,12-18:2 and 20:4	[81]
TMR diet 70:30 forage to concentre supplemented with: **1. Corn meal plus a protein mix containing soybean meal and sunflower meal** **2. Corn meal plus flaxseed meal** **3. Liquid molasses plus a protein mix containing soybean meal and sunflower meal.** **4. liquid molasses plus flaxseed meal**	Flaxseed meal: ↓ milk yield, milk fat and milk lactose Liquid molasses plus flaxseed meal: ↑ Saturated fatty acids ↑ Δ9-desaturase index Corn meal plus soybean meal-sunflower meal protein mix: ↑ C4:0 and C18:0	[82]
TMR diet supplemented with: **1. Control - no sunflower oil and no monensin** **2. Diet containing (dry matter basis) 42 g/kg sunflower oil** **3. Control with monensin (16 mg/kg of DM)** **4. Diet containing (DM basis) 42 g/kg sunflower oil and16 mg/kg monensin**	Monensin supplementation: ↓18:0 and 22:0↑ cis9-17:1 Sunflower oil supplementation: ↓ short-chain (8:0 to 13:0) and most medium-chain (14:0, 15:0, 16:0, 17:0, cis9-17:1) cis9,12,15–18:3, cis8,11,14–20:3 and cis5,8,11,14–20:4. ↑ 18:0, total trans 18:1, cis9-18:1,19:0, cis9,12-18:2, cis9,trans11-18:2, trans10,cis12-18:2 and 22:0	[83]

1. Control group TMR Diet- 9.5 kg of concentrate mainly constituted by corn, soy, barley flour, and bran, 5 kg of corn grains, and 6.5 kg of vetch and oat hay **2. Flaxseed group – Control diet, 1kg of concentrate was substituted with the same amount of whole flaxseed**	Flaxseed supplementation: ↑saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids ↑ C18:3n-3 and Omega 3 fatty acids ↓ Atherogenic and thrombogenic indices	[84]
Grass silage-based diet (forage:concentrate ratio 58:42, on a dry matter basis) supplemented with **1. 0g of fish oil** **2. 75g of fish oil** **3. 150g of fish oil** **4. 300g of fish oil**	Supplementation with Fish oil: ↓milk fat content and yield ↑ fish oil = ↑20:5n-3 and 22:6n-3 fatty acids ↑ fish oil = ↑total conjugated linoleic acid, trans, and polyunsaturated fatty acid concentrations.	[85]
1.A lipid-free emulsion medium infused in the rumen (CTL) **2. Soybean oil as a source of polyunsaturated fatty acids infused in the rumen (RSO)** **3. Saturated fatty acids (38% 16:0, 40% 18:0) infused in the rumen (RSF)** **4. Saturated fatty acids infused in the abomasum (ASF). Fat supplements were provided continuously as emulsions at a rate of 450 g/d.**	The yields of energy-corrected milk, fat, and protein were greater with RSF compared with RSO. The concentration of odd-chain fatty acids was decreased by RSO, whereas even-chain iso fatty acids were not affected. Milk fat concentration of 17:0 + cis-9 17:1 was higher for RSF than for RSO	[86]
1. Corn silage-based diet with no additional lipid (control), **2. Control supplemented with extruded linseeds (5% of additional fat-in-dry matter intake)** **3. Diet 2 and vitamin E (7500 IU per cow per day of DL-a-tocopherol; ELE),** **4. Diet 3 and plant extracts rich in carotenoids and polyphenols (1% of dry matter intake)**	Linseed supplementation: ↑unsaturated fatty acid content Diet 3: ↓ milk and cheese 4- to 16-carbon saturated FA ↑ 18:1 cis-9, 18:3n-3 and total trans Fatty acids Vitamin E supplementation: ↑ α-tocopherol concentration,	[87]

1.10 An Alternative to Raw milk: Low Temperature Milk Processing

The consumption of raw milk poses a very real and unnecessary health risk, especially to those who may have a weak or compromised immune system such as the young, elderly and pregnant women, therefore, alternative technologies for the removal of harmful bacteria without the use of high temperatures have been developed. Moreover, post heat treatment of milk, the lysed bacterial cells remain behind along with with their potential active enzymes which can result in alterations to milks during storage, reducing shelf and stability, so the complete removal of the bacteria offers an interesting alternative. Microfiltration and bactofugation have been identified as potential reduced temperature processing methods for the removal of bacteria from milks [88]. The fat content of milk has in the past caused issues with filtration processes. However, microfiltration of homogenised milk using a 0.8 - 1.4 μm mean pore size has been demonstrated to remove bacteria and spores [89]. Microfiltration of milk for the removal of bacteria initially proposed by Holm*, et al.* [90] has led to development of industrial plants for the microfiltration of milks at 50 °C using a 1.4 μm pore sized membrane known as Bactocatch® by Tetra Leval Group, producing extended shelf-life milks, with averaged decimal reductions of pathogenic bacteria of 3.5 - 4.0 $\log_{10}$ and >4.5 $\log_{10}$ reduction in sporeforming bacteria [91]. It has been concluded therefore, that microfiltered milk can be considered as safe as pasteurised milk [88].

1.11 Milk Components as Gene Regulators in Human Health

MicroRNAs (miRNAs) are short single stranded non-coding RNA molecules (~22 nucleotides in the mature form) which affect gene expression by binding to specific sequences within the 3' UTR of messenger RNA (mRNA). miRNAs function through direct prevention of translation or by targeting mRNA for degradation [92, 93]. Since their discovery, they have been extensively researched, revealing a vast array of regulatory roles covering a range of biological processes [94]. Along with regulating normal gene expression, they have also been

connected to a myriad of pathologies including cancer [95-97], autoimmune disorders [95, 98, 99], and diseases of the gastrointestinal tract [100-102].

A recently published study indicated the detection of miRNA (miR)-168a from Oryza sativa, the rice plant, in human and animal sera [103]. The study reported that a rice based diet led to decreased expression of LDL receptor adaptor protein 1 in mouse liver through the activity of the exogenous miRNA [103], however the work remains controversial [104-107]. Immune related microRNAs have been detected in breast milk [108], and found to be present at high amounts during the first six months of lactation [109]. Indeed, milk contains the highest concentration of microRNAs of all bodily fluids [110]. It certainly would be a fitting narrative wherein maternal microRNAs could potentially be transferred to new-borns, leading to direct regulation of gene expression beyond the known immune modulation by the transfer of immune molecules, growth factors and nutrients [111]. As previously noted, miRNAs can affect health both positively and negatively. A study in recent years has shown miRNAs of bovine origin with identical sequence homology to human miRNAs in milk which have impacted expression of genes in human cells [112]. They have also given a probable mechanism of miRNA entry into intestinal enterocytes after milk consumption [113]. The work remains controversial [114], but suggests potential health benefits and potential health hazards of milk consumption through complex genetic regulatory pathways. A recent study has demonstrated no change in miRNA levels during cold storage of pasteurised whole milk, 2% milk, and skim milk, but a 63% (±28%) and 67% (±18%) decrease in concentration of miR -200c and miR-29b, respectively, was recorded following pasteurisation of raw bovine milk [115]. These authors speculate that disruption of milk exosomes results in degradation of the miRNAs in milk, as previously demonstrated through sonication of exosomes [112]. Examination of other milk treatments and milk fermentation on miRNA levels in bovine milk could be of interest in the future in this currently unfolding area of research.

1.12 Conclusion

Milk, raw or processed, is a highly nutritious food item being an excellent source of proteins, minerals and lipids, particularly important for the developing individual. Past negative reports on the fat content of milk have failed to view this complex ingredient collectively rather than as individual fatty acids. As such several benefits have been reported with the consumption of milk. Several factors can affect the nutritional composition of milks, however modification of the cows feeding system offers a simple method to achieve a more desirable nutritional profile. Raw milk consumption poses a very serious and unnecessary health risk, particularly to young, elderly and immunocompromised individuals owing to the potential presence of pathogenic bacteria. Pasteurisation processing at 72 °C for 15s does not have any significant negative effect on the nutritional quality of milk, but alleviates the potential risk of ingestion of pathogens. Despite this, many different benefits of raw milk consumption have been put forward but these lack a significant scientific basis. A number of *in vitro* studies have shown that heat treatment can alter raw milk's immunomodulatory properties and epidemiological studies have reported an inverse correlation between raw milk consumption and the incidence of certain allergic diseases. Further research is required to identify particular raw milk components responsible for such effects which may then be isolated and used in a functional food setting. In addition, milk processes such as microfiltration offer the opportunity to produce milk which is deemed as safe as pasteurised milk in terms of bacterial load but which has undergone only mild heat treatment. Overall, greater efforts to educate the general public are required to further alleviate food borne illnesses associated with raw milk consumption and combat erroneous myths that are widespread on the internet and in non-scientific material today.

1.13 Acknowledgements

Tom F. O'Callaghan is the recipient of a Teagasc Walsh Fellowships. The financial support of the following is gratefully acknowledged: Teagasc, JPI Food Processing for Health funded 'Longlife', Science Foundation Ireland (SFI) under Grant Number SFI/12/RC/2273 and the Dairy Levy Fund administered by Dairy Research Ireland.

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Chapter 1
(Part 2)

The gut microbiome as a virtual endocrine organ with implications for farm and domestic animal endocrinology

Published in *Domestic Animal Endocrinology,* 2016, 56:S44-S55

DOI: 10.1016/j.domaniend.2016.05.003

1.2.1 Abstract

The gut microbiome exerts a marked influence on host physiology and manipulation of its composition has repeatedly been shown to influence host metabolism and body composition. This virtual endocrine organ also has a role in the regulation of the plasma concentrations of tryptophan, an essential amino acid and precursor to serotonin, a key neurotransmitter within both the enteric and central nervous systems. Control over the hypothalamic-pituitary-adrenal axis also appears to be under the influence of the gut microbiota. This is clear from studies in microbiota-deficient germ-free animals with exaggerated responses to psychological stress that can be normalized by monocolonization with certain bacterial species including *Bifidobacterium infantis*. Therapeutic targeting of the gut microbiota may thus be useful in treating or preventing stress-related microbiome-gut-brain axis disorders and metabolic diseases, much the same way as redirections of metabolopathies can be achieved through more traditional endocrine hormone-based interventions. Moreover, the implications of these findings need to be considered in the context of farm and domestic animal physiology, behaviour and food safety.

1.2.2 Introduction

Research in the area of microbiome science has in recent years received a large amount of interest. This has been supported by major advances in the methods used for the characterisation and identification of microbes typically not culturable by traditional plate culturing methods. Such advancements in technology include the development of sequencing based measurements, genetic fingerprinting, fluorescent labelled oligonucleotide probes, quantitative PCR, multilocus gene typing and metagenomic sequencing approaches [1-3]. The new discoveries emerging from such substantial investment in this field has prompted a changing perception of the importance of this symbiotic relationship between a host and the trillions of microbes that make up its microbiome [4, 5].

The gut microbiota has a wide range of functions of a protective, structural and metabolic nature influencing the health of the host by aiding the processing of foods, synthesis of vitamins (such as B_{12} and K), prevention of pathogen colonisation and digestion, and harvest of energy from complex carbohydrates ingested but not digestible by the host [6, 7]. As noted [8], mammals in particular lack an enzymatic stock to efficiently harvest energy and nutrients from plant structural carbohydrates. This is especially important in ruminants on a herbivorous diet. Indeed through the use of compartmentalisation, the microbiome of the ruminants gastrointestinal tract (GIT) aids in the digestion of this plant material with the fermentation products yielding up to 70% of total dietary energy [9]. The gut microbiota also has effects on the intestinal epithelium by supporting the growth of intestinal microvilli [10] and has been shown to play an essential role in the development of innate and adaptive immune responses mediated by a number of signalling molecules and metabolites derived from the microbiota [7, 10].

The gut microbiota has also been shown to impact the function of distal systems and organs via the production of potent hormones that are released into the bloodstream through

interstitial tissue and then transported to act not just locally in the enteric nervous system (ENS) but also at remote sites such as the liver [11, 12] and even the brain [13]. The use of microbiota-deficient germ-free animals in particular has been the catalyst behind some fundamental advances in our grasp of the extent to which the gut microbiota influences brain and behaviour [14]. This expanding repertoire currently includes anxiety-like behaviours [15-17], regulation of the stress response [18], social development [19, 20], neurotransmitter synthesis and precursor availability [21, 22], microglia activation [23], neurogenesis [24], neurotrophic support [25], transcriptional regulation [26] and maintaining the integrity of the blood brain barrier (BBB) [27]. This impact is likely achieved by recruitment of the scaffolding provided by the gut-brain axis, a bidirectional communication system with immune, endocrine and neural infrastructure [28]. Lagging behind our expanding knowledge of the key roles played by the gut microbiota in health and disease are mechanistic insights into how it achieves such a vast range of functions. Conceptualizing the gut microbiota as a virtual organ, attributed to its vast metabolic activity, regulatory potential and ability to influence the function of local and non-local organs and systems, provides a framework to help understand this complex area [6, 29-31]. For example, an increased understanding of how the neonatal microbiome of animals is developed and maintained can help clarify and promote its function in influencing health effects on the host, such as the mucosal immune system [8]. This extends to a major role in orchestrating the communication between the immune and neuroendocrine systems, key to the maintenance of homeostasis across the lifespan [32].

In this review, we discuss this endocrine capacity and the main features of the gut microbiome that facilitate its impact on host physiology, brain and behaviour. To date, research efforts have been concentrated on the importance of the gut microbiota in human health and disease, ably supported by parallel laboratory animal studies. We also consider the substantial

relevance and implications from this emerging field for farm and domestic animal physiology and behaviour.

1.2.3 The Gut Microbiome as a Virtual Endocrine Organ

Typical endocrine organs or systems have the capability to produce a single or small number of hormones. Conversely the gut microbiota has the potential to produce a plethora of different hormonal products, which have the ability to affect the status of the gut and when taken up by the blood stream and transported throughout the body these hormones can have an effect on the function of remote organs and systems [6] (see Fig. 1.2.1). Although the gut microbiome is multicellular with numerous constituent genomes [33], it does satisfy many of the classical criteria required for consideration as an organ. This includes the fact that microbes in the GIT are responsive to signals from other organs and in turn influence the function of other organs within the host [34] (see Fig. 1.2.2).

The gut microbiota from a morphological and biochemical viewpoint is much larger and more heterogeneous than that of any other endocrine organ in man, even exceeding the biochemical complexity of the brain [35]. A comprehensive review by Smith, *et al.* [36] on the use of germ free (GF) animals has further demonstrated the numerous effects this essential organ can have on host health and quality of life. This enormous biochemical capacity results from the vast and diverse array of microbial cells that are located within the gut.

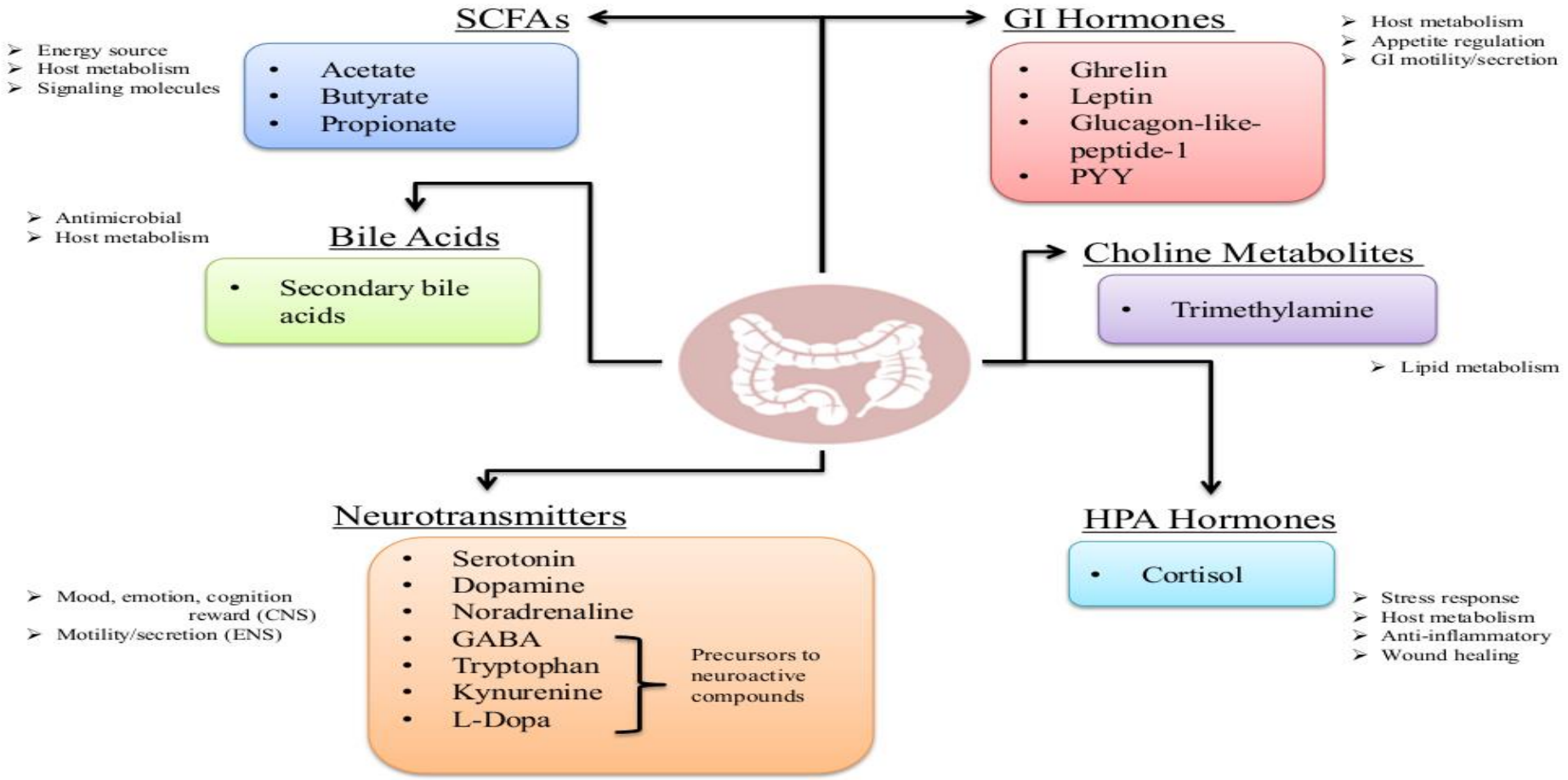

SCFAs
Energy source
Host metabolism
Signaling molecules
Acetate
Butyrate
Propionate
GI Hormones
Host metabolism
Appetite regulation
GI motility/secretion
Ghrelin
Leptin
Glucagon-like-peptide-1
PYY
Antimicrobial
Host metabolism
Bile Acids
Secondary bile acids
Choline Metabolites
Trimethylamine
Lipid metabolism
Neurotransmitters
Mood, emotion, cognition reward (CNS)
Motility/secretion (ENS)
Serotonin
Dopamine
Noradrenaline
GABA
Tryptophan
Kynurenine
L-Dopa
Precursors to neuroactive compounds
HPA Hormones
Cortisol
Stress response
Host metabolism
Anti-inflammatory
Wound healing

Figure 1.2.1 Candidate outputs of the gut microbiota. The gut microbiota has the potential to produce or regulate numerous different hormonal products, which have the ability to affect the status of the gut and when taken up by the bloodstream and transported throughout the body, these hormones can have an effect on the function of remote organs and systems. This includes hormones such as cortisol as well as neurotransmitters and their precursors such as serotonin (5-HT) and tryptophan. Bacterial SCFA production is considered particularly important, not just as an energy source but also as signalling molecules. CNS, central nervous system; ENS, enteric nervous system; GABA, γ-aminobutyric acid; GI, gastrointestinal; HPA, hypothalamic-pituitary-adrenal axis; SCFAs, short-chain fatty acids.

It is worth mentioning however, due to its functions being derived from its diverse microbial composition, perhaps the gut microbiota should be considered as a system instead, much like the immune system is comprised of different cells with various functions and roles [30]. Nevertheless, a functional understanding of key microbiota genes is essential to beneficially exploit the endocrine features of the gut microbiota in promoting host health.

1.2.4 The Gut Microbiome: Development, Composition, and Functional Relevance

After birth the human GIT is colonised with 10^{13} -10^{14} microorganisms [37, 38]. The microbiome is a diverse environment which is dominated by bacteria mainly consisting of strict anaerobes but also contains viruses, protozoa, archaea and fungi. Simpson*, et al.* [39] described the differing microbiota between food animal species. The gut microorganisms of monogastric animals such as pigs, chicken, rabbits and humans, are dominated by *Bacteroides, Clostridium, Bifidobacterium, Eubacterium, Lactobacillus, Enterobacteriaceae, Streptococcus, Fusobacterium, Peptostreptococcus* and *Propionibacterium*. On the other hand in polygastric animals including cows and sheep, the rumen is dominated with fiber degrading species of *Fibrobacter, Ruminococcus, Butrivibrio* and *Bacteroides* with other major groups including *Prevotella, Selenomonas, Streptococcus, Lactobacillus* and *Megasphaera*, in addition to a large presence of methanogens in the rumen [40]. Given the dietary differences as well as the disparate mechanics and adaptations of the respective digestive processes, such differences are not surprising. Although the rumen microbiome has been extensively studied in isolation, integration of this knowledge with the findings of newly emerging concepts from rodent and human microbiome research is a neglected area. The challenges in translating microbiome findings even between monogastric species has been noted [41] such that further extrapolation to ruminants will undoubtedly be a difficult process. Nevertheless, there are sufficient similarities in terms of establishment, development and core functions to suggest this is a viable prospect [42]. Moreover, there are indications that specific bacterial members of the rumen microbiome are associated with high

and low milk production efficiency [43]. From an economic perspective, increasing our understanding of the complex process of rumen microbial-based fiber digestion and its functional consequences with a view to optimising host health is certainly a worthwhile venture.

A number of different factors can influence the development of the human and animal microbiome ranging from the host diet, a variety of maternal factors, gestational age, genetics and mode of delivery [8, 44]. The variable dynamics of this process may have implications for neurodevelopment such that interventions during this early-life period may hold promise for mental health outcomes and stress responsivity in adulthood [45]. At birth the neonate is essentially sterile and exits the maternal womb with a GIT which is both structurally and functionally immature [46]. Postnatal bacterial colonisation and development of a host GIT occurs following birth, and the initial microbial composition of the hosts GIT can depend on the method of the birthing process [47]. The initial composition and developmental trajectory of the microbiome may have a marked influence on host health status at a later stage in life [48, 49]. Preclinical research suggests, for example, that appropriate microbial colonisation of the GIT is critical for postnatal development [50] and normal neuronal excitability [51] in the ENS as well as epithelial proliferation [52].

It has been shown that a natural birthing process exposes the infant to its first microbes present in the birth canal of the mother and infants delivered via the birth canal acquire a gut microbiota which resemble that of the maternal vaginal mucosa. However, it has also been reported that infants which are born via caesarean-section are not directly exposed to maternal microbes but instead encounter their first microbes from the mother's skin and environment of the birthing place. This was neatly illustrated by Dominguez-Bello*, et al.* [47] in an important study which showed that infants delivered by caesarean-section have a gut microbiota which resembles that of the mothers epidermal microbiota, dominated by *Lactobacillus*, *Prevotella* and

Sneathia spp. Existing data also shows that babies delivered by caesarean section harbouring a different microbiota than vaginally delivered infants have abnormal short term immune responses and a greater long term risk of developing immune diseases [47, 53].

The developing infant microbiome is also heavily influenced by early nutritional regimen, particularly in the context of breast vs formula feeding. Maternal colostrum, for example, is a nutrient rich milk produced by mammals post birthing which contains numerous beneficial components for the infant. Such nutrients include antimicrobial proteins, immunoglobulin's, cytokines, growth factors, and leukocytes that transfer passive immunity to the immunologically deprived infant [54]. Many of these nutrients are not reproducible in artificially manufactured infant milk formulae's used as a breast milk replacer [55]. The initial composition of a hosts GIT microbiota has also been seen to influence the quality of life of animals at a later stage. Calves fed colostrum for a 14 day period have displayed 108 less days per treatment group of gastrointestinal distress as indicated by the presence of diarrhoea than calves fed milk replacer [56]. Bacteria have also been identified in colostrum and it has been noted that bovine colostrum is dominated by *Lactobacillus* and *Bifidobacterium* phylotype which are a common bacteria used as probiotics in animals and humans [57]. Piglets which were fed colostrum or infant formula had a similar cecal bacterial composition at the phyla level, but significant differences were seen between the groups at the lower taxa level [58]. The functional implications of these compositional differences are currently unclear. Similar influences could also be seen for humans with suckling infants having a GIT dominated with *Bifidobacterium*, while formula fed infants had similar proportions of *Bifidobacterium* and *Bacteroidetes* [8].

Postnatal colonisation of the host gut is essential for the development of an innate and adaptive immune system, which enables the host to develop a tolerance to a number of microbial antigens. The ability to discriminate between commensal organisms and pathogenic bacteria is a complex and sophisticated immunoregulatory process. This results in reduced

allergic and inflammatory responses, further enabling the maintenance of physiologically normal steady state of inflammation in the gut throughout life [59, 60]. Demonstrating this phenomenon, GF animals, who have reduced development of the gastrointestinal associated lymphoid tissue (GALT) with distortion of the T-helper cell balance compared to specific pathogen free (SPF) controls. Colonisation of the intestinal mucosa by pathogenic organisms causes a robust inflammatory response, while commensals on the other hand have been seen to induce a self-limiting inflammatory process, without causing damage to tissue with resultant immunological tolerance [7, 61]

The epithelial tissue of the gut has many important functions in addition to acting as a physical barrier against both endogenous commensal microorganisms of the gut microbiota, and enteropathogenic bacteria and viruses. The gut epithelium is responsible for the release of compounds such as chemokines and cytokines that have a role in the recruitment of inflammatory and immune cells, which play a major role in the restraint of exposure to these harmful agents [62]. Innate effectors play a role in the regulation of the gut microbiota composition, regulating colonisation through the secretion of IgA into the intestine. Defensins are produced and released by the mucosal epithelial cells, which act as natural antimicrobials against Gram negative and Gram positive bacteria and have a marked activity against fungi, and certain enveloped viruses [7, 63]. The interaction between bacteria in the gut also plays a central role in controlling the guts bacterial composition. Bacterial metabolic activities, such as short chain fatty acids (SCFA's) production, alteration of potential redox potentials and production of bacteriocins, has the potential to alter the gut environment making it more suitable for certain bacterial genera over others [7]. Diet composition also has a significant influence on GIT function, microbiota composition and their metabolic products in animals. Lejeune*, et al.* [64] demonstrated the effects of diets on calves, with those outdoors on pastures having a significantly different immunological profile when compared to heifers housed

indoors on a grain based diet. This profile included lower interferon gamma (IFNγ) production by concanavalin A (ConA) stimulated leukocytes and significant differences in cytokines profiles from small intestine tissues. Targeting the gut microbiome to regulate the proinflammatory profile may be an interesting strategy in calves given the importance of controlling the immune response to ensure optimal growth and metabolism [65].

After 1-3 years in humans, an adult like complex microbiota is present, that is functionally specialized for energy harvest from the diets [66], and is stable over time in healthy adults. Homeostatic mechanisms within the microbiota do however become less effective in elderly individuals [67]. Fischbach and Sonnenburg [68] described how after 2 years of life, the adult gut is predominantly colonised by *Bacteroidetes* and *Firmicutes*.

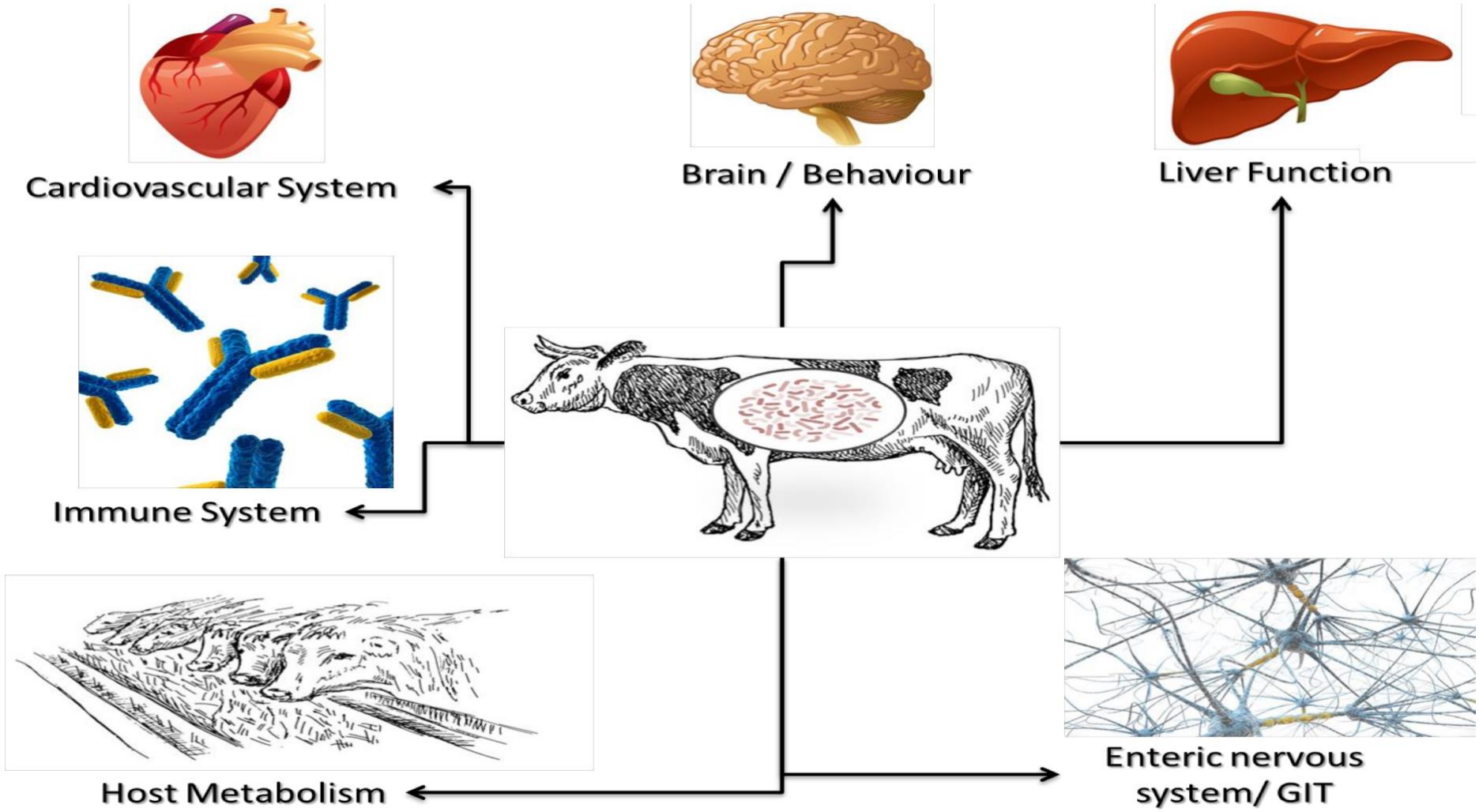

Cardiovascular System
Brain / Behaviour
Liver Function
Immune System
Host Metabolism
Enteric nervous system/ GIT

Figure 1.2.2 The potential impact of the rumen and/or gut microbiota on host physiology and well-being. Hormonal products of the gut microbiota taken up by the bloodstream and transported throughout the host have the ability to interact and affect the function, regulation and behaviour of distal organs and systems of the body. This includes important physiological effects on organs such as the liver, cardiovascular system and has implications for host metabolism. Recent research indicates that the reach of the gut microbiota also extends to an impact on brain and behaviour. GIT, gastrointestinal tract.

Bacteroidetes are anaerobic, gram negative spore forming bacteria enriched in carbohydrate degrading enzymes. *Firmicutes* on the other hand are gram positive anaerobic spore formers, which are known to ferment simple sugars, producing a variety of SCFA's such as propionate, butyrate and acetate. A significant amount of data on the functions of gut microbiota has been acquired through studies utilising GF animals, particularly mice and rats [30, 69]. Such functions include providing the host with energy in the form of SCFA's. These SCFA act as signalling molecules important for gut health and also reach the systemic circulation to modulate host metabolism and possibly impact on central nervous system (CNS) function (see below) [6]. Comparisons of GF animals and SPF animals have also highlighted the central role that the gut microbiota plays in development of the host GIT. For the GIT to mature it needs to develop efficient peristaltic motility, sufficient surface area and blood supply via capillary networks to enable nutrient absorption all of which have been observed to be altered in GF animals [70, 71].

1.2.5 Candidate Effector Molecules and Hormones of the Gut Microbiota

1.2.5.1 A Focus on Short Chain Fatty Acids

A major contribution that the gut microbiota makes to host function is the production of essential SCFAs particularly butyrate, acetate and propionate via the fermentation of dietary fibres, carbohydrates and proteins in the gut [30, 72]. Certain carbohydrates in the diet are not digested by the upper part of the GIT due to lack of the required digestive enzymes and as a result, these resistant carbohydrates (which include cellulose, hemicellulose, inulin and resistant starches) reach distal parts of the GIT, where they act as substrates for microbial fermentation [73]. The main site for SCFA production and absorption is the proximal large intestine mainly due to greater carbohydrate availability. Bacteria that produce SCFA include but are not limited to *Bacteroides*, *Bifidobacterium*, *Propionibacterium*, *Eubacterium*, *Lactobacillus*, *Clostridium*, *Roseburia* and *Prevotella* [74]. This fermentation process is essential to host health since it contributes to

more efficient extraction of energy from the diet by producing metabolites that can be utilised as energy sources [75]. This has important implications for the control of host body weight [76] and SCFAs have been shown to display a repertoire of other beneficial functions to the host such as anti-inflammatory properties and a cascade of apoptosis and innate immunity related processes [77, 78]. Fine-tuning the rumen microbiome to produce specialised SCFA outputs is an appealing, if demanding, strategy.

Butyrate is a SCFA of particular importance to host health as it is utilised as an energy source by the epithelial cells of the gut and has also been shown to have anti carcinogenic and anti-inflammatory properties. Butyrate is also known to exhibit numerous physiological functions in eukaryotic cells [74], particularly by its effects on histone acetylation where the inhibition of histone deacetylase facilitates hyper acetylation of histone proteins, facilitating the access of DNA repair enzymes [79]. A study by Schroeder*, et al.* [80] also suggested that sodium butyrate has an antidepressant effect on the murine brain by inducing a short lived transient acetylation of histones in the frontal cortex and hippocampus in conjunction with changes in expression of brain derived neurotropic factor (BDNF). In bovine cells, butyrate induces the expression of genes associated with cell growth, immune response and signal transduction [81]. Acetate is used as an energy source for the synthesis of complex molecules by muscle, liver and other peripheral tissues. Propionate has anti-inflammatory potential, is utilised by the liver and adipose tissue, plays an essential role in the satiety sensation and improves insulin sensitivity [75]. Bindels*, et al.* [82] has demonstrated in mice that propionate can suppress the proliferation of cancer cells in the liver.

There are many factors which can affect the bacteria metabolism and the production of SCFA in the gut, including diet composition, age of the host, neuroendocrine system activity, stress, disease, drugs, antibiotics, gut transit time and epithelial cell turnover times [74]. Increased

carbohydrate utilisation from non-digestible plant derived polysaccharides is known to result in a greater production of SCFAs that in turn contribute to total energy in the host [6].

1.2.5.2 Regulation of neurotransmitters

The link between the gut microbiota and the brain, having a resulting influence on host behaviour has received much attention in recent years [83, 84]. The neurotransmitter 5-HT or serotonin is a metabolite of particular interest as it is hypothesized to have a key role in the regulation of learning, sleep, anxiety, mood and other stress-related psychiatric disorders [30]. It is an important signalling molecule in the gut-brain axis (for review see Cryan and Dinan [85]), with critical roles at both the CNS and ENS level [22, 86, 87]. Tryptophan, an amino acid precursor for a range of different metabolites of interest to host health is also metabolised by the gut microbiota. Studies have shown that the gut microbiota also has a regulatory effect on the availability of tryptophan in the circulation (essential as a CNS tryptophan supply for serotonin production) [17, 88] as well as on tryptophan metabolism to serotonin in the ENS [89, 90]. The fact that oral administration of certain bacterial species (as demonstrated in rodents) can modulate tryptophan levels offers confidence that the gut microbiota may be successfully targeted to ensure optimal availability of this critical precursor [88, 91]. Microorganisms such as *Candida* spp., *Streptococcus* spp., *Escherichia* spp and *Enterococcus* spp have shown the ability to produce serotonin [92] while human intestinally derived *Lactobacillus* spp and *Bifidobacterium* spp have been shown to produce γ-aminobutyric acid (GABA) [93], both target neurotransmitter systems for anxiolytic psychotropics.

1.2.6 Gut Microbiota: Host Metabolism and Obesity

The ability of the gut microbiota to produce a large range of hormonal agents allows it to interact with host metabolism and energy homeostasis [6] as well as play key roles in regulating insulin sensitivity, fat storage, body weight, and adiposity [94]. It is widely understood that metabolic diseases such as type-2 diabetes and obesity, the latter of which, has been described

as "a prolonged imbalance of energy intake and energy expenditure" [94], can occur as a result of environmental factors and host genetics [95].

Antibiotic-induced weight gain in farm animals is well established (see section 1.2.7). The links between the gut microbiota, host metabolism and body condition was further confirmed with the observation that GF animals require a much higher calorie diet to maintain the same bodyweight of their SPF counterparts [1]. This was also demonstrated by Bäckhed and colleagues [96], where GF mice, despite the consumption of a higher calorie diet, had less total body fat than conventional mice raised on a similar diet. However, upon colonisation with a conventional mouse microbiota, such mice displayed a vigorous increase in body fat and developed insulin resistance without any increase in food consumption or energy expenditure. Similarly, it has been demonstrated that the inoculation of GF mice with microbiota from obese animals resulted in the emergence of an obese phenotype while loss of weight could be observed as a result of a microbiota transplant from mice that displayed rapid weight loss traits post gastric bypass surgery [97, 98].

Differences in intestinal microbiota composition have been shown to cause alterations in the rate of energy harvest from the diet. Obesity in human subjects is associated with alterations of the gut microbiota composition, including decreases in the abundance of *Bacteroidetes* and relative increases in the *Firmicutes* populations when compared to their lean counterparts [99, 100]. Although many studies have reported similar alterations in the *Firmicutes* to *Bacteroidetes* ratio in obese individuals, other studies have found contradicting alterations in the gut microbiota composition in obese individuals [101, 102]. Such inconsistencies emphasize the importance of considering all host and environmental factors as well as the methodology and techniques utilised in gut microbiota profiling when comparing studies [72].

1.2.7 Microbiota and the Stress Response: Relevance to Animal and Food Safety

There are many factors in any animal production system that can cause stress in animals, including handling practises, housing systems, overcrowding, transport, excess heat or cold and food or water deprivation. Stress can result in reduced feed intake, decreased activity as well as physiological, hormonal, and immunological deficiencies [103], which in turn result in reduced animal performance standards and can also have negative effects on quality of animal derived food products [104]. During stressful periods, the endocrine system secretes various hormones to act as a stress response, primarily glucocorticoids and catecholamine's [105]. The adrenal glands, due to their involvement in the symphatho-adrenomedullary system and the hypothalamic-pituitary-adrenocortical axis, play a key role in the secretion of hormones in response to stress [106]. The GIT plays host to many different bacteria including pathogenic bacteria. Many studies have shown that the presence of catecholamines alters the growth and virulence of many pathogens. During stressful periods catecholamine's are released by the GIT ENS causing a significant local increase of the hormone [107]. The presence of catecholamines has been shown to liberate iron from the high affinity ferric iron binding proteins lactoferrin and transferrin [108]. As a result increased growth capacity of Gram negative bacteria has been associated with the presence of the catecholamine hormones, attributed to their ability to supply the bacterium with iron [109, 110]. The colonisation of farmed animals with enteric pathogenic bacteria such as *E. coli* O157:H7 and *Salmonella enterica* and their subsequent spreading into the human food chain is a major health concern for meat producing industries [107].

It is now clear that there is a bidirectional relationship between the gut microbiota and the stress response with various stressors applied during different time windows (as shown in rodents) inducing an alteration in the composition of the gut microbiota [111-113] with implications for host physiology [114]. Conversely, GF animals have an exaggerated stress

response in terms of corticosterone output highlighting the critical role played by the gut microbiota in regulating appropriate stress responses [18, 21]. Interestingly, this aberrant stress response could be normalized by monocolonization with certain bacterial species including *Bifidobacterium infantis* [18]. From a behavioural perspective, the influence of the gut microbiota on anxiety is one of the most robust features of research in this area [15, 17, 21]. As we have indicated, managing stressful situations for farm and domestic animals is an important objective with many potential benefits. It remains to be seen if this can be achieved by targeting the gut microbiome or if the findings from a recent study illustrating that dietary-induced changes in the hindgut microbiota of horses influenced behaviour will generalise to other farm or domestic animals [115].

1.2.8 Potential Intervention Strategies: Probiotics for Animal Health, Production and Food Safety

Probiotics are "live organisms, which when administered in adequate amounts can confer a health benefit on the host" [116]. Animals are often subjected to environmental stresses that can result in an imbalance in the intestinal microbiota homeostasis. Probiotics have been investigated as a alternative remedy to maintain gut homeostasis and to the use of antibiotics, particularly as growth promoters, whose use in recent years has widely been restricted or banned due to food safety concerns and the promotion of antibiotic resistance. Indeed, there has been a substantial rise in probiotic use for farm and domestic animals in the past 15-20 years [117, 118].

1.2.8.1 Ruminants

Uses of probiotics during particularly stressful periods for an animal have been shown to impart many beneficial effects. Stressful periods in particular for ruminants include weaning, start of lactation and a dietary shift from forage to readily fermentable carbohydrates [119]. Many studies have shown the use of probiotics for the reduction of diarrhoea in calves. Agarwal*, et al.* [120] reported a reduction in diarrhoea through the feeding of calves, milk

fermented with a mix Lactic Acid Bacteria, *Lactobacillus acidophilus* 15 or *Saccharomyces cerevisiae* NCDC49, administration of *E. coli* strain Nissle 1917, was also seen to have an obvious effect on the prophylaxis and treatment of neonatal calf diarrhoea [121]. The most marketed probiotic product for ruminants includes live yeasts particularly those containing *S. cerevisiae*. Such live yeasts have been seen to have many beneficial effects on animals performance including increased dry matter intake, milk production and growth parameters in beef cattle [122-124]. Probiotics have also been shown to have a beneficial effect against digestive disorders in cattle. Ruminal acidosis is a common digestive disorder in dairy cattle [119], reportedly occurring due to a lactic acid overload in the rumen or the accumulation of volatile fatty acids [125]. There is also evidence that probiotic yeast strains can be administered to stabilise rumen pH and decreases the risk of ruminal acidosis occurring. One strain of *S. cerevisiae* was reported by Brossard*, et al.* [126] to be capable of preventing a drop in ruminal pH through stimulation of specific populations of ciliate protozoa, capable of rapidly engulfing starch; this in effect creates competition for lactate producing bacteria, aiding in the maintenance of a constant lactic acid level [119].

Methane production by livestock has also been a growing concern due to its contribution to global warming, estimated to be in the region of 3-5%. Chaucheyras*, et al.* [127], through the use of *in vitro* studies, showed that by the addition of a live yeast strain, hydrogen utilisation and acetate production by a ruminal acetogen bacterial isolate, was improved.

1.2.8.2 Poultry

There are many practises in modern broiler production systems that can cause excess stress in poultry, leading to a change in the gut homeostasis and weakening of the immune system, thus resulting in a greater risk for colonisation by pathogenic bacteria. Such stresses include high density housing, changes to feeding regimes, transport and management practises [128]. Pathogenic colonisation poses major risk to the poultry health and negatively affects food safety. Competitive exclusion is the most common approach for probiotic administration in

poultry, and this strategy has been described as the most effective and harmless method to control intestinal disturbances in poultry [129]. This strategy has also been shown to reduce the colonisation of many pathogenic bacteria including, *Salmonella*, and protect chicks from *Campylobacter jejuni*, *Listeria monocytogenes*, pathogenic *E. coli*, and *Clostridium perfringens* [129, 130].

Probiotics have also been widely used as replacers for growth promoting antibiotic treatments. Supplementation of twelve *Lactobacillus* strains in broiler diets had beneficial effects on the feed conversion rate, body weight gain and reduction in abdominal fat deposition in the birds [131]. Probiotic blends isolated from the gut of healthy chickens added to water and feed has also been shown to cause similar growth promoting effects as that of an avilamycin treatment [128].

1.2.8.3 Pigs

There is a large body of research on the use of probiotics in pig production in amelioration of gastrointestinal disorders, reduction of pathogenic load and as alternatives to preventative use of antibiotics in feed, which has been banned in Europe since 2003 [128, 132]. The most common probiotics used for pigs and other monogastric animals are yeasts particularly *Saccharomyces boulardii* and bacteria *Lactobacillus* spp, *Enterococcus* spp, *Pediococcus* spp, and *Bacillus* spp [119]. *Lactobacillus* strains have been identified as a key component of the pigs' microbiota and, with that, they have been widely used as probiotic interventions. *Lactobacillus planatarum* with maltodextrin and fructose oligosaccharides resulted in a reduction of *E. coli* 08: K88 counts in piglets' jejunum and colon and *Lactobacillus sorbrius* had a significant effect at reducing the levels of enterotoxigenic *E. coli* (ETEC) in the ileum when fed to piglets post weaning [133, 134]. Konstantinov*, et al.* [134] also demonstrated how supplementation with the probiotics *L. sobrius* had a beneficial effect on daily weight gain. Further examples of study findings for the use of probiotics as microbiota-based interventions for health and maintenance of domestic/ farm animals can be seen in Table 1.2.1.

Table 1.2.1 Study findings for the use of probiotics as microbiota-based interventions for health maintenance of domestic and/ or farm animals.

Strain:	Species:	Study Findings:	Reference:
Enterococcus faecium	Pig	Reduction of diarrhoea in piglets Increased daily weight gain	[135]
Lactobacillus plantarum	Pig	Increased gut populations of lactobacilli	[136]
Lactobacillus sobrius	Pig	Reduction of ETEC in the piglets ileum Improved daily weight gain	[134]
***Lactobacillus rhamnosus* GG**	Pig	Amelioration of post weaning diarrhoea in piglets	[137]
***Bifidobacterium lactis* HN019**	Pig	Reduction of post weaning diarrhoea	[138]
Bifidobacterium animalis* ssp., *lactis	Pig	Increased growth performance in weaning piglets Improved ratio of *Biffidobacteria* to *E. coli* in the gut	[139]
Bacilus licheniformis* & *Bacillus subtilus	Pig	Reduction of morbidity and mortality, Improved performance of fattening pigs, Improved carcass quality	[140]
Mix:Lactobacillus murinus, Lactobacillus salivarius subsp. salivarius, Lactobacillus pentosus & Pediococcus pentosaceous	Pig	Reduced incidence, severity, and duration of diarrhoea. Increased weight gain.	[141]
Lactobacillus johnsonii	Poultry	Observed to compromise colonisation of *E. coli* 078K80 and *Clostridium perfringens*	[142]
Mix: ***Lactobacillus acidophilus, Lactobacillus casei, bifidobacterium thermophilis, E. faecium***	Poultry	Reduction in presence of C. *jejuni*	[143]
Bacillus cereus var. toyoi	Poultry	Reduction in *S. enteritidus* colonisation and invasion	[144]
Bacillus subtilus	Poultry	Suppression of *S. enteritidis* and *C. perfringens* persistence and colonisation	[145]
Mix: ***Lactobacillus acidophillus, L. casei, biffidobacterium thermophilis, Enterococcus faecium***	Poultry	Reduced feed costs and improved egg size in laying hens	[146]
Saccharomyces cerevisiae boulardii ***Pediococcus acidilatici***	Poultry	Secretion if IgA Reduction of translocation of ETEC	[147]

***Lactobacillus acidophilus*-15 *Saccharomyces cerevisiae* NCDC49**	Ruminants	Reduction of incidence of diarrhoea in calves	[120]
***E. coli Nissle* 1917**	Ruminants	Beneficial effect on treatment neonatal calf diarrhoea	[121]
***Lactobacillus acidophilus* NP51**	Ruminants	Decrease faecal shedding of *E. coli* O157:H7	[148]
Saccharomyces cerevisiae	Ruminants/ goat	Increased dry matter intake Increased milk production	[122, 124, 149]
***Lactobacillus rhamnosis* GG**	Dog	Alleviation or prevention of atopic dermatitis	[150]
Mix: ***Lactobacillus farciminis, Pediococcus acidilactis, Bascillus subtillus, Bacillus lichenformis, L. acidophilis***	Dog	Reduced convalescence time in acute self-limiting gastro enteritis	[151]
***Enterococcus faecium* SF68**	Cat	Decreased episodes of diarrhoea	[152]
Lactobacillus ingluiviei	Mice	Weight gain	[153]
***Lactobacillus plantarum* PL62**	Mice	Reduction in bodyweight	[154]
***Lactobacillus rhamnosus* PL60**	Mice	Reduction in bodyweight	[155]
Lactobacillus salivarius	Mice	Reduction in bodyweight	[156]

1.2.9 Conclusions

It is clear that the gut microbiota has a marked influence on the health and physiology of the host through its ability to produce a large range of hormonal agents that can play a regulatory role in the activity of local and non-local systems and organs. This includes an important impact on brain and behaviour, the stress response, host metabolism, the immune system, the cardiovascular system and liver function. This has implications across the lifespan and even prenatally where it is possible that a suboptimal stressed maternal microbiome may be passed on at birth with deleterious consequences for offspring metabolism and development [157-159]. Relevant hormone-like compounds, including SCFAs, and the ability to regulate the supply of tryptophan, an essential amino acid and precursor to serotonin, a key neurotransmitter within the ENS and CNS. The influence of the gut microbiota on host weight is now well recognized through the regulation of metabolic activity. Unlike other organs however, the gut microbiota exhibits compositional plasticity and the associated functionality can be subject to fluctuation as a result of stressors or dietary factors. Differences in the intestinal microbiota composition have been shown to affect efficiency of energy harvest from the diet. Stress in animals can have a negative effect on animal performance and in some cases food safety, resulting from the release of catecholamines or glucocorticoids by the gut during times of stress. This review points to a myriad of novel findings pertaining to the broad influence of the gut microbiota on host physiology and behaviour of laboratory animals and clinical populations. Although probiotic use is already widespread for farm and domestic animals, these findings suggest a much wider reach on host physiology than previously envisaged. Further research into the effects of different feeding systems (pasture vs conventional indoor systems) of production animals on the gut/ rumen bacterial composition and metabolic output would provide valuable information for the agricultural and farming industries. Probiotic supplementation of animal feed also requires further research, not just in the context of a substitute for the growth-promoting effects of antibiotics but also with regard

to animal health, welfare and quality of resulting food derived from animals. Mechanistic insights are also urgently required to ensure that the promising body of preclinical literature can be successfully exploited. It is likely that approaches such as culturomics, metatranscriptomics and metabolomics will be informative in this regard. A greater understanding of the gut/rumen microbiota of farm and domestic animals and methods to control its functionality with the use of intervention strategies could improve animal health, wellbeing, the stress response, ability to meet production targets and act as a means to prevent metabolic dysfunction and stress related disorders.

1.2.10 Acknowledgements

The APC Microbiome Institute is funded by Science Foundation Ireland (SFI) (Grant Number 12/RC/2273). Tom F. O'Callaghan is the recipient of a Teagasc Walsh Fellowship award. The Centre has conducted studies in collaboration with several companies including GSK, Pfizer, Wyeth and Mead Johnson. GC is supported by a NARSAD Young Investigator Grant from the Brian and Behaviour Research Foundation (Grant Number 20771). The content of this article was neither influenced nor constrained by this support.

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Chapter 2

Effect of pasture versus indoor feeding systems on raw milk composition and quality over an entire lactation

Published in; *Journal of Dairy Science*, 2016, 99:9424-9440.

DOI: 10.3168/jds.2016-10985

2.1 Abstract

The aim of this study was to investigate the effects of different feeding systems on milk quality and composition. Fifty-four multiparous and primiparous Friesian lactating cows were divided into 3 groups (n = 18) to study the effects of 3 feeding systems over a full lactation. Group 1 was housed indoors and offered a total mixed ration diet (TMR), group 2 was maintained outdoors on a perennial ryegrass pasture (GRS), and group 3 was also grazed outdoors on a perennial ryegrass/white clover pasture (CLV). Bulk milk samples were collected from each group at morning and afternoon milkings once weekly from March 11 to October 28 in 2015. Milk from pasture- fed cows (GRS and CLV) had significantly higher concentrations of fat, protein, true protein, and casein. The pasture feeding systems induced significantly higher concentrations of saturated fatty acids C11:0, C13:0, C15:0, C17:0, C23:0, and unsaturated fatty acids C18:2n-6 trans, C18:3n-3, C20:1, and C20:4n-6 and a greater than 2-fold increase in the conjugated linoleic acid C18:2 cis-9,trans-11 content of milk compared with that of the TMR feeding system. The TMR feeding system resulted in milks with increased concentrations of C16:0, C18:2n-6 cis, C20:3n6, C22:1n-9, and C18:2 cis-10,trans-12. Principal component analysis of average fatty acid profiles showed clear separation of milks from the grazed pasture-based diets to that of a TMR system throughout lactation, offering further insight into the ability to verify pasture-derived milk by fatty acid profiling.

2.2 Introduction

Farming practises are primarily dictated by a regions' climate and resources. The Irish dairy industry, like New Zealand, has a temperate climate and is based around the use of pasture as a low cost primary feed source [1]; as a result, temperate regions have a seasonal milk supply. Typically, in pasture based feeding systems, cows are maintained outdoors grazing fresh pasture during the warmer months and are dried off and housed indoors in the winter months leading up to the spring calving period. Dairy products derived from pasture-based systems are considered by consumers to be "more natural" as result of increased animal welfare and protection of the environment [2]. Pasture systems also offer cows a more natural environment, which allows the expression of normal behaviours [3, 4]. Total mixed ration (TMR), year round indoor housing systems are widely practiced in the United States and parts of Europe as the major farming systems [5, 6]. Such systems involve feeding cows a TMR diet, composed of a mix of grass/maize/corn silage, carbohydrates and concentrates, which better enable high milk production per cow, through greater control of feed intake quality and increased daily dry matter intake (DMI) [4]. Indoor TMR systems also offer the cows protection from environmental extremes such as heat, cold and wetness [3]. Such systems have been linked with animal welfare concerns such as increased lameness, reduced comfort and an increased prevalence of mastitis, all of which can have an effect on animal production [7, 8].

The effect of cow dietary system on milk composition has received much attention in the past and it is widely accepted that feeding system has significant effects on milk fatty acid (FA) composition with particular emphasis on the health-benefiting unsaturated FA components, particularly conjugated linoleic acid (CLA). Examples of feeding systems that have been studied for their effects on milk include consumption of TMR [9], red clover [10], red clover and grass silage [11], fresh alfalfa [12], alfalfa silage [13], linseed [14], fresh forage and marine algae [15], camelina [16], fish oil [17], fish oil and extruded soybeans [18], rapeseed supplementation [19] and various proportions of fresh grass [20]. Research has clearly

identified that incorporating white clover into pasture based diets has many benefits on dairy cows performance, due to its increased nutritive value over perennial ryegrass [21-23]. Feeding pure white clover, however, is not a feasible practise due to difficulties in maintaining such swards and increased risk of bloat occurring [24]. The clover level needed to induce a beneficial response on a cow's performance has also been studied with mixed results. Thomson [25] indicated that clover content needed to be at least 30 %, Egan*, et al.* [21] found benefits at sward clover contents of 23 %, whereas studies performed in New Zealand reported 50-60 % clover content to be more appropriate to increase milk yields significantly [24, 26]. Caradus*, et al.* [27] outlined the major benefits associated with clover feeding, which include its improved sward quality, improved forage dry matter intake (DMI) and utilisation rates in animals, and effectiveness at fixing nitrogen (N) in the soil.

It is understood that milk from cows consuming significant quantities of grazed grass contains higher proportions of unsaturated FA and CLA than cows which are offered diets dominated by conserved forages, concentrates and grains [28]. Much of this research, however, was conducted over a short period using cross-over studies or replicated Latin square designs. There is limited information available for the comparison of pasture-based and TMR feeding systems on the composition and quality of raw milk over an entire lactation season.

The objective of this study was to examine and assess the effects of three widely practised feeding systems, namely a TMR diet indoors, perennial ryegrass (*Lolium perenne* L.) outdoors (GRS) and perennial ryegrass/white clover (*Trifolium repens* L.) outdoors (CLV) on the composition and quality of raw milk throughout an entire lactation, and to identify potential attributes of milks which could be used to verify pasture derived milks.

2.3 Materials and Methods

2.3.1 Reagents

Hexane, heptane, formic acid, and 25 % sodium methoxide were purchased from Sigma Aldrich (Dublin, Ireland). Diethyl ether was purchased from Fisher Scientific (Dublin, Ireland). Internal standard trinonadecanoin (C19:0; part number: T-165) and a standard mix of conjugated linoleic acid C18:2 c9t11 and *C18:2 c12t10* (part number: UC-59M) were purchased from Nu-Chek Prep Inc. (Elysian, MN; c = cis; t = trans). Fatty acid methyl ester standard mix containing C4:0 to C24:0 methyl esters (part no: 18919–1AMP) was purchased from Sigma Aldrich (Dublin, Ireland).

2.3.2 Experimental Design and Sample Collection

Fifty-four spring calving Friesian cows were allocated to three groups (n=18) at the Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland. The experiments were conducted between the 11th of March and the 28th of October 2015. Groups were randomised based on milk yield, milk solids yield, calving date (mean calving date 19th February 2015) and lactation number. Three feeding systems were compared over a full lactation; Group 1 was housed indoors and fed a total mixed ration diet (TMR), Group 2 was maintained outdoors on perennial ryegrass only pasture (GRS) while Group 3 was also maintained outdoors on a perennial ryegrass/white clover pasture (CLV). The TMR diet consisted of, on a dry matter basis (DM) 7.15 kg of grass silage, 7.15 kg of maize silage and 8.3 kg concentrates (see Table 2.1 and 2.2). Cows within the TMR system were fed at 08:30 h daily into electronically controlled Griffith Elder Mealmaster individual feed bins (Griffith Elder and Company Ltd, Suffolk, England) and was available *ad-libitum*. Pasture based cows consumed ~ 18 kg DM/day (see Table 2.3) measured by pre and post grazing sward heights daily using the rising plate meter (Jenquip, Fielding, New Zealand) while pre grazing herbage mass was measured with an Etesia mower (Etesia UK Ltd, Warwick, UK). The CLV sward contained 20 % clover and was measured according to Egan, *et al.* [29]. Milking took

place at 07:30 and 15:30 h daily and milk yields were recorded using DairyMaster milk meters (DairyMaster, Kerry, Ireland). In order to obtain a representative sample of milk, the cows in each of the three feeding systems were milked separately into designated 5000 L refrigerated tanks. The evening milk was stored at 4 °C overnight, to which the morning milk was then added to and agitated prior to collection. Bulk milk samples were collected post-morning milking weekly throughout lactation (n=32) and stored at 4 °C prior to analysis.

Ethical Approval. Teagasc has both an animal welfare body (AWB) and animal ethics committee. The AWB are a legal requirement of Article 26 of Directive 2010/63/EU and Regulation 50 of S.I. No. 543 of 2012. The Health Products Regulatory Authority (HPRA) provides project authorization and the HPRA Licence number for this project is: AE19132/P019.

2.3.3 Milk and Feed Compositional Analysis

Total nitrogen (TN), crude protein (CrP), non-protein nitrogen (NPN), non-casein nitrogen (NCN) and true protein (TP) were determined as outlined in ISO [30, 31] using the Kjeldahl method and a nitrogen-to-milk protein conversion factor of 6.38. These N values were then used to calculate true protein (TP), casein protein (C_p) and whey protein (W_p) contents as outlined by Auldist, *et al.* [32] where TP= TN-NPN × 6.38, C_p= (TN-NCN) × 6.38 and W_p = (NCN-NPN) × 6.38. Milk samples were analysed for fat, lactose and total solids contents by infrared absorption spectroscopy using a FT6000 Milkoscan (Foss Ireland Ltd, Dublin, Ireland). Feed samples were collected throughout lactation from paddocks at time of grazing. Grass silage samples were collected weekly. Samples were dried at 60 °C for 48 h, milled and stored prior to analysis. Samples were analysed using near infrared reflectance spectroscopy using a FOSS 6500 (FOSS Ireland Ltd, Dublin, Ireland). The UFL, PDIA, PDIE, PDIN have been calculated according to the INRA feeding system equations [33]. Analysis of Maize silage was carried out by FBA Laboratories Ltd (Co. Waterford, Ireland) Table 2.2 and 2.3.

2.3.4 Milk Fatty Acid Analysis

Lipid Extraction. Lipid extraction was performed as per the procedure outlined by De Jong and Badings [34]. Briefly, 10 mL of ethanol (98 % purity) was added to 10 mL of milk, and 1 mL of 2.5 M H_2SO_4 was added to each sample mixture. This mixture was extracted three times with 15 mL diethyl ether/heptane (1:1) and each time the solution was clarified by centrifugation at 1500 × g for 5 min at room temperature. The collected extracts were pooled and dried down at 55 °C under N gas.

Methyl Ester Derivitisation of Triglycerides. A volume of 4.8 mL of $C_{19:0}$ TAG (500 mg/L) in heptane was added to 60 mg of the extracted lipid sample after which 200 µl of 2 M Sodium Methoxide solution was added and the sample was mixed vigorously for about 30 s. Then, 1 g of sodium hydrogen sulfate monohydrate (Sigma Aldrich, Dublin, Ireland) was added to the solution and shaken vigorously. After the salt had settled, the upper layer containing the methyl esters was poured into a clean test tube and diluted with 8 mL of heptane. Fatty acid methyl esters (FAME) were stored at -20 °C prior to GC analysis in 2 mL amber vials which were capped with PTFE/white silicone septa.

Instrument Conditions for Analysis of FAME. Fatty acid methyl esters analysis was performed on an Agilent 7890B gas chromatograph, equipped with a GC80 autosampler (Agilent Technologies, Little Island, Cork, Ireland) and flame ionisation detector. The column was a Select FAME capillary column (100 m × 250 µm I.D., 0.25 µm phase thickness, part number: CP7420, Agilent Technologies). The injector was held at 250 °C for the entire run and was operated in split mode using a split ratio of 1:10 and the injection volume was 1 µl. The inlet liner was a split gooseneck liner (Part no.: 8004-0164, Agilent Technologies). The column oven was held at 80 °C for 8 min and raised to 200 °C at 8.5 °C/ min, and held for 55 min. The total run time was 77.12 min. The FID was operated at 300°C. The carrier gas was helium

and was held at a constant flow of 1.0 mL/min. Results were processed using OpenLab CDS Chemstation edition software version Rev.C.01.05 (Agilent Technologies).

Standard curves for FAME analysis along with in-run quality control samples were prepared using an Agilent 7696A Sample Prep Workbench instrument (Agilent Technologies).

Nutritional indices and fatty acid ratios. Several FA's ratios and nutritional indices of milks from each of the feeding systems are reported. The summation of omega 6 (*n*6; linolelaidic acid, linoleic acid (LA), eicosatrienoic acid and arachidonic acid) omega 3 (*n*3; α-linolenic acid (ALA)) and omega 9 (*n*9; oleic acid and erucic acid) are reported. Other longer chain *n*3 fatty acids include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been found in milk at low concentrations, but were not present at quantifiable levels in our analysis. Similar to Benbrook*, et al.* [35], to more fully reflect variations in levels of health promoting dairy FA's we have also included total *n*3 and CLA and as Benbrook*, et al.* [35] described with this in mind, we also include the ratio of *n*6 fatty acids to *n*3 + total CLA to fully reflect these variations. The atherogenecity index (AI) and thrombogenicity index (TI) outlined by Ulbricht and Southgate [36] are dietary risk indices for cardiovascular disease. The AI indicates the relationship between fatty acids with pro-atherogenic and those with anti-atherogenic properties, showing the inhibition of aggregation of plaque and diminishing the levels of esterified FA, cholesterol and phospholipids, while TI shows the relationship between pro-thrombogenic (saturated) and anti-thrombogenic fatty acids; indicating the tendency to form clots in the blood [37].

Atherogenecity index (AI) and thrombogenicity index (TI) have been calculated as described by Ulbricht and Southgate [36];

$$\text{AI} = \frac{C12{:}0+(4\ x\ C14{:}0)+C16{:}0}{n6\ \text{PUFA}+n3\ \text{PUFA}+\text{MUFA}}$$

$$\text{TI} = \frac{C14{:}0+C16{:}0+C18{:}0}{(0.5\ \text{x MUFA})+(0.5\ x\ n6PUFA)+(3\ x\ n3\ PUFA)+(\frac{n3PUFA}{n6\ PUFA})}$$

Desaturase index (DI) was calculated as described by Kay, *et al.* [38] :

$$\frac{(\text{product of } \Delta 9 - \text{desaturase})}{(\text{product of } \Delta 9 - \text{desaturase} \ + \ \text{substrate of } \Delta 9 - \text{desaturase})}$$

Therefore, DI = $\frac{(C14:1+C16:1+C18:1)}{[(C14:0+C16:0+C18:0)+(C14:1+C16:1+C18:1)]}$

2.3.5 Statistical Analysis

Statistical analysis was performed using SPSS v18.0 (IBM Statistics Inc., Armonk, NY, USA). A Between- and Within-Subjects repeated measures ANOVA with post hoc Tukey test was used to compare chemical compositions and fatty acid content of milks from herds on different feeding systems (TMR, GRS and CLV) throughout lactation (March to October). *P*-values < 0.05 were considered significant. The strength of statistically significant results are also reported as the partial eta^2 effect size (η^2) where, effects sizes are small ($0.01 \leq \eta^2 < 0.06$), medium ($0.06 \leq \eta^2 < 0.14$) and large ($\eta^2 \geq 0.14$). Principal component analysis (PCA) of milk fatty acids averages data set was performed using The Unscrambler® X multivariate analysis program, v10.3 (CAMO ASA, Trondheim, Norway). Analysis of milks was performed on a weekly basis in duplicate throughout lactation; monthly and lactation figures reported below are the mean and standard deviation (SD) of all weeks within that period.

Table 2.1. Typical ingredient formulation (% as fed) and chemical composition (%) of TMR concentrate

TMR Ingredients	**% as fed**
Maize	13.0
Beet pulp molassed	15.5
Soyabean meal 48% CP	30.0
Maize distillers	12.0
ACID BUF	0.7
Maize/Beet Min Balancer	2.5
Salt	0.5
Barley (rolled)	15.0
Rapeseed meal	7.5
Megalac	3.3
Chemical Composition	**%**
Organic Matter	93.5 ± 0.9
Dry Matter	86.8 ± 0.8
Protein	23.7 ± 3.7
Fibre	7.8 ± 1.9
Starch	21.5 ± 1.9
Total Sugar	9.6 ± 0.4
Ash	6.5 ± 0.9
Moisture	13.2 ± 0.8
NCGD	83.4 ± 1.2

ACID BUF = acid buffer; NCGD = neutral cellulase plus gamanase digestibility; Megalac, Volac Ireland, Co. Cavan, Ireland.

Table 2.2. Chemical composition (g/kg of DM; mean ± SD) and nutritional content of silages from TMR diet (grass silage and maize silage) collected weekly throughout lactation analyzed by near-infrared spectroscopy

	g/ kg of DM	
	Grass Silage	**Maize Silage**
Dry Matter	389.37 ± 61.35	343.03 ± 43.45
Organic Matter (OM)	917.94 ± 7.45	972.53 ± 3.22
Crude protein	114.55 ± 12.55	68.97 ± 9.91
Starch	NA	285.37 ± 28.81
ADF	296.82 ± 23.40	NA
NDF	452.02 ± 39.31	434.80 ± 49.57
ASH	82.06 ± 6.75	27.47 ± 3.22
UFL (/kg DM)	0.93 ± 0.04	1.00 ± 0.02
PDIA (/kg DM)	24.55 ± 1.63	14.97 ± 2.20
PDIE (/kg DM)	68.07 ± 2.23	66.57 ± 3.14
PDIN (/kg DM)	72.52 ± 5.11	42.37 ± 6.05

UFL = unité fourragère lait; PDIA = sum of the feed protein ruminally undegraded and truly digested in the small intestine; PDIE = sum of PDIA and the microbial true protein that is truly digested in the small intestine (PDIM) when energy is limiting; PDIN = sum of PDIA and PDIM when nitrogen is limiting. NA = not available.

Table 2.3. Chemical composition (g/kg of DM; mean ± SD) and nutritional content of pasture systems forages (grass and clover) collected weekly throughout lactation, analyzed by near-infrared spectroscopy

	g/ kg of DM	
	GRS	**CLV**
Organic Matter (OM)	928.00 ± 9.31	931.49 ± 7.18
OM Digestibility	764.43 ± 19.34	769.22 ± 18.97
Crude protein	210.90 ± 23.71	220.67 ± 14.05
ADF	218.89 ± 16.91	220.67 ± 14.05
NDF	427.62 ± 23.83	423.46 ± 18.94
ASH	72.00 ± 9.31	68.51 ± 7.18
UFL (/kg DM)	0.99 ± 0.03	1.00 ± 0.03
PDIA (/kg DM)	41.79 ± 3.03	44.80 ± 2.99
PDIE (/kg DM)	100.91 ± 3.38	104.48 ± 3.50
PDIN (/kg DM)	135.96 ± 15.73	151.67 ± 15.52

UFL = unité fourragère lait; PDIA = sum of the feed protein ruminally undegraded and truly digested in the small intestine; PDIE = sum of PDIA and the microbial true protein that is truly digested in the small intestine (PDIM) when energy is limiting; PDIN = sum of PDIA and PDIM when nitrogen is limiting.

Table 2.4. Mean daily milk yield data of individual cows from TMR, grass, and clover feeding systems throughout the duration of the lactation trial and live weight of cows at the end of the trial in October

	Feeding System				
	TMR	**GRS**	**CLV**	**SE**	***P*-value**
Milk Yield L/d	27.71	20.98	24.59	0.14	< 0.001
Milk Solids kg/d*	2.24	1.78	1.99	0.01	< 0.001
Protein kg/d	0.94	0.76	0.87	0.01	< 0.001
Fat kg/d	1.31	1.02	1.12	0.03	< 0.001
Lactose kg/d	1.32	1.01	1.18	0.01	0.716
Live Weight kg	591.51	532.11	550.45	13.15	< 0.001

*Milk solids = sum of protein and fat

2.4 Results

2.4.1 Milk Chemical Composition

The results of this study demonstrate that the feeding system had a significant effect ($P = < 0.05$) on daily milk yield of cows throughout lactation. The TMR cows had the highest daily milk yields which were significantly higher than GRS and CLV systems ($P = < 0.001$), and CLV cows daily milk yield was also significantly higher than GRS cows ($P = < 0.001$) (see Table 2.4). Average weekly, monthly and total lactation milk chemical composition for the TMR, GRS and CLV feeding systems are shown in Figure 2.1, 2.2 and Table 2.5.

Total lactation average milk solids content from cows on the GRS system was significantly higher than that of TMR ($P = < 0.001$) and CLV ($P = < 0.001$) systems. There was no significant difference in average lactation total solids contents between TMR and CLV milk (Fig. 2.2A). Average total solids content for early, mid and late lactation were (mean ± SD) 13.06 ± 0.25, 13.02 ± 0.19 and 14.00 ± 0.26 % for TMR, 13.60 ± 0.23, 13.56 ± 0.20 and 14.58 ± 0.41 % for GRS and 13.14 ± 0.15, 13.21 ± 0.18 and 13.99 ± 0.36 % for CLV systems, respectively. Maximum solids contents were recorded in September and minimum contents were observed during early lactation (March/ April) for each diet (Fig. 2.1A). The cows from the GRS feeding system produced milk with significantly higher ($P = < 0.001$) total lactation average milk fat content than that of TMR and CLV systems (Fig. 2.2B). There was no significant difference in total lactation fat contents between TMR and CLV milk. Average milk fat content for early, mid and late lactation were (mean ± SD) 4.23 ± 0.18, 4.24 ± 0.18 and 4.65 ± 0.13 % for TMR, 4.56 ± 0.26, 4.46 ± 0.19 and 4.90 ± 0.29 % for GRS and 4.04 ± 0.18, 4.21 ± 0.17, 4.57 ± 0.24 % for CLV, respectively. Maximum fat concentrations were recorded in October and minimum fat concentrations were observed in March/ April for each diet (Fig. 2.1B).

Table 2.5. Monthly and yearly average (percentage ± SD) physicochemical analysis of weekly bulk raw milk samples from cows on different feeding systems of TMR, perennial ryegrass (grass), and perennial ryegrass/white clover (clover)

		Early-Lactation			Mid-Lactation			Late-Lactation		Yearly Average		
	Diet	March	April	May	June	July	August	September	October		*P:*	η^2:
Protein	TMR	3.15 ± 0.02	3.06 ± 0.11	3.16 ± 0.06	3.32 ± 0.10	3.32 ± 0.07	3.58 ± 0.02	3.66 ± 0.04	3.80 ± 0.04	3.38 ± 0.26	.001	.911
	GRS	3.37 ± 0.06	3.29 ± 0.10	3.34 ± 0.12	3.53 ± 0.18	3.65 ± 0.04	3.86 ± 0.11	4.02 ± 0.13	4.19 ± 0.13	3.65 ± 0.26		
	CLV	3.28 ± 0.12	3.34 ± 0.07	3.36 ± 0.06	3.41 ± 0.06	3.56 ± 0.04	3.64 ± 0.05	3.78 ± 0.14	4.09 ± 0.06	3.56 ± 0.27		
Fat	TMR	4.36 ± 0.12	4.10 ± 0.11	4.28 ± 0.17	4.29 ± 0.15	4.16 ± 0.15	4.56 ± 0.16	4.72 ± 0.06	4.67 ± 0.06	4.39 ± 0.25	.001	.917
	GRS	4.48 ± 0.31	4.64 ± 0.17	4.42 ± 0.17	4.56 ± 0.23	4.40 ± 0.11	4.54 ± 0.06	5.12 ± 0.18	5.03 ± 0.11	4.65 ± 0.32		
	CLV	4.05 ± 0.12	4.02 ± 0.07	4.14 ± 0.12	4.24 ± 0.14	4.24 ± 0.20	4.33 ± 0.21	4.68 ± 0.19	4.69 ± 0.09	4.30 ± 00.30		
Lactose	TMR	4.96 ± 0.10	4.95 ± 0.03	4.98 ± 0.03	4.96 ± 0.06	4.89 ±0.05	4.84 ± 0.03	4.82 ± 0.01	4.75 ± 0.02	4.89 ± 0.09	.516	-
	GRS	5.02 ± 0.14	4.93 ± 0.02	4.99 ± 0.05	4.92 ± 0.05	4.86 ± 0.03	4.82 ± 0.02	4.76 ± 0.07	4.67 ± 0.02	4.87 ± 0.13		
	CLV	5.02 ± 0.19	4.99 ± 0.03	4.96 ± 0.08	4.88 ± 0.03	4.84 ± 0.03	4.81 ± 0.04	4.76 ± 0.05	4.65 ± 0.02	4.86 ± 0.14		
Total Solids	TMR	13.23 ± 0.23	12.89 ± 0.12	12.98 ± 0.16	13.09 ± 0.09	13.00 ± 0.26	13.82 ± 0.31	14.24 ± 0.07	13.96 ± 0.07	13.40 ± 0.52	.001	.938
	GRS	13.58 ± 0.29	13.62 ± 0.15	13.39 ± 0.16	13.76 ± 0.13	13.53 ± 0.10	14.14 ± 0.23	14.93 ± 0.23	14.67 ± 0.27	13.95 ± 0.57		
	CLV	13.20 ± 0.16	13.09 ± 0.11	13.06 ± 0.20	13.33 ± 0.09	13.25 ± 0.13	13.65 ± 0.34	14.24 ± 0.27	14.07 ± 0.10	13.48 ± 0.47		
NPN	TMR	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.03	.001	.868
	GRS	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.01		
	CLV	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.01		
NCN	TMR	0.10 ± 0.01	0.11 ± 0.01	0.10 ± 0.00	0.10 ± 0.01	0.1 ± 0.01	0.12 ± 0.00	0.12 ± 0.01	0.12 ± 0.00	0.11 ± 0.01	.020	.582
	GRS	0.11 ± 0.00	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.13 ± 0.00	0.14 ± 0.01	0.14 ± 0.00	0.12 ± 0.02		
	CLV	0.12 ± 0.02	0.11 ± 0.01	0.12 ± 0.01	0.10 ± 0.02	0.13 ± 0.01	0.13 ± 0.00	0.14 ± 0.01	0.14 ± 0.01	0.12 ± 0.02		
True Protein	TMR	2.97 ± 0.03	2.85 ± 0.11	2.95 ± 0.04	3.123 ± 0.08	3.12 ± 0.07	3.39 ± 0.03	3.46 ± 0.04	3.63 ± 0.12	3.19 ± 0.27	.001	.798
	GRS	3.18 ± 0.07	3.10 ± 0.10	3.14 ± 0.12	3.37 ± 0.18	3.44 ± 0.05	3.66 ± 0.11	3.82 ± 0.11	4.01 ± 0.12	3.46 ± 0.33		
	CLV	3.08 ± 0.14	3.12 ± 0.10	3.15 ± 0.08	3.18 ± 0.07	3.32 ± 0.04	3.43 ± 0.04	3.55 ± 0.14	3.90 ± 0.09	3.34 ± 0.28		
Casein	TMR	2.48 ± 0.07	2.38 ± 0.12	2.51 ± 0.05	2.66 ± 0.05	2.61 ± 0.09	2.83 ± 0.02	2.88 ± 0.05	3.20 ± 0.36	2.69 ± 0.29	.010	.640
	GRS	2.69 ± 0.06	2.63 ± 0.09	2.66 ± 0.10	2.81 ± 0.19	2.87 ± 0.11	3.26 ± 0.39	3.15 ± 0.07	3.52 ± 0.40	2.95 ± 0.37		
	CLV	2.50 ± 0.23	2.63 ± 0.10	2.60 ± 0.03	2.74 ± 0.14	2.74 ± 0.06	3.05 ± 0.36	2.94 ± 0.16	3.43 ± 0.35	2.83 ± 0.35		
Whey	TMR	0.48 ± 0.05	0.47 ± 0.02	0.45 ± 0.02	0.47 ± 0.03	0.51 ± 0.07	0.55 ± 0.02	0.59 ± 0.02	0.58 ± 0.01	0.51 ± 0.06	.068	-
	GRS	0.49 ± 0.02	0.47 ± 0.04	0.48 ± 0.02	0.56 ± 0.03	0.57 ± 0.09	0.60 ± 0.04	0.67 ± 0.04	0.67 ± 0.02	0.56 ± 0.09		
	CLV	0.58 ± 0.12	0.49 ± 0.04	0.55 ± 0.08	0.44 ± 0.10	0.58 ± 0.08	0.58 ± 0.02	0.62 ± 0.03	0.64 ± 0.02	0.56 ± 0.09		

Statistical analysis was by repeated-measures ANOVA, and η2 is the significance effect size.

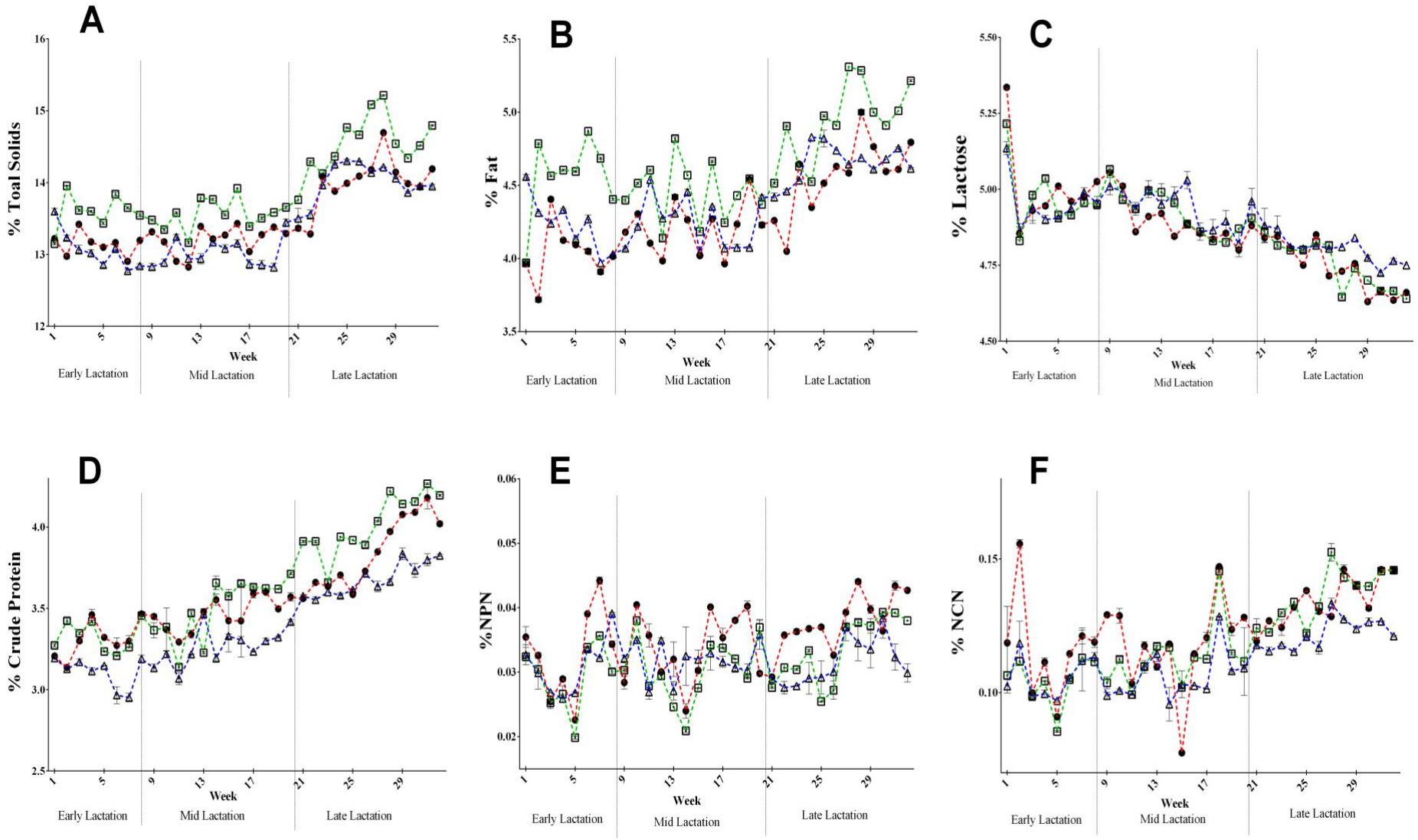


Figure 2.1. Seasonal variation of weekly chemical composition of milks (mean ± SD) derived from different feeding systems: (TMR (▲), perennial ryegrass (■), perennial ryegrass/white clover (●)). NPN nonprotein nitrogen, NCN = noncasein nitrogen.

The cows in both the GRS and CLV feeding systems produced milk with significantly higher protein concentration than that of cows on the TMR system (P = < 0.001) (Fig. 2.2D and 2.2E). There was no significant difference in the average lactation milk true protein contents between GRS and CLV systems. However, on a % of total protein basis, GRS derived milks had significantly higher lactation average % true protein of total protein content (94.59 ± 0.82 %) than TMR (P, 0.003, 94.02 ± 0.72 %) and CLV (P = < 0.001) (93.65 ± 1.16 %), which was also significantly greater than TMR (P = 0.036, Fig. 2.2F). Crude and true protein contents also varied throughout lactation; whereby average crude protein contents for early, mid and late lactation were (mean ± SD) 3.11 ± 0.09, 3.27 ± 0.11 and 3.68 ± 0.10 % for TMR, 3.33 ± 0.09, 3.50 ± 0.18 and 4.02 ± 0.17 % for GRS and 3.31 ± 0.11, 3.45 ± 0.10 and 3.84 ± 0.21 % for CLV, respectively. Maximum milk protein concentrations were recorded in October and minimum protein concentrations were observed in March/ April for each diet (Fig. 2.1D). Milk casein content from the TMR system was lower than both GRS (P = 0.008) and CLV systems. There was, however, no significant difference in casein contents of GRS and CLV milks. Casein and whey contents increased throughout lactation in each system. Maximum milk casein and whey concentrations were recorded during late-lactation in October and minimum concentrations for each were observed in March/ April for each diet.

Lactose concentration did not differ (P = > 0.05) between feeding systems throughout lactation, but did, however, vary by time particularly in late lactation. The lactose content of milks remained relatively stable in early and mid-lactation at 4.95 ± 0.08 and 4.94 ± 0.06 % for TMR, 4.97 ± 0.11 and 4.92 ± 0.07 % for GRS and 5.00 ± 0.13 and 4.84 ± 0.07 % for CLV, respectively. There was a reduction in milk lactose concentrations in late lactation for all three systems; milk lactose concentrations in October were 4.75 ± 0.02 %, 4.67 ±0.02 % and 4.65 ± 0.02 % for TMR, GRS and CLV, respectively. Highest lactose concentrations were recorded during the early lactation period in March and lowest figures were recorded in October (Fig. 2.1C).

There was no significant difference in average yearly non-protein N (NPN) concentration between TMR and GRS milk. The NPN concentration of CLV milk was significantly higher ($P = < 0.001$) than that of TMR and GRS milk samples for yearly production (Fig. 2.2I). Non casein N (NCN) content was highest in CLV milk which was significantly higher ($P = 0.017$) than that of TMR milk (Fig. 2.2H).

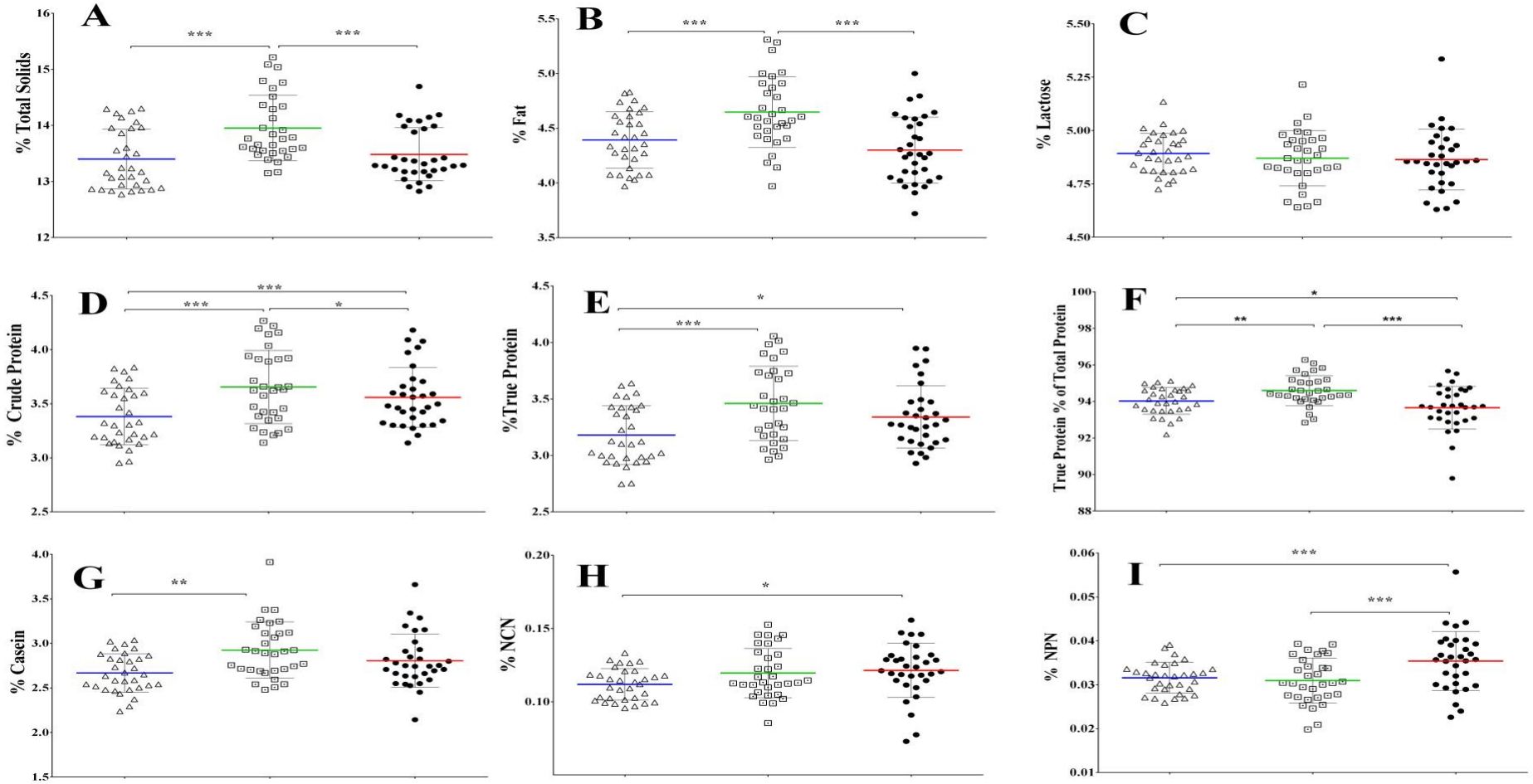


Figure 2.2. Mean and standard deviation of weekly chemical analysis of milks, for an entire lactation, derived from different feeding systems: (TMR (▲), perennial ryegrass (■), perennial ryegrass/white clover (●)). Statistical analysis by repeated measures ANOVA, *P < 0.05, **P < 0.01, and ***P < 0.001. NPN nonprotein nitrogen, NCN = noncasein nitrogen.

2.4.2 Milk Fatty Acid Composition

A total of 26 FA triglycerides were quantified (g/100 g of milk fat) from raw milks each week throughout lactation by GC-flame ionization detector. Overall, 15 of these FA varied significantly (P = < 0.05) between the different feeding systems. A list of milk FA contents (mean ± SD) during early (March–April), mid (May– July), and late lactation (August–October) is displayed in Table 2.6.

The most abundant FA in milk from each feeding system throughout lactation were palmitic acid (C16:0) and oleic acid (C18:1n-9 cis). Average palmitic acid content for the year was highest in TMR at 24.39 ± 2.45 g/100 g of milk fat, which was significantly higher than GRS at 20.78 ± 2.65 g/100 g of milk fat ($P = 0.008$) and CLV at 20.17 ± 2.76 g/100 g of milk fat ($P = 0.003$). No significant difference was found between the palmitic acid content of GRS and CLV milk samples. Total mixed ration milk had the highest average oleic acid content for lactation at 14.59 ± 2.83 g/100 g of milk fat, which was not significantly higher than GRS or CLV at 13.99 ± 3.02 and 13.23 ± 2.46 g/100 g of milk fat, respectively.

Among SFA, significant differences ($P = < 0.05$) between feeding systems were recorded for undecanoic acid (C11:0), tridecanoic acid (C13:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), heptadecanoic acid (C17:0), and tricosanoic acid (C23:0). Undecanoic acid was significantly lower in TMR at 0.04 ± 0.02 than GRS ($P = < 0.001$) at 0.06 ± 0.03 and CLV ($P = 0.012$) at 0.05 ± 0.02 g/100 g of milk fat for the year. Tridecanoic acid was lower in TMR, than that of GRS ($P = 0.007$) and CLV milk with average concentrations of 0.07 ± 0.02, 0.08 ± 0.03, and 0.08 ± 0.02 g/100 g of milk fat, respectively. Pentadecanoic acid was lower in TMR than that of GRS ($P = 0.003$) and CLV ($P = 0.011$) milk with average concentrations of 0.78 ± 0.16, 0.95 ± 0.21, and 0.92 ± 0.12 g/100 g of milk fat, respectively. Heptadecanoic acid content was lower in TMR milk than in GRS ($P = 0.009$) and CLV ($P = 0.05$) milk with average concentrations of 0.43 ± 0.08, 0.49 ± 0.09, and 0.47 ± 0.07 g/100 g of milk fat,

respectively. The GRS and CLV milks had a greater concentration of tricosanoic acid (0.04 ± 0.03 and 0.05 ± 0.03 g/100 g of milk fat, respectively) throughout lactation than TMR (P = < 0.001), which was devoid of FA with a concentration of 0.00 ± 0.01 g/100 g of milk fat. Tricosanoic acid, also, was present but not quantified in GC chromatograms during the first month of this study due to levels being too low to quantify.

Among MUFA eicosenoic acid (C20:1) and erucic acid varied significantly (P = < 0.05) between diets. Pasture feeding system resulted in significantly (P = < 0.001) higher concentrations of eicosanoic acid than that of TMR. Erucic acid content was highest (P = < 0.001) in TMR milk samples.

Among the PUFA, linolelaidic acid (C18:2n-6 trans), linoleic acid (C18:2n-6 cis), α-linolenic acid (C18:3n-3), Eicosatrienoic Acid (C20:3n6), C18:2 CLA (cis-9,trans-11), CLA (cis-10,trans-12), and arachidonic acid (C20:4n-6) varied significantly (P = < 0.05) between feeding systems. Linolelaidic acid was highest in CLV and GRS milks with concentrations of 0.36 ± 0.04 and 0.33 ± 0.06 g/100 g of milk fat, which were 2.5 times greater than TMR concentration (P = < 0.001) of 0.15 ± 0.07 g/100 g of milk fat. Linoleic acid was highest in TMR with concentrations of 1.31 ± 0.28 g/100 g of milk fat, almost 2-fold higher content than that of GRS and CLV concentrations (P = < 0.001), 0.55 ± 0.21 and 0.64 ± 0.17 g/100 g of milk fat, respectively. Eicosatrienoic acid was present but not quantified in GC chromatograms during the first month of this study due to levels being too low to quantify. Total lactation average TMR eicosatrienoic acid concentration was 0.10 ± 0.06 g/100 g of milk fat, which was greater (P = < 0.001) than GRS and CLV milk, which had little or no eicosatrienoic acid with concentrations of 0.00 ± 0.01 and 0.01 ± 0.02 g/100 g of milk fat, respectively. α-linolenic acid was lowest (P = < 0.001) in TMR at 0.27 ± 0.10 g/100 g of milk fat and CLV was also greater than GRS (P = < 0.001) with concentrations of 0.68 ± 0.10 and 0.53 ± 0.09 g/100 g of milk fat, respectively. The biologically active isomer of CLAcis-9trans-11 was present at highest

concentrations in GRS and CLV at 1.44 ± 0.37 and 1.32 ± 0.25 g/100 g of milk fat, respectively, greater than 2-fold higher (P = < 0.001) than the average TMR CLA cis-9,trans-11 content of 0.58 ± 0.15 g/100 g of milk fat. Total mixed ration had the highest CLA trans-10,cis-12 content of 0.09 ± 0.02. Arachidonic acid was absent in TMR throughout lactation but present in GRS and CLV milks at concentrations of 0.05 ± 0.03 and 0.06 ± 0.02 g/100 g of milk fat, respectively (P = < 0.001). Pasture-derived milks had significantly higher n-3 FA (n-3) content than that of TMR (P = <0.001), whereas TMR milk had a significantly higher concentration of n-6 FA (n-6) than GRS and CLV. The CLV n-6 content was also significantly higher than that of GRS (P = 0.045). As a result of this, the ratio of n-6:(n-3+CLA) was significantly lower (P < 0.001) in pasture-derived milk than in TMR milk. The feeding system had a significant effect on the desaturase index, with increased desaturase activity associated with GRS-derived milks over TMR (P = 0.026). Although there was no significant effect of feeding system on the atherogenicity index of milks, there was a significant effect of feeding system on the thrombogenic index, where TMR scores were significantly higher than that of pasture-derived milks (P = < 0.001).

Table 2.6. Mean fatty acid composition of milks (g/100 g of milk fat ± SD) throughout lactation from cows on different feeding systems of TMR, perennial ryegrass (GRS), and perennial ryegrass/white clover (CLV)

	Early-Lactation			Mid-Lactation			Late-Lactation			Yearly Average			Significance:	
	TMR	**GRASS**	**CLOVER**	**TMR**	**GRASS**	**CLOVER**	**TMR**	**GRASS**	**CLOVER**	**TMR**	**GRASS**	**CLOVER**	***P*,**	**η^2**
Butyric Acid (C4:0)	3.71 ± 0.36	3.90 ± 0.53	3.98 ± 0.44	4.24 ± 0.79	3.78 ± 0.81	3.67 ± 0.39	3.76 ± 0.70	3.45 ± 0.34	3.43 ± 0.37	3.93 ± 0.71	3.69 ± 0.63	3.66 ± 0.45	0.257	-
Caproic Acid (C6:0)	1.80 ± 0.19	1.97 ± 0.33	2.08 ± 0.25	2.15 ± 0.39	1.99 ± 0.43	1.91 ± 0.19	2.00 ± 0.36	1.77 ± 0.16	1.76 ± 0.19	2.01 ± 0.37	1.90 ± 0.35	1.90 ± 0.24	0.410	-
Caprylic Acid (C8:0)	0.93 ± 0.09	1.07 ± 0.20	1.17 ± 0.15	1.13 ± 0.21	1.10 ± 0.26	1.07 ± 0.12	1.11 ± 0.19	0.98 ± 0.08	0.98 ± 0.10	1.08 ± 0.19	1.05 ± 0.20	1.06 ± 0.14	0.872	-
Capric Acid (C10:0)	1.94 ± 0.26	2.31 ± 0.56	2.60 ± 0.41	2.49 ± 0.47	2.55 ± 0.69	2.53 ± 0.38	2.45 ± 0.39	2.17 ± 0.18	2.18 ± 0.21	2.34 ± 0.46	2.35 ± 0.55	2.42 ± 0.38	0.567	-
Undecanoic Acid (C11:0)	0.03 ± 0.01	0.06 ± 0.02	0.07 ± 0.02	0.04 ± 0.01	0.06 ± 0.04	0.05 ± 0.02	0.06 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.02	0.06 ± 0.03	0.05 ± 0.02	0.001	0.769
Lauric Acid (C12:0)	2.15 ± 0.29	2.54 ± 0.64	2.88 ± 0.44	2.77 ± 0.52	2.91 ± 0.82	2.89 ± 0.47	2.86 ± 0.44	2.55 ± 0.22	2.55 ± 0.25	2.65 ± 0.53	2.68 ± 0.64	2.76 ± 0.43	0.678	-
Tridecanoic Acid (C13:0)	0.05 ± 0.01	0.08 ± 0.03	0.09 ± 0.02	0.07 ± 0.01	0.09 ± 0.04	0.08 ± 0.02	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.02	0.08 ± 0.03	0.08 ± 0.02	0.009	0.650
Myristic Acid (C14:0)	6.81 ± 0.82	7.38 ± 1.55	7.81 ± 1.07	8.84 ± 1.57	8.55 ± 1.86	8.41 ± 0.83	8.73 ± 1.59	8.30 ± 0.75	8.31 ± 0.79	8.29 ± 1.67	8.17 ± 1.53	8.22 ± 0.92	0.943	-
Myristoleic Acid (C14:1)	0.48 ± 0.06	0.52 ± 0.14	0.52 ± 0.07	0.70 ± 0.13	0.77 ± 0.18	0.70 ± 0.09	0.89 ± 0.18	0.92 ± 0.15	0.85 ± 0.14	0.72 ± 0.21	0.76 ± 0.22	0.71 ± 0.17	0.459	-
Pentadecanoic Acid (C15:0)	0.61 ± 0.06	0.80 ± 0.20	0.84 ± 0.14	0.83 ± 0.12	1.01 ± 0.26	0.96 ± 0.11	0.84 ± 0.17	0.99 ± 0.09	0.93 ± 0.08	0.78 ± 0.16	0.95 ± 0.21	0.92 ± 0.12	0.003	0.732
Palmitic Acid (C16:0)	21.46 ± 2.45	20.78 ± 2.65	20.17 ± 2.76	24.98 ± 4.50	20.28 ± 4.20	19.29 ± 1.54	25.75 ± 4.92	21.15 ± 2.21	20.55 ± 2.30	24.39 ± 4.60	20.73 ± 3.21	19.98 ± 2.26	0.002	0.743
Palmitoleic Acid (C16:1)	1.06 ± 0.12	1.19 ± 0.11	1.09 ± 0.17	1.17 ± 0.19	1.14 ± 0.24	1.05 ± 0.09	1.36 ± 0.30	1.25 ± 0.20	1.16 ± 0.18	1.22 ± 0.25	1.19 ± 0.21	1.10 ± 0.16	0.094	-
Heptadecanoic Acid (C17:0)	0.45 ± 0.07	0.56 ± 0.06	0.52 ± 0.09	0.44 ± 0.06	0.48 ± 0.10	0.48 ± 0.06	0.39 ± 0.09	0.44 ± 0.06	0.43 ± 0.05	0.43 ± 0.08	0.49 ± 0.09	0.47 ± 0.07	0.009	0.646
Stearic Acid (C18:0)	7.86 ± 1.02	8.502 ± 0.84	7.99 ± 1.12	7.79 ± 1.15	6.72 ± 1.29	6.68 ± 0.80	6.29 ± 1.37	6.43 ± 0.76	6.39 ± 0.78	7.25 ± 1.42	7.06 ± 1.32	6.90 ± 1.09	0.280	-
Oleic Acid (C18:1n9c)	16.19 ± 2.64	17.49 ± 2.16	15.54 ± 3.04	14.73 ± 2.36	13.08 ± 2.59	12.60 ± 1.62	13.38 ± 2.82	12.57 ± 1.88	13.32 ± 1.61	14.59 ± 2.83	13.99 ± 3.02	13.23 ± 2.46	0.064	-
Linolelaidic Acid (C18:2n6t)	0.12 ± 0.08	0.29 ± 0.07	0.35 ± 0.06	0.16 ± 0.09	0.33 ± 0.05	0.37 ± 0.03	0.15 ± 0.03	0.35 ± 0.04	0.37 ± 0.04	0.15 ± 0.07	0.33 ± 0.06	0.36 ± 0.04	0.001	0.968
Linoleic Acid (C18:2n6c) (LA)	1.30 ± 0.22	0.82 ± 0.21	0.86 ± 0.18	1.27 ± 0.28	0.47 ± 0.09	0.58 ± 0.05	1.37 ± 0.29	0.43 ± 0.07	0.56 ± 0.08	1.31 ± 0.28	0.55 ± 0.21	0.64 ± 0.17	0.001	0.972
α-Linolenic Acid (C18:3n3) (ALA)	0.29 ± 0.05	0.56 ± 0.05	0.67 ± 0.09	0.30 ± 0.14	0.53 ± 0.10	0.68 ± 0.08	0.22 ± 0.05	0.50 ± 0.08	0.69 ± 0.12	0.27 ± 0.10	0.53 ± 0.09	0.68 ± 0.10	0.001	0.973
CLA (c9t11)	0.51 ± 0.07	0.99 ± 0.21	1.07 ± 0.24	0.64 ± 0.17	1.51 ± 0.29	1.41 ± 0.21	0.55 ± 0.13	1.66 ± 0.25	1.41 ± 0.16	0.58 ± 0.15	1.44 ± 0.37	1.32 ± 0.25	0.001	0.956
CLA (c12t10)	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.10 ± 0.02	0.09 ± 0.02	0.09 ± 0.02	0.09 ± 0.02	0.08 ± 0.02	0.08 ± 0.02	0.09 ± 0.02	0.08 ± 0.02	0.08 ± 0.02	0.021	0.578
Eicosenoic Acid (C20:1)	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.03	0.05 ± 0.02	0.08 ± 0.03	0.10 ± 0.02	0.05 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.05 ± 0.02	0.08 ± 0.03	0.09 ± 0.03	0.001	0.842

Eicosatrienoic Acid (C20:3n6)	0.01 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.03	0.01 ± 0.01	0.02 ± 0.03	0.10 ± 0.06	0.00 ± 0.01	0.01 ± 0.02	0.001	0.979
Behenic Acid (C22:0)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.685	-
Erucic Acid (C22:1n9)	0.02 ± 0.01	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.02	0.00 ± 0.01	0.01 ± 0.01	0.05 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.001	0.888
Tricosanoic Acid (C23:0)	0.00 ± 0.00	0.01 ± 0.02	0.02 ± 0.03	0.00 ± 0.02	0.04 ± 0.03	0.05 ± 0.03	0.00 ± 0.00	0.06 ± 0.01	0.07 ± 0.01	0.00 ± 0.01	0.04 ± 0.03	0.05 ± 0.03	0.001	0.876
Arachidonic Acid (C20:4n6)	0.00 ± 0.00	0.02 ± 0.02	0.04 ± 0.03	0.01 ± 0.02	0.06 ± 0.02	0.07 ± 0.01	0.00 ± 0.00	0.07 ± 0.02	0.08 ± 0.01	0.00 ± 0.01	0.05 ± 0.03	0.06 ± 0.02	0.001	0.970
Saturated	47.82 ±4.75	49.96 ±6.58	50.21 ± 5.72	55.77 ± 9.51	49.58 ± 10.46	48.08 ± 4.07	54.34 ± 10.09	48.46 ± 4.43	47.73 ± 4.78	53.25 ± 9.38	49.25 ± 7.72	48.48 ± 4.90	0.093	-
Unsaturated	20.11 ± 3.01	22.00 ± 2.26	20.27 ± 3.38	19.26 ± 2.88	18.08 ± 3.36	17.66 ± 1.75	18.28 ± 3.80	17.95 ± 2.56	17.66 ± 2.18	19.11 ± 3.36	19.01 ± 3.31	18.31 ± 2.66	0.445	-
MUFA	17.80 ± 2.76	19.26 ± 2.19	17.21 ± 3.19	16.69 ± 2.63	15.08 ± 2.90	14.47 ± 1.65	15.74 ± 3.29	14.84 ± 2.18	14.45 ± 1.88	16.61 ± 3.03	16.03 ± 3.10	15.15 ± 2.51	0.080	-
PUFA	2.31 ± 0.29	2.74 ± 0.28	3.06 ± 0.40	2.57 ± 0.32	3.00 ± 0.53	3.19 ± 0.26	2.54 ± 0.52	3.10 ± 0.41	3.20 ± 0.32	2.50 ± 0.41	2.97 ± 0.46	3.16 ± 0.33	0.001	0.774
Short Chain C4-14	17.91 ± 2.01	19.83 ± 3.94	21.20 ± 2.74	22.43 ± 4.01	21.81 ± 5.00	21.33 ± 2.28	21.94 ± 3.83	20.27 ± 1.77	20.18 ± 1.95	21.11 ± 4.00	20.74 ± 3.89	20.87 ± 2.35	0.923	-
Medium Chain C15-17	23.59 ± 2.65	23.33 ± 2.92	22.61 ± 3.03	27.42 ± 4.82	22.92 ± 4.78	21.78 ± 1.76	28.35 ± 5.44	23.84 ± 2.52	23.07 ± 2.56	26.81 ± 5.02	23.37 ± 3.64	22.47 ± 2.49	0.005	0.691
Long Chain C18-24	26.43 ± 3.88	28.80 ± 2.98	26.67 ± 4.26	25.19 ± 3.71	22.93 ± 4.29	22.63 ± 2.38	22.34 ± 4.59	22.29 ± 2.96	22.14 ± 2.56	24.43 ± 4.44	24.16 ± 4.43	23.45 ± 3.55	0.444	-
Omega 3 (n3)	0.29 ± 0.05	0.56 ± 0.05	0.67 ± 0.09	0.30 ± 0.14	0.53 ± 0.10	0.68 ± 0.08	0.22 ± 0.05	0.50 ± 0.08	0.69 ± 0.12	0.27 ± 0.10	0.53 ± 0.09	0.68 ± 0.10	0.001	0.973
Omega 6 (n6)	1.43 ± 0.21	1.13 ± 0.17	1.25 ± 0.20	1.53 ± 0.23	0.87 ± 0.16	1.01 ± 0.08	1.55 ± 0.38	0.88 ± 0.11	1.13 ± 0.37	1.51 ± 0.30	0.94 ± 0.18	1.11 ± 0.27	0.001	0.948
Omega 9 (n9)	16.21 ± 2.65	17.50 ± 2.16	15.55 ± 3.04	14.76 ± 2.37	13.09 ± 2.5	12.61 ± 1.62	13.43 ± 2.83	12.58 ± 1.89	12.34 ± 1.61	14.62 ± 2.83	14.00 ± 3.03	13.24 ± 2.46	0.060	-
CLA	0.59 ± 0.07	1.06 ± 0.22	1.14 ± 0.25	0.75 ± 0.17	1.60 ± 0.30	1.50 ± 0.21	0.64 ± 0.15	1.74 ± 0.25	1.49 ± 0.16	0.67 ± 0.16	1.52 ± 0.38	1.41 ± 0.25	0.001	0.954
n3 + CLA	0.88 ± 0.09	1.62 ± 0.24	1.82 ± 0.32	1.04 ± 0.31	2.13 ± 0.39	2.18 ± 0.22	0.86 ± 0.19	2.24 ± 0.32	2.18 ± 0.20	0.94 ± 0.24	2.04 ± 0.41	2.09 ± 0.29	0.001	0.962
n3/n6	0.20 ± 0.04	0.51 ± 0.06	0.55 ± 0.08	0.21 ± 0.16	0.61 ± 0.03	0.67 ± 0.05	0.13 ± 0.01	0.58 ± 0.04	0.68 ± 0.04	0.18 ± 0.011	0.57 ± 0.06	0.64 ± 0.08	0.001	0.973
LA/ALA+CLA	1.48 ± 0.18	0.52 ± 0.16	0.49 ± 0.15	1.29 ± 0.31	0.22 ± 0.02	0.27 ± 0.03	1.60 ± 0.17	0.19 ± 0.02	0.25 ± 0.03	1.45 ± 0.27	0.29 ± 0.16	0.32 ± 0.13	0.001	0.998
n6/(n3+CLA)	1.63 ± 0.14	0.71 ± 0.14	0.71 ± 0.15	1.56 ± 0.33	0.41 ± 0.03	0.47 ± 0.05	1.96 ± 0.21	0.39 ± 0.03	0.47 ± 0.04	1.73 ± 0.31	0.48 ± 0.16	0.53 ± 0.13	0.001	0.998
Desaturase-Index:	0.33 ± 0.02	0.34 ± 0.03	0.32 ± 0.03	0.29 ± 0.01	0.30 ± 0.02	0.29 ± 0.02	0.28 ± 0.01	0.29 ± 0.02	0.29 ± 0.01	0.29 ± 0.03	0.31 ± 0.03	0.30 ± 0.02	0.031	0.536
Atherogenic index	2.65 ± 0.42	2.56 ± 0.54	2.90 ± 0.49	3.41 ± 0.28	3.48 ± 0.36	3.48 ± 0.39	3.62 ± 0.19	3.55 ± 0.29	3.50 ± 0.24	3.30 ± 0.49	3.27 ± 0.57	3.34 ± 0.45	0.654	-
Thrombogenic index	3.42 ± 0.38	2.97 ± 0.35	3.07 ± 0.26	4.08 ± 0.41	3.49 ± 0.24	3.29 ± 0.22	4.31 ± 0.24	3.63 ± 0.25	3.37 ± 0.22	4.00 ± 0.49	3.41 ± 0.38	3.27 ± 0.26	0.001	0.964

Statistical analysis was by repeated-measures ANOVA, and $\eta 2$ is the significance effect size. c = cis; t = trans.

2.4.3 Principal Component Analysis (PCA)

The similarity plot defined by principal components PC-1 and PC-2 showed a clear discrimination of samples according to both feeding system and stage of lactation (Figure 2.3). All samples from early lactation are located on the positive side of the plot, whereas mid and late lactation samples did not appear to cluster based on season but are very clearly separated according to feeding system. All TMR samples clustered on the positive side of the plot; however, the majority of GRS and CLV samples are located in the negative section. Early lactation samples were characterized by oleic acid and stearic acid, whereas mid and late lactation TMR samples are characterized by eicosatrienoic acid, erucic acid, palmitic acid, and linoleic acid. In contrast, however, mid and late lactation GRS and CLV samples are characterized closely by pentadecanoic acid, tridecanoic acid, tricosanoic acid, α-linolenic acid, CLA (C18:2 cis-9,trans-11), arachidonic acid, undecanoic acid, and linolelaidic acid content, which is in agreement with Table 2.6.

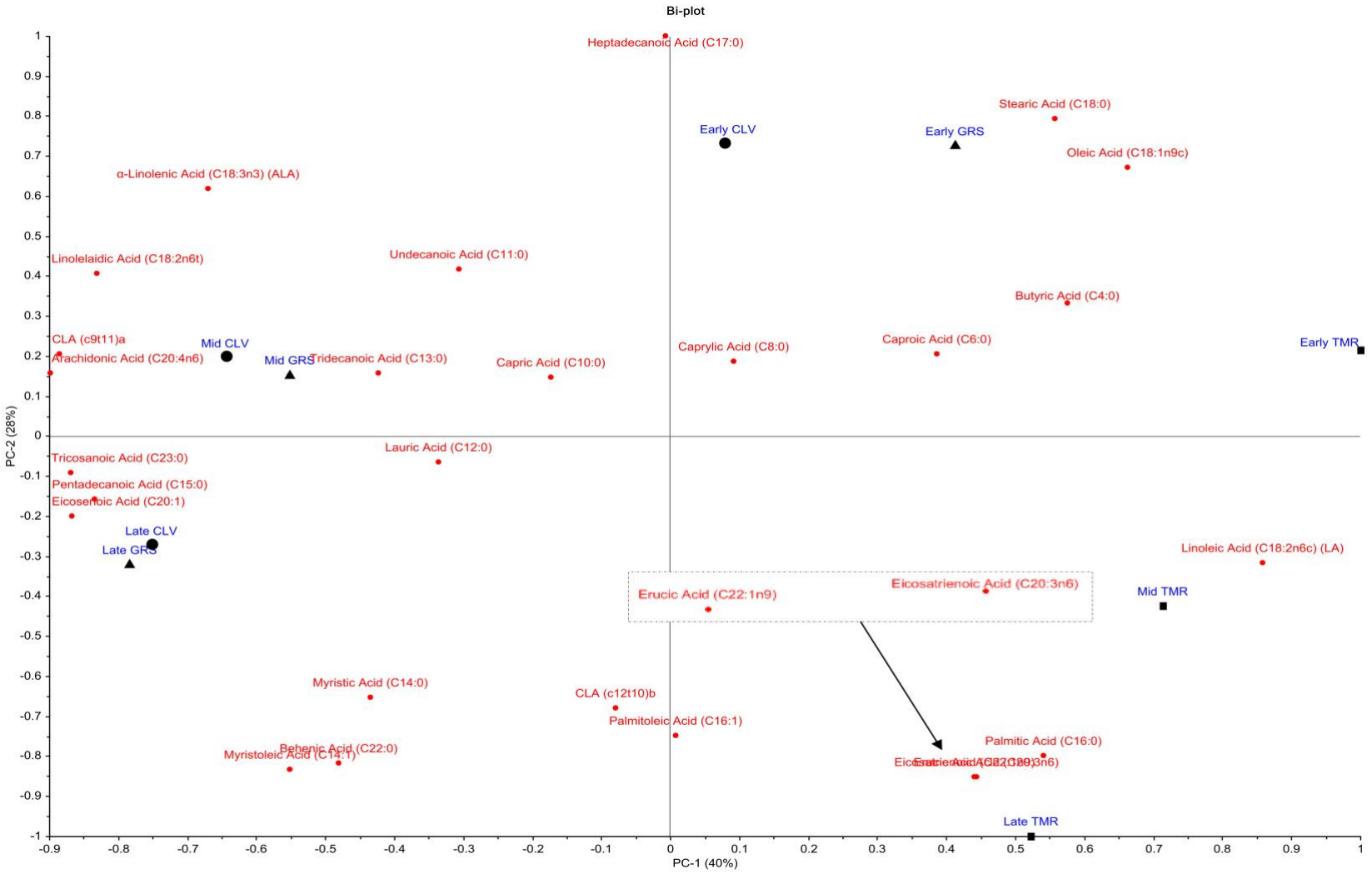


Figure 2.3. Principal coordinate analysis plot of mean raw milk FAME results throughout lactation, indicating clear separation of milks from pasture and TMR feeding systems (TMR (▲), perennial ryegrass (■), perennial ryegrass/white clover (●)), and stage of lactation (early, mid, and late). c = cis; t = trans

2.5 Discussion

Several studies in the past have reported that TMR and white clover diets are associated with increased milk yields from the cow when compared to pasture-only diets. Studies investigating the use of TMR feeding systems have reported an increased DMI and due to the high protein and dietary fat nature of a TMR diet, increased net energy intake has resulted in significantly greater milk yields than that of a pasture based system [28, 39-42]. In contrast, white clover perennial ryegrass has a lower resistance to chewing than other grasses with increased daily DMI (10-35 %) being reported [27]. This coupled with its high nutritive and feeding value, higher digestible protein and faster rate of passage through the rumen, means white clover feeding systems have resulted in increased milk yield, compared to perennial rye pastures not containing any clover [26, 43, 44]. With this in mind, the present study investigated the effects on milk composition from feeding cows a diet of TMR, perennial ryegrass only or perennial ryegrass/white clover throughout an entire lactation. Our data revealed similar trends for milk yield to those mentioned above (Table 2.4).

Seasonal variations in milk composition of pasture based systems have been well described in the past. Milk composition from each feeding system in this study all followed similar lactation trends to each other which are in agreement with those of previous studies conducted in both Ireland and New Zealand [32, 45] (Fig. 2.1). Concentrations of total solids and macronutrient components of milks (e.g. fat protein, casein, whey) were lowest in early lactation and increased as lactation progressed. This trend is likely due to a concentrating effect as a result of reduced milk yield as cows progressed from the mid to late stage of lactation [32]. The GRS milk had significantly higher yearly average total solids content than that of TMR and CLV milks which can be attributed to GRS's significantly higher fat and protein contents, indeed, among milk composition, fat and protein are the two components most subjected to change due to feeding system [46].

The GRS milk had significantly higher fat content than TMR and CLV feeding systems (4.65, 4.39 and 4.30 % fat, respectively) which is consistent with past studies where milk fat percentage from cows grazing pasture was increased compared to that of a TMR feeding system [28, 38, 39]. Reynolds [47] reported that grains such as maize used in TMR diets can provide a high proportion of starch for digestion in the small intestine leading to an increase in milk yield and a decrease in milk fat concentration. The CLV diet resulted in a reduced milk fat content which concurs with a study by Harris*, et al.* [26] where increased proportions of white clover in the diet increased daily milk yield and reduced the fat content of milk compared to cows consuming no clover. Similar studies in the past have found that use of a TMR feeding system can produce milk with higher fat contents [20, 48]. Feeding of TMR diets high in unsaturated FA has been linked with a reduction in milk fat content as unsaturated FA are toxic to many rumen bacteria, particularly those responsible for fiber degradation resulting in reduced activity of acetyl CoA carboxylase enzyme and *de novo* synthesis [49, 50]

Pasture based diets (GRS and CLV) had significantly higher yearly average protein content than that of the TMR diet (3.65, 3.56 and 3.38 % protein respectively), and possibly, more importantly, from a manufacturing perspective, true protein and casein content also followed this trend. Although Harris*, et al.* [24] found no significant difference in crude protein contents of milks from pasture with and without inclusion of white clover, there was a significant difference in the crude protein contents of GRS and CLV diets here, which could be attributed to increased milk yield associated with CLV system. Increased protein content of pasture milk over TMR milk has also been seen by Couvreur*, et al.* [20] who reported a linear increase in milk protein content with increasing pasture content of cows' diet. Couvreur*, et al.* [20] attributed the increase in protein content to a modification of energy provided to the udder by an increase in propionic acid supplied to the rumen from GRS diets. Dhiman*, et al.* [48] and Schroeder*, et al.* [51], however, have reported increased protein contents in milks derived from increased protein and energy intake with TMR feeding systems. In contrast,

studies have shown that protein content may be negatively influenced by a high intake of dietary fat [52].

The GRS milk was shown to have significantly higher quality protein with highest true protein content of total protein (Fig 2.2F). Significant differences in the true protein and casein contents of milks could be of concern to milk manufacturers as previous studies have attributed improved cheese making properties and rennet coagulation characteristics to milk with increased protein and casein concentration [1, 53]. It has been reported that a 0.1 % reduction in total casein concentration can cause a reduction in cheddar cheese yield potential by 0.5 kg/100 kg of milk [54], resulting in major losses for cheese manufacturers [53]. The CLV derived milk had a higher NPN concentration (0.04 %) than that of GRS or TMR which were the same (0.03 vs 0.03 % NPN) and CLV NCN concentrations were significantly higher than those of TMR milk (0.124 vs 0.112 % NCN). Such NPN and NCN results are similar to those of Harris*, et al.* [24] who reported that increased clover proportions in the diet resulted in higher urea concentrations which can account for up to 48 % of NPN content [55]. Increased proportions of NPN and NCN could be of concern to dairy manufacturers whose typical payment scheme is on a crude protein basis, with increased NPN and NCN resulting in poorer quality protein and potential reduction in product yields.

Milk fat is primarily composed of two major fractions; long chain FA (50 – 70 %) and short chain fatty acids (30 - 50 %). Long chain FA are typically derived from dietary system, short chain FA, however, are synthesized *de novo* by the mammary gland utilizing precursors such as acetate and butyrate [46]. There was no significant difference in total saturated FA content in each milk - pasture-derived milks had insignificantly lower amounts of saturated FA compared to TMR milk which correlates with a similar study by Baltušnikienė*, et al.* [56] comparing milk FA content from TMR and pasture diets. Lower saturated FA content would be a beneficial attribute for human health as consumption of saturated fat has been associated with a number of human diseases especially cardiac problems in the past [57]. However there is increasing

evidence available that dietary SFA, in the context of dairy foods, have a neutral or inverse association with cardiovascular disease [58] The pasture diets had significantly higher concentrations of undecanoic acid and pentadecaoic acid similar to results reported by Adler*, et al.* [59] who compared organic vs TMR farming systems effects on milk. Baltušnikienė*, et al.* [56] reported that cows consuming a TMR diet produced milk with higher levels of palmitic acid which was also observed in this study. Increased levels of linolelaidic and α-linolenic acid in pasture derived milks and increased linoleic acid content in TMR milks was also observed by Couvreur*, et al.* [20] when examining the effects of 100% corn silage diets versus 100% grass diet on milk composition. Two processes which contribute to the development of ischaemic heart disease include atherosclerosis and thrombosis, both of whose occurrence can be contributed to through consumption of dietary fats [60]. Alterations in the fatty acids of milks between feeding systems resulted in TMR derived milks having significantly higher thrombogenicity indices than those of pasture derived milks.

Omega-6 (*n*6) and omega-3 (*n*3) poly unsaturated fatty acids have been described as precursors to eicosanoids which are potent lipid mediating signaling molecules which play a role in regulation of inflammation. In general *n*6 derived eicosanoids are pro-inflammatory while *n*3 derived eicosanoids are anti-inflammatory [61]. The ratio of *n*3 to *n*6 fatty acids in dietary products has received much attention in recent years and there is increasing evidence that the dietary balance of *n*3 and *n*6 FA is perhaps as important as the dietary proportions of SFA, MUFA, PUFA and total fat etc., [35]. The nutritionally optimum intake ratio of *n*6 to *n*3 fatty acids for humans has been reported to be near 1-4:1; however in recent years the western diet has resulted in significant increases in *n*6 fatty acids to undesirable levels of as high as 15:1. Coinciding with this increased intake of *n*6 fatty acids in the western diet are increases in inflammatory related diseases, see review by Patterson*, et al.* [61]. TMR derived milks had significantly higher *n*6 fatty acid content than that of pasture derived milks while pasture derived milks *n*3 fatty acid content was significantly higher than that of TMR derived milk.

This data corroborates results reported by Benbrook*, et al.* [35], who performed a nationwide study of FA content of milks from conventional and organic dairy farms in the United States. The beneficial modulation of the level of *n*6 and *n*3 fatty acid content of milks from pasture based cows could be beneficial for combating this negative trend of high *n*6 and low *n*3 fatty acid intake in developed societies [35].

The bioactive isomer, CLA has been shown to exert potent physiological functions such as antihypertensive, antiobesity, antidiabetic and anti-carcinogenic properties [62]. Conjugated linoleic acid is formed in the rumen as an intermediate in the biohydrogenation pathway of linoleic acid to stearic acid by *Butyrivibrio fibrisolvens* [28, 56, 62]. Many studies in the past have reported the positive linear response of CLA concentration in cows milk to intake of fresh pasture [9, 19, 28, 48, 63]. We revealed that pasture fed cows in the current study produced greater than two-fold concentration of CLA_{c9t11} than that of TMR (1.38 vs 0.58 g/100g of milk fat respectively). This result is similar to that reported in past studies [9, 20, 28] and is a much higher increase than that reported by Baltušnikienė*, et al.* [56]. Milk fat CLA is affected by intake of unsaturated FA. In this respect, fresh-pasture associated increases in milk CLA content have been attributed to increased α-linolenic acid content in grasses which is extensively biohydrogenated in the rumen [64]. The GRS milk also had a higher desaturase index than TMR milk indicating increased activity of stearoyl CoA desaturase which is involved in CLA production [65], this result is also in agreement with results by Lock and Garnsworthy [66] which also suggested that fresh grass promotes the synthesis of CLA through an increase in Δ9-desaturase activity. Data from the current study revealed that there was greater variation in the CLA content of GRS and CLV samples throughout lactation, than that of TMR (standard deviations of 0.15, 0.37 and 0.25 g/ 100g of milk fat, respectively). Similar variations in CLA content have been reported in the past by Kelly*, et al.* [28].

Principal component analysis of average milks FA contents showed clear separation throughout entire lactation between TMR fed and pasture based milk. Overall, this analysis

shows that the FA composition of milks from TMR diets and pasture-based diets were quite distinct, whereas the GRS and CLV pasture diets were much less differentiated. Similarly, early lactation milks were very different to mid and late lactation samples, however, there was little distinction between mid and late lactation samples within TMR and pasture feeding systems, indicating that FA profiling could be used as a tool for verification of pasture derived feeding systems over TMR systems. This result is in agreement with results reported by Capuano*, et al.* [67] which concluded that fatty acid profiling may be used for the verification of fresh grass feeding of cows.

2.6 Conclusion

The novelty of this study was the real-time comparison of three distinct feeding systems widely practised throughout the world on dairy cows over a full lactation period. Observed variations in milk composition could be linked to both stage of lactation and feeding system utilized. In conclusion, pasture-based feeding systems have been shown to produce milk with increased concentrations of fat and protein. Moreover, the GRS feeding systems produced milks with better quality protein with increased true protein concentrations. The use of a TMR feeding system resulted in significant decreases in protein, fat, casein and whey concentrations. The inclusion of CLV appeared to produce milk with more comparable compositional concentrations to that of GRS. The feeding system utilised also had a direct effect on milk fatty acid composition. GRS feeding appeared to beneficially alter the nutritional status of milks with greater than two fold increases in total concentration of CLA particularly the health benefitting isomer CLA_{c9t11} offering further confirmation to previous studies that revealed an association between increased milk CLA and fresh grass feeding. Pasture feeding systems resulted in significantly higher contents of Omega 3 fatty acids and significantly lower contents of Omega 6 fatty acids than that of TMR milk which also had a significantly higher thrombogenic index than that of pasture derived milks. Finally, this study further indicated the possibility of fatty acid profiling of milk for verification of fresh pasture feeding systems over that of TMR systems.

2.7 Acknowledgements

This publication has emanated from research conducted with the financial support of Science Foundation Ireland (SFI) under Grant Number SFI/12/RC/2273, Teagasc and the Dairy Levy Fund administered by Dairy Research Ireland. Tom F. O'Callaghan is the recipient of a Teagasc Walsh Fellowship grant. The valuable input of Elaine Patterson and David Mannion is gratefully acknowledged and Jimmy Flynn (Teagasc Moorepark, Ireland) for technical assistance over the course of the experiment. The input of Sean Lacey (Department of Mathematics, Cork Institute of Technology, Ireland) for statistical analysis is very much appreciated. The authors sincerely thank the technical and farm staff at Moorepark for their excellent care of the experimental cows and assistance during the experiment

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Chapter 3

Quality characteristics, chemical composition and sensory properties of butter from cows on pasture versus indoor feeding systems

Published in *Journal of Dairy Science* 2016, 99:9441-9460

"Editor's Choice" Article, December, 2016

DOI:10.3168/jds.2016-11271

3.1 Abstract

This study evaluated the effects of three widely-practiced cow feeding systems, in the US, Europe and southern hemisphere regions, on the characteristics, quality and consumer perception of sweet cream butter. Fifty-four multiparous and primiparous Friesian cows were divided into three groups (n=18) for an entire lactation. Group 1 was housed indoors and fed a total mixed ration diet (TMR) of grass silage, maize silage and concentrates, Group 2 was maintained outdoors on perennial ryegrass only pasture (GRS), while Group 3 was also maintained outdoors on a perennial ryegrass/white clover pasture (CLV). Mid lactation butter was manufactured in triplicate with milk from each group in June 2015 (137 ± 7 days in milk) and was analyzed over a six-month storage period at 5 °C for textural and thermal properties, fatty acid composition, sensory properties and volatile compounds. The nutritional value of butters was improved by pasture feeding; having significantly lower thromboenecity index scores than that of TMR butters. With this, pasture-derived milks (GRS and CLV) produced butter with significantly higher concentrations of CLA (c9t11) and trans β-carotene than that of TMR. Alterations in the fatty acid composition of butter contributed to significant differences in textural and thermal properties of the butters. Total mixed ration derived butters had significantly higher hardness scores at room temperature than those of GRS and CLV. Onset of crystalisation (T-onset) for TMR butters also occurred at significantly higher temperatures than pasture butters. Volatile analysis of butter by GC-MS identified 25 compounds present in each of the butters, five of which differed significantly based on feeding system including acetone, 2-butanone, 1-pentenol, toluene and β-pinene. Toluene was very significantly correlated with pasture-derived butter. Sensory analysis revealed significantly higher scores for GRS-derived butter in several attributes including 'liking' of appearance, flavour and colour over that of TMR butter. Partial least square regression plots of fatty acid profiles showed clear separation of butter derived from grazed pasture-based diets (GRS and

CLV) from that of a TMR system, offering further insight into the ability of fatty acid profiling to verify such pasture-derived dairy products.

3.2 Introduction

The consumption of milk fat has in the past been a concern for consumers compared to spread and margarine alternatives, due to its high levels of saturated fatty acids (SFA) - whose intake has been linked with high cholesterol, atherosclerosis, and heart disease [1, 2]. However, recent reviews and meta-analysis of the topic have concluded that there is at least a neutral effect of milk intake on multiple health outcomes, and alternatively, cows' milk consumption may be beneficial in combating osteoporosis, cardiovascular disease, stroke, type 2 diabetes, and some cancers [3, 4].

The different farming and feeding systems of dairy cows practiced throughout the world are dictated by a number of factors including land availability, climate and dairy cow requirements. It is widely understood that the feeding system of dairy cows can have a direct impact on the composition of milk, particularly the fatty acid (FA) composition of milk fat [5]. The resulting milk FA profile can in-turn, have profound effects on the sensory, textural, nutritional and shelf life properties of dairy fat products such as butter [6, 7]. Indeed, the level of saturated fatty acids (SFA) and unsaturated fatty acids (UFA) in milk fat is closely dependent on the nature of the cows diet [8]. Fresh grass feeding systems, which are widely practiced in Ireland and New Zealand, produce a milk fat with higher proportions of unsaturated FA compared to those derived from total mixed ration (TMR) systems [7] widely practiced in the US, Asia and parts of Europe. Previous studies have shown that the texture and hardness of butter is related to the levels of saturated and unsaturated fatty acids, where the lower melting point of unsaturated FA produces a less firm and more spreadable butter [9]. Feeding system can also have an effect on the natural color of products, maize silage and TMR diets have been shown to produce dairy products that are much whiter in colour than those of pasture feeding systems which have a characteristic yellow color [10]. With this in mind, it has been reported that there is a consumer perception of the spreadability of butters associated with their colour [11].

Consumer acceptability of butter is influenced by its sensory properties, which are dependent on several factors including flavor, aroma, textural, appearance and rheological factors. The volatile composition of butter has been well studied with over 287 volatile compounds being identified, [12]. Feeding regimen has been shown to have an effect on the sensory and volatile properties of cows' milk, with milk from TMR systems reportedly having considerably different flavor profiles than pasture-derived milk [13].

The benefits of including white clover in pasture swards has been documented and is associated with increased milk production [14]. However, there is limited information available however for the effects of inclusion of white clover with perennial rye pastures on the characteristics and sensory properties of dairy products. There is at present a consumer perception that dairy products from cows maintained outdoors consuming fresh grass is "more natural" than that of TMR farming systems [15]. This perception has become a major marketing scheme for countries such as Ireland and New Zealand, practicing fresh grass feeding, for promotion of dairy products. There is however, little information available to substantiate this notion at present or for the verification of dairy products derived from fresh grass feeding systems. While many studies have been conducted in this area in the past, many of these have been from non-continuous, cross over designs. There is limited information available from studies using a larger herd size and cows which have been maintained on their respective diets throughout an entire lactation, in a commercial farm setting, utilizing larger bulk milk volumes, which would provide a better reflection of milks being provided to the dairy manufacturer.

The objective of this study was to investigate the effect of three widely practiced feeding systems of a TMR diet indoors, perennial ryegrass (*Lolium perenne* L.) outdoors (GRS), and perennial ryegrass/white clover (*Trifolium repens* L.) outdoors (CLV) on the chemical composition, sensory properties and quality of mid lactation sweet cream butter, and

investigate potential attributes that may be used for the verification of such pasture derived dairy products from those of a TMR system.

3.3 Materials and Methods

3.3.1 Reagents

Hexane, heptane, formic acid, 25 % sodium methoxide, valeric acid ($C_{5:0}$), undecylic acid ($C_{11:0}$) and margaric acid ($C_{17:0}$) were purchased from Sigma Aldrich (Dublin, Ireland). Diethyl ether was purchased from Fisher Scientific (Dublin, Ireland). Certified free fatty acid (FFA) standard (STD) mix containing $C_{4:0}$-$C_{22:0}$ free acids (GLC Reference standard 74 "Free acid"), Trinonadecanoin ($C_{19:0}$) (part number: T-165) and a standard mix of conjugated linoleic acid C18:2,c9t11 and C18:2,c12t10 (part number: UC-59M) were purchased from Nu-Chek-prep, Inc. (Minnesota, USA). Fatty acid methyl ester (FAME) standard mix containing C4:0-C24:0 methyl esters (part no: 18919-1AMP) was purchased from Sigma Aldrich (Dublin, Ireland). 500 mg aminopropyl cartridges (part no. 12102041) were obtained from Agilent technologies (Little Island, Cork, Ireland).

3.3.2 Experimental Design

Fifty-four spring calving Friesian cows were allocated to three groups (n=18) at the Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland. Groups were randomized based on milk yield, milk solids yield, calving date (mean calving date 19th February 2015) and lactation number. Group 1 was housed indoors and fed a TMR diet, Group 2 was maintained outdoors on perennial ryegrass only pasture (GRS) while Group 3 was also maintained outdoors on a perennial ryegrass/white clover pasture (CLV). For further information on the chemical and nutritional values of each of the diets see O'Callaghan, *et al.* [16] and supplementary tables 3.1, 3.2 and 3.3. Briefly, the TMR diet consisted of, on a dry matter basis (DM) 7.15 kg of grass silage, 7.15 kg of maize silage and 8.3 kg concentrates. Cows within the TMR system were fed at 08:30 h daily into electronically

controlled Griffith Elder Mealmaster individual feed bins (Griffith Elder and Company Ltd, Suffolk, England) and was available *ad-libitum*. Pasture based cows consumed ~ 18 kg DM/day measured by pre and post grazing sward heights daily using the rising plate meter (Jenquip, Fielding, New Zealand) while pre grazing herbage mass was measured with an Etesia mower (Etesia UK Ltd, Warwick, UK). CLV sward contained 20 % clover and was measured according to Egan*, et al.* [17]. Milking took place at 07:30 and 15:30 h daily. In order to obtain a representative sample of milk, the cows in each of the three feeding systems were milked separately into designated 5000 L refrigerated tanks. The evening milk was stored at 4 °C overnight, to which the morning milk was then added, tanks were maintained at 4 °C and agitated prior to sample collection. Milk was collected from each of the groups in the trial for butter manufacture on three separate occasions over a three week period in mid-June 2015, when cows were 137 ± 7 days in milk, each batch of butter were produced 7 days apart, and each of the butters within each batch were manufactured on the same day.

3.3.3 Sample Collection and Butter Manufacture

Butter making trials were performed in Moorepark Technology Ltd. (MTL, Moorepark, Fermoy Co. Cork, Ireland) with milk from each of the three groups in the study. For each batch, 400-450 L of milk from each group was pasteurized using a Unison pasteurizer (Unison Engineering Ltd, Limerick, Ireland) at 72 °C for 15 s, skimmed at 50 °C using a cream separator (Westfalia separator d-4740, GEA, Naas, Ireland) and standardized to produce a cream containing 38-40 % fat content. Pearson's square was used to assess the amount of skim milk required to obtain a desired cream fat content. Cream was then stored for 72 h at 5 °C in sealed containers to facilitate crystallization of milk fat.

The evening before cream processing, the butter churn was washed with hot water and stored in a Super-Sil detergent solution (Biocel Ltd, Little Island, Cork, Ireland) overnight and rinsed with chilled reverse osmosis treated water (RO) prior to cream processing.

For butter manufacture, 4.5–5.0 kg of cream was churned at 7 °C in a 19 °C room using a "Milky" Butter churn model FJ-10 (Sebright Supplies Limited, Buckinghamshire, England). Cream was churned at 150 RPM until the "break" was observed and butter grains were formed. Buttermilk was separated from the butter grains and butter was washed three times with cold RO water (~10 °C) in the churn until drained water was clear and free from traces of butter milk. Washed butter was then weighed and 2 % salt was added in 40-50 % salt slurry. The salt slurry was poured over the washed butter and mixed in by kneading the butter at 60 RPM. The butter was then worked at 30 RPM for several minutes to reduce free water content. Butter was packaged by hand using a spatula into 100 ml containers (Sarstedt, Co. Wexford, Ireland).

Pots of butter were then stored at 5 °C over a 6 month period, during this period they were analyzed for chemical composition, thermal and textural properties, FA composition, sensory properties and analysis of volatile compounds by GC-MS.

All analyses of butters from individual batches unless otherwise stated were analyzed in duplicate and results reported are the mean and standard deviation (S.D.) of results.

3.3.4 Chemical Analysis

Total solids content of butter samples was measured by recording the weight lost from samples (initial weight of 10 ± 0.5 g) post drying in an oven at 102 °C for at least 15 h. Fat content of cream and butter samples was analysed by the Rose Gotleib method [18].

3.3.5 Butter Hardness

Hardness of butter from each of the feeding systems was analyzed using a TA-HDi texture analyzer (Stable Micro Systems Ltd, Surrey, UK) equipped with a 100 kg load cell and a 30° conical probe in a room maintained at 20 °C. Triplicate butter sample molds were prepared for hardness analysis at both 5 °C and 20 °C using a cylindrical aluminum press, with a 3 cm diameter and 3 cm height, samples were removed from the mold and wrapped in aluminum foil and placed in a 5 °C storage room prior to analysis. An extra sample was prepared and used as a temperature reference block with a digital thermometer inserted in its center. Analysis

was performed by lowering a 30° conical probe perpendicularly, at 1 mm/s to a depth of 12 mm from the surface of the sample, as described by Bobe*, et al.* [19]. One measurement per sample was performed. Hardness values for each sample were determined as the highest peak force recorded during each analysis.

Samples for analysis at 20 °C were placed in a temperature controlled room and left to acclimatize (~2 h) as indicated by the reference butter. Samples for analysis at 5 °C were removed from refrigerated storage immediately prior to analysis. For each butter at any given sampling time there were samples for analysis at both 5 °C and 20 °C, where the temperature of each butter sample was also measured immediately post analysis. Texture analysis was performed on butter after 1 month (M), 3 M and 6 M of storage.

3.3.6 Colour

Colour measurements were taken from the surface of newly opened cups of butter following 1 w, 1 M, 3 M and 6 M of storage at 4 °C. Five replications of L*, A* and B* values were taken at random locations across the surface of the butters using a Minolta Chroma-Meter CR-400 (Mason Technology Ltd, Dublin, Ireland). L* value defines the position of the sample on the lightness-darkness axis, A*on the green-red axis and B* on the blue-yellow axis as described by Lighting International Commission [20]. The mean of 5 replications was calculated and used as a unit for 1 replicate butter trial in statistical analysis.

3.3.7 β-Carotene Analysis

Butter samples were analyzed in triplicate for *trans* β-carotene content by Eurofins Food Testing Ireland Ltd (Dublin, Ireland). Trans-β-carotene was saponified using ethanolic potassium hydroxide solution for 16 h at room temperature and extracted once with EtOH:hexane (4:3 v/v) and two times with hexane. Trans β-Carotene analysis was performed by reverse phase high performance liquid chromatography (RP-HPLC) with an ultraviolet diode array detector (UV/DAD) at 452 nm. For quantification, a 3-point calibration curve was used. The calibration standards used were pure compounds from Sigma Aldrich (Dublin,

Ireland), purity > 98 %. The purity of the standard for each calibration was determined by a series of spectrophotometric measurements (UV 340/455/483 nm).

3.3.8 Thermal Analysis

Thermal properties of butters produced from each feeding system were examined by differential scanning calorimetry (DSC) using a TA Q2000 calorimeter (Waters Chromatography, Dublin, Ireland). Thirty milligrams of butter melted at 40 °C was weighed into a hermetic Tzero aluminum pan (Waters Chromatography, Dublin, Ireland) and press sealed. An empty hermetic pan was used as reference. For analysis, samples were equilibrated to 25 °C for 1 min, heated at 20 °C/min to 60 °C and held for 5 min to completely melt the fat and eliminate all crystal nuclei, cooled at 5 °C/min from 60 to -10 °C and then heated at 2 °C/min to a final temperature of 60 °C. Data from thermal analysis were analyzed using TA Universal Analysis 2000 program v4.5A (TA Instruments- Waters LLC). Cooling profiles were analyzed for temperature at beginning of fat crystalisation (T-onset) and each crystalisation peak. Final melting temperatures (T-offset) were recorded from each sample heating profile as outlined by Couvreur*, et al.* [7]. Each sample was analyzed in duplicate and results shown are the mean and S.D. of each set of analysis.

3.3.9 Fatty Acid Analysis

Lipid Extraction. Lipid extraction was performed as per the procedure outlined by De Jong and Badings [21] with modifications. Briefly, 5 g of butter was added to 10 mls of ethanol (98 % purity), 1 ml of 2.5 M H_2SO_4 and 1 ml of internal free acid standard (ISTD) ($C_{5:0}$, $C_{11:0}$,$C_{17:0}$ at 1000 ppm in heptane) were added to each sample mixture. This mixture was extracted three times with 15 ml diethyl ether/heptane (1:1 v/v) and each time the solution was clarified by centrifugation at 1500 g × 5 min. The collected extracts were pooled for solid phase extraction.

Solid Phase Extraction. The 500 mg aminopropyl columns (Agilent Technologies Ltd, Cork Ireland) were pre-conditioned with 10 ml heptane. The lipid extract was applied to the

column, a vacuum was applied and the triglycerides were eluted. Residual triglycerides were removed using 10 ml of 20 % diethyl ether/hexane (v/v) wash step and the entire triglyceride extract was stored at -20 °C. The FFA were eluted using 5 ml 2 % formic acid/diethyl ether (v/v) in glass test tubes. At no point were the columns left to dry. The entire FFA extract was immediately separated and stored in 2 ml amber vials (Part no: 5182-0716, Agilent Technologies Ltd) which were capped with PTFE/white silicone septa (part no: 5185-5864, Agilent Technologies Ltd). For FFA analysis, 0.5 µl of this solution was directly injected (on-column injection) on the GC. For FAME analysis, samples of extracted triglyceride fractions were dried down under nitrogen and 60 mg of extracted fat was methylated.

Methyl Ester Derivitisation of Triglycerides. Triglycerides were derivitised by adding 4.8 ml of C19:0 TAG (500 mg/L) in heptane to ~60 mg of extracted fat sample - 200 µl of 2 M sodium methoxide solution was then added and sample was mixed vigorously for about 30 s. Subsequently, 1 g of sodium hydrogen sulfate monohydrate (Sigma Aldrich, Dublin, Ireland) was added to the solution and shaken vigorously. After the salt had settled, the upper layer containing the methyl esters was decanted into a clean test tube, and diluted with 8 ml of heptane. Methylated FAME were stored at -20 °C prior to analysis in 2 ml amber vials which were capped with PTFE/white silicone septa.

Instrumentation. Free fatty acids analysis was performed on a Varian CP3800 gas chromatograph (Aquilant, Dublin 22, Ireland) equipped with a CP8400 autosampler and flame ionisation detector and a 1079 universal capillary injector. The column was a Zebron™ ZB-FFAP capillary column (30 m x 0.32 µm I.D., 0.25 µm phase thickness) (Part no: 7HM-G009-11, Phenomenex Inc, Cheshire, UK). Total FA triglyceride analysis (FAME) was carried out on an Agilent 7890B gas chromatograph (equipped with a GC80 autosampler), and flame ionization detector and a multimode inlet injector (Agilent Technologies Ltd, Little Island, Cork, Ireland). The column was a Select FAME capillary column (100 m × 250 µm I.D., 0.25 µm phase thickness) (product no. CP7420, Agilent Technologies Ltd).

Solid phase extraction was performed using Vac Elut 20 Manifold and adaptor caps (parts no. 12234104 & 12131001) from Agilent Technologies Ltd and a KNF vacuum pump (Scientific & Chemical Supplies Ltd, Carrigtwohill, Co. Cork, Ireland).

Standard curves for both FFA and FAME analysis, along with in-run quality control samples were prepared using Agilent 7696A Sample Prep Workbench (Agilent Technologies Ltd).

Instrument Conditions for Analysis of FFA's. The injector was maintained at 25 °C for 1 min - this was raised to 240 °C at 30 °C/min. The injector and auto-sampler were operated in on-column mode using an injection volume of 0.5 µl. The column oven was held at 40 °C for 2 min and raised to 240 °C at 7.5 °C/ min, this was held for 23.33 min. The total runtime was 52 min. The FID was operated at 300 °C. The carrier gas was helium and was held at a constant flow of 1.2 ml/min.

Instrument Conditions for Analysis of FAME. The injector was held at 250 °C for the entire run and was operated in split mode using a ratio of 1:10 and an injection volume of 1µl. The inlet liner was a split gooseneck liner (Part no.: 8004-0164, Agilent Technologies Ltd). The column oven was held at 80 °C for 8 min and raised to 200 °C at 8.5 °C/min, and held for 55 min. The total runtime was 77.12 min. The FID was operated at 300 °C. The carrier gas was helium and was held at a constant flow of 1.0 ml/min. Results were processed using OpenLab CDS Chemstation edition software version Rev.C.01.05 (Agilent Technologies Ltd.)

3.3.10 Analysis of volatile compounds by GC-MS

For analysis of volatile compounds in butter samples, 2 g of the butter was placed in a 20 ml screw capped headspace vial with a silicone/PTFE liner (Apex Scientific Ltd., Maynooth, Co.Kildare, Ireland) and equilibrated to 40 °C for 10 min with pulsed agitation of 5 s at 500 rpm. The samples were analysed in triplicate using a Shimadzu AOC-5000 Plus injection system (Shimadzu UK Ltd, Milton Keynes, UK). A single 75 µm divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber was used for

analysis. The SPME fiber was exposed to the headspace above the samples for 20 min at a depth of 1.2 cm. The fibre was retracted and injected into the GC inlet and desorbed for 2 min at 250 °C. Injections were made on a Shimadzu 2010 plus GC with an Agilent DB-5 (60 m × 0.25 mm × 0.25 µm) column using a multipurpose injector with a merlin microseal. The temperature of the column oven was set at 35 °C, then increased at 6.5 °C/min to 230 °C, and further increased at 15 °C/min to 320 °C, yielding a total GC run time of 41.5 min. The carrier was helium at a constant pressure of 23 psi. The detector was a Shimadzu TQ8030 MSD triple quadrupole mass spectrometer (Shimadzu UK Ltd.) and was used in single quadrupole mode. The ion source temperature was 220 °C and interface temperature was set at 280 °C and the MS mode was electronic ionization (-70v) with the mass range scanned between 35 and 250 amu. Data files were processed using Targetview™ software (v4.0.0.18, Markes International, Llantrianst, U.K.) and compounds were identified based on match spectra matches against those in the NIST 2011 database, and an internal library created on Targetview based on linear retention indices (LRI) using the van den Dool and Kratz procedure [22]. An auto-tune of the GCMS was performed prior to the analysis to ensure optimal GCMS performance. A set of external standards was also analysed at the start and end of the sample set and abundances were compared to known amounts to ensure that both the SPME extraction and MS detection was performing within specification.

3.3.11 Hedonic sensory analysis and ranking descriptive analysis of butters

Butter samples for sensory analysis were aged at 5 °C for 48 h and then stored frozen at -80 °C prior to analysis date. Twenty-six naïve assessors aged between 23-50 years old were recruited in University College Cork, Ireland. Inclusion criteria for assessors were availability, good health, motivation to participate on all days of the experiment and that they were butter consumers. Sensory acceptance testing was conducted using these untrained assessors [23, 24]. The experiment was conducted in panel booths, in a fluorescent lighted room which

conformed to International Standards [25]. Assessors used the sensory hedonic descriptors provided to them, for three different butter samples (TMR, GRS and CLV) (see supplementary Table 3.4), presented in duplicate and from two separate production batches. Samples were held at refrigeration temperatures overnight (5 ºC), before monadic presentation to the naïve assessor panel at ambient temperatures (~21 °C) and coded with a randomly selected 3 digit code. The butter was immediately served to assessors. Each assessor was provided with deionized water and instructed to cleanse their palates between tastings. Additionally, each assessor was asked to indicate their degree of liking on a 10 cm line scale ranging from 0 (extremely dislike) at the left to 10 (extremely like) at the right and rating subsequently scored in cm from left. The order of the presentation of all test samples was randomized to prevent first order and carryover effects and all samples were presented in duplicate. The assessors then participated in ranking descriptive analysis (RDA) [26-28] using the consensus list of sensory descriptors provided to them which was also measured on a 10 cm line scale. All samples were again presented in duplicate [29].

3.3.12 Nutritional indices and Fatty acid ratios.

Several FA's ratios and nutritional indices of milks from each of the feeding systems are reported. The summation of omega 6 (*n*6; linolelaidic acid, linoleic acid (LA), eicosatrienoic acid and arachidonic acid) omega 3 (*n*3; α-linolenic acid (ALA)) and omega 9 (*n*9; oleic acid and erucic acid) are reported. Other larger chain *n*3 fatty acids include eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been found in milk at low concentrations, but were not detected in our analysis. Similar to Benbrook*, et al.* [30], to more fully reflect variations in levels of health promoting dairy FA's we have also included total *n*3 and CLA and as Benbrook*, et al.* [30] described with this in mind, we also include the ratio of *n*6 fatty acids to *n*3 + total CLA to fully reflect these variations . The atherogenecity index (AI) and thrombogenecity index's (TI) outlined by Ulbricht and Southgate [1] are a dietary risk indices for cardiovascular disease. AI indicates the relationship between fatty acids with pro-

atherogenic and those with anti atherogenic properties, showing the inhibition of aggregation of plague and diminishing the levels of esterified FA, cholesterol and phospholipids. While TI shows the relationship between pro-thrombogenic (saturated) and anti-thrombogenic fatty acids; showing the tendency to form clots in the blood [31].

Atherogenecity index (AI) and thrombogenicity index (TI) have been calculated as described by Ulbricht and Southgate [1];

$$\text{AI} = \frac{C12{:}0+(4\ x\ C14{:}0)+C16{:}0}{n6\ \text{PUFA}+n3\ \text{PUFA}+\text{MUFA}}$$

$$\text{TI} = \frac{C14{:}0+C16{:}0+C18{:}0}{(0.5\ \text{x MUFA})+(0.5\ x\ n6PUFA)+(3\ x\ n3\ PUFA)+(\frac{n3PUFA}{n6\ PUFA})}$$

3.3.13 Statistical Analysis

Statistical analysis was performed using SPSS v18.0 (IBM Statistics Inc., Armonk, NY, USA). Datasets were analyzed for normality using the Shapiro-Wilk's test and for homogeneity of variance using the Levene's test.

Analysis which was carried out at only one time point and were normally distributed were analyzed using one-way ANOVA (β-Carotene, FAME, FFA, thermal properties, sensory analysis) with post hoc Tukey test. For data whose distribution appeared to be non-normal, a Kruskall–Wallis test was performed (Volatile analysis). If the Kruskall–Wallis test result was significant, post-hoc Mann–Whitney test was used to analyze for differences between individual groups and significant results were adjusted using the Bonferrroni correction.

Datasets from analysis which was carried out at several time points throughout the study (colour analysis and texture analysis) a Between- and Within-Subjects repeated measures ANOVA with post hoc Tukey test was used to compare butters from herds on different feeding systems (TMR, GRS and CLV) throughout storage period (6 months).

Multivariate data analysis (partial least squares regression; PLSR) was applied to investigate relationships between FAME, sensory analysis and volatiles analysis data and the

experimental treatments (different feeding regimes) using The Unscrambler® X multivariate analysis program, v10.3 (CAMO ASA, Trondheim, Norway).

P-values < 0.05 were considered significant, results of statistical tests with *P* values of 0.000 are represented as *P* < 0.001.

3.4 Results

Raw milks from each of the feeding systems were separated to produce creams with similar (*P* > 0.05) fat contents (mean ± S.D.) of 40.29 ± 2.35 % for TMR, 40.64 ± 1.08 % for GRS and 43.29 ± 2.32 % for CLV. Churning times varied between creams; 45.9 ± 8.9 min for TMR, 40.9 ± 0.5 min for GRS and 41.2 ± 4.9 min for CLV creams. Butters from each of the feeding systems had a mean fat and moisture content of 83.60 ± 0.67 % and 13.92 ± 0.53 % for TMR, 83.27 ± 0.80 % and 14.19 ± 1.12 % for GRS and 83.00 ± 1.06 % and 13.08 ± 1.8% for CLV, which were not significantly different.

3.4.1 Fatty Acid Composition

A total of 26 FA were quantified (g/100 g of butter fat) from each butter sample. Overall, 12 of these FA varied significantly (P ≤ 0.05) between feeding systems. A full list of butter FA contents (mean ± SD) is displayed in Table 3.1. Among SFA, significant differences between butters were recorded for pentadecanoic acid (C15:0), palmitic acid (C16:0), and tricosanoic acid (C23:0). Pentadecanoic acid was significantly higher (*P* = 0.023) in GRS butter than in TMR butter. The TMR butter had the highest palmitic acid content, which was greater (*P* = 0.046) than that of the GRS butter and significantly (*P* = 0.002) more than that of the CLV butter. The GRS and CLV butters had higher concentrations of tricosanoic (*P* = < 0.001) acid than TMR butter, which was devoid of the FA.

Among MUFA, eicosenoic acid (C20:1 cis-11) and erucic acid (C22:1n-9) varied significantly (*P* = < 0.05) between feeding system. Eicosenoic acid was present at the greatest

concentrations in GRS and CLV butters but was almost absent ($P = < 0.001$) in TMR samples. Erucic acid content was highest ($P = 0.012$) in TMR samples.

Significant ($P = <0.05$) differences in PUFA were also recorded between feeding systems for linolelaidic acid (C18:2n-6 trans), linoleic acid (C18:2n-6 cis), α-linolenic acid (C18:3n-3), eicosatrienoic acid (C20:3n6 cis8,11,14), CLA (C18:2 cis-9,trans-11), and arachidonic acid (C20:4n-6 cis-11,-14,-17). Linolelaidic acid was highest in pasture-derived butters, significantly ($P = < 0.001$) higher than in TMR butter. Linoleic acid was highest in TMR butter, with a greater than 2-fold increase ($P = < 0.001$) in content compared with GRS and CLV concentrations. Pasture derived butters had significantly higher α-Linolenic acid content than TMR ($P = < 0.001$). α-Linolenic acid was highest in CLV butters, significantly higher than GRS ($P = < 0.001$) and TMR ($P \leq 0.001$) butters.

Table 3.1. Relationship between cow feeding system and the fatty acid triglyceride content of butter. Mean and SD of FAME as g/100 g of butter fat of butters derived from cows on different feeding systems.

	Feeding System			
	TMR	**GRS**	**CLV**	***P*-value**
Butyric Acid ($C_{4:0}$)	3.81 ± 0.35	3.54 ± 0.29	3.33 ± 0.28	0.070
Caproic Acid ($C_{6:0}$)	1.98 ± 0.19	1.86 ± 0.18	1.72 ± 0.12	0.073
Caprylic Acid ($C_{8:0}$)	1.06 ± 0.10	1.04 ± 0.13	0.97 ± 0.07	0.371
Capric Acid ($C_{10:0}$)	2.30 ± 0.23	2.37 ± 0.35	2.20 ± 0.19	0.625
Undecanoic Acid ($C_{11:0}$)	0.04 ± 0.01	0.05 ± 0.01	0.04 ± 0.00	0.442
Lauric Acid ($C_{12:0}$)	2.59 ± 0.27	2.74 ± 0.47	2.52 ± 0.25	0.615
Tridecanoic Acid ($C_{13:0}$)	0.07 ± 0.01	0.08 ± 0.02	0.06 ± 0.01	0.069
Myristic Acid ($C_{14:0}$)	8.36 ± 0.83	8.36 ± 0.89	7.74 ± 0.56	0.375
Myristoleic Acid ($C_{14:1}$)	0.73 ± 0.09	0.82 ± 0.14	0.70 ± 0.09	0.231
Pentadecanoic Acid ($C_{15:0}$)	0.81 ± 0.10	1.00 ± 0.13	0.88 ± 0.06	0.027
Palmitic Acid ($C_{16:0}$)	23.87 ± 2.42	20.46 ± 2.23	18.42 ± 1.28	0.003
Palmitoleic Acid ($C_{16:1}$)	1.15 ± 0.12	1.16 ± 0.19	1.03 ± 0.07	0.323
Heptadecanoic Acid ($C_{17:0}$)	0.41 ± 0.04	0.48 ± 0.07	0.45 ± 0.05	0.155
Stearic Acid ($C_{18:0}$)	7.06 ± 0.59	6.50 ± 0.80	6.56 ± 1.22	0.572
Oleic Acid ($C_{18:1n9c}$)	13.79 ± 1.30	12.29 ± 1.14	11.79 ± 1.63	0.088
Linolelaidic Acid ($C_{18:2n6t}$)	0.14 ± 0.04	0.35 ± 0.02	0.35 ± 0.03	0.001
Linoleic Acid ($C_{18:2n6c}$) (LA)	1.23 ± 0.11	0.47 ± 0.05	0.57 ± 0.06	0.001
α-Linolenic Acid ($C_{18:3n3}$) (ALA)	0.27 ± 0.04	0.55 ± 0.05	0.73 ± 0.07	0.001
CLA ($_{c9t11}$)	0.58 ± 0.04	1.71 ± 0.06	1.35 ± 0.12	0.001
CLA ($_{c12t10}$)	0.09 ± 0.02	0.09 ± 0.01	0.09 ± 0.01	0.830
Eicosenoic Acid ($C_{20:1c11}$)	0.00 ± 0.00	0.10 ± 0.01	0.11 ± 0.01	0.001
Eicosatrienoic Acid ($C_{20:3n6\ cis8,11,14}$)	0.13 ± 0.01	0.00 ± 0.00	0.01 ± 0.02	0.001
Behenic Acid ($C_{22:0}$)	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.418
Erucic Acid ($C_{22:1n9}$)	0.04 ± 0.04	0.02 ± 0.01	0.02 ± 0.01	0.006
Tricosanoic Acid ($C_{23:0}$)	0.00 ± 0.00	0.05 ± 0.00	0.06 ± 0.00	0.001
Arachidonic Acid ($C_{20:4n6\ cis5,8,11,14}$)	0.00 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.001
Saturated	52.39 ± 4.98	48.55 ± 5.06	44.98 ± 3.29	0.062
Unsaturated	18.15 ± 1.65	17.63 ± 1.54	16.83 ± 1.79	0.463
MUFA	15.71 ± 1.49	14.38 ± 1.38	13.65 ± 1.64	0.123
PUFA	2.43 ± 0.17	3.26 ± 0.17	3.18 ± 0.22	0.001
Short Chain C_{4-14}	20.94 ± 2.04	20.86 ± 2.45	19.29 ± 1.41	0.368
Medium Chain C_{15-17}	26.24 ± 2.68	23.09 ± 2.61	20.79 ± 1.45	0.007
Long Chain C_{18-24}	23.35 ± 2.00	22.23 ± 2.08	21.73 ± 2.96	0.222
Omega 3 (n3)	0.27 ± 0.04	0.55 ± 0.05	0.73 ± 0.07	0.001
Omega 6 (n6)	1.49 ± 0.12	0.90 ± 0.08	1.01 ± 0.11	0.001
Omega 9 (n9)	13.83 ± 1.31	12.31 ± 1.16	11.81 ± 1.64	0.085
Sum CLA	0.69 ± 0.04	1.80 ± 0.06	1.45 ± 0.13	0.001
n3 + CLA	0.95 ± 0.08	2.36 ± 0.09	2.17 ± 0.14	0.001
n3 / n6	0.18 ± 0.02	0.62 ± 0.03	0.72 ± 0.02	0.001
n6/(n3+CLA)	1.58 ± 0.12	0.38 ± 0.02	0.46 ± 0.04	0.001
Atherogenecity Index	3.43 ± 0.11	3.58 ± 0.20	3.41 ± 0.39	0.541
Thrombogenic Index	4.10 ± 0.12	3.56 ± 0.15	3.21 ± 0.07	0.001
Spreadability Index ($C_{16:0}/C_{18:1}$)	1.73 ± 0.08	1.67 ± 0.10	1.58 ± 0.20	0.255

Eicosatrienoic acid was depleted in pasture-derived butters, whereas eicosatrienoic acid content of TMR butter was significantly ($P = < 0.001$) higher. Pasture derived butters had a greater than 2-fold increase in the biologically active isomer of CLA (C18:2 cis- 9,trans-11) compared with TMR butter. Conjugated linoleic acid was present at the greatest concentration in GRS butter, which had significantly ($P = < 0.001$) higher amounts than CLV and TMR butters. The CLV butter CLA content was also significantly higher than that of TMR ($P = < 0.001$). Arachidonic acid was absent in TMR butter but present in GRS and CLV butters ($P = < 0.001$). The TMR butter had a greater thrombogenicity index score than CLV ($P = < 0.001$) and GRS ($P = < 0.001$), the CLV butters thrombogenecity index was also significantly lower than that of the GRS ($P = 0.005$) butters, at 4.10 ± 0.12, 3.56 ± 0.15, and 3.21 ± 0.07 scores, respectively. The ratio of C16:0 to C18:1, which has been used as an index for butter spreadability in the past, was highest in TMR butters. Following 6-mo storage at 5°C, each of the butters had a FFA content ($P > 0.05$) of 0.13 ± 0.01, 0.13 ± 0.01, and 0.11 ± 0.02 g/100 g of butter fat for TMR, GRS, and CLV butters, respectively.

3.4.2 Partial Least Squares Regression (PLSR)

The PLSR plot of FAME showed clear discrimination of samples according to feeding system (Figure 3.1). The TMR samples were clustered on the outer right side of the plot; however, the GRS and CLV samples were located on the outer left side. The TMR butter samples were found to be more associated with eicosatrienoic acid, erucic acid, palmitic acid, and linoleic acids. In contrast, GRS and CLV samples were associated with tricosanoic acid, CLA (C18:2 cis-9,trans-11), arachidonic acid, eicosanoic acid, α-Linolenic acid and linolelaidic acid content, which is in agreement with the FA data shown in Table 3.1.

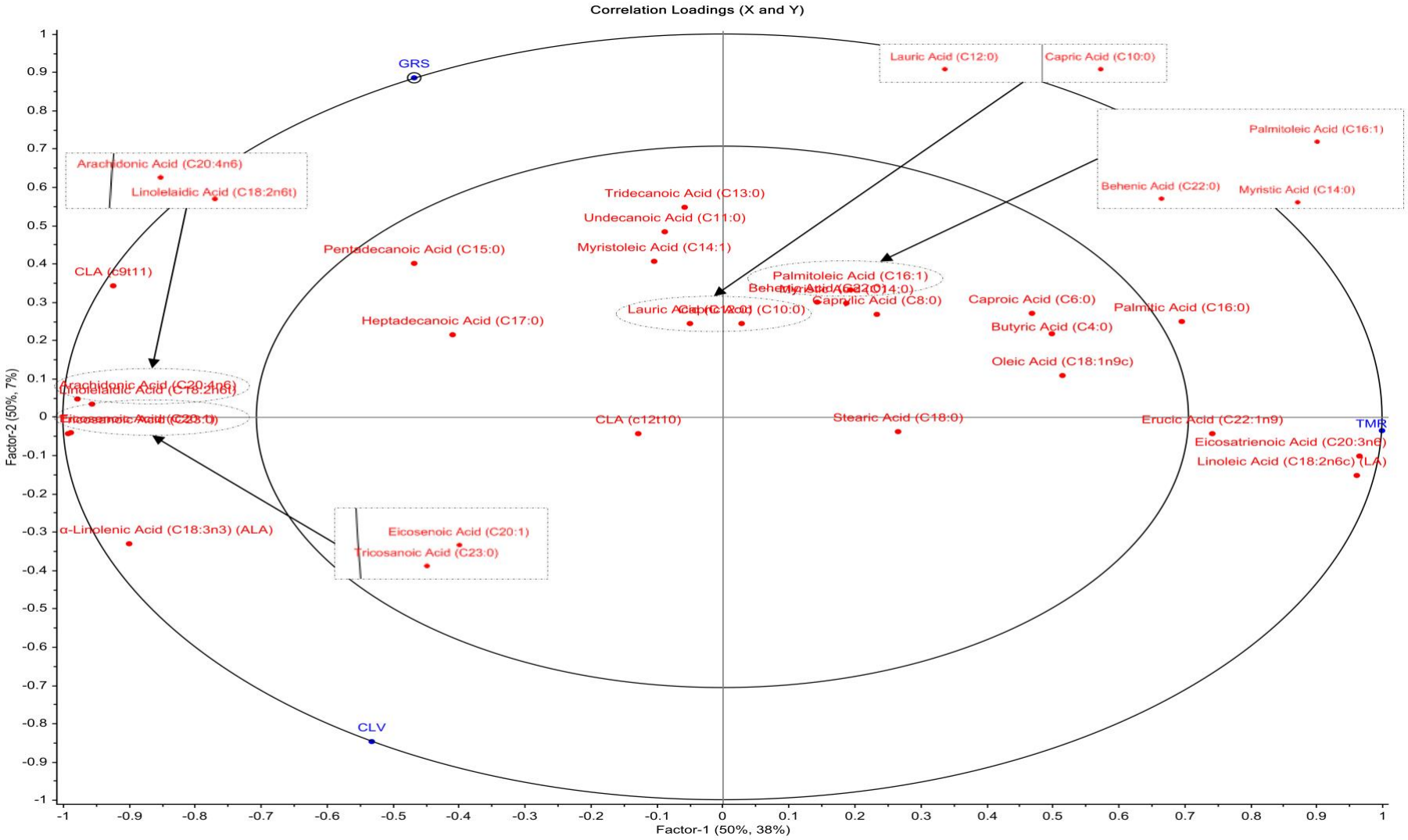


Figure 3.1. Multivariate data analysis partial least squares regression (PLSR) plot of fatty acid methyl esters from butter of different feeding systems of TMR, perennial ryegrass (Grass), and perennial ryegrass and white clover (Clover). Zoom in squares shown to clarify overlapping areas.

3.4.3 Thermal Properties

Crystallization onset temperature (T-onset) for each of the butters differed significantly between feeding systems (Fig. 3.2 and Table 3.2). The TMR butters had the highest T-onset temperature at mean ± S.D. of 17.24 ± 0.30 °C which was significantly higher than GRS (*P*, 0.004) and CLV (*P*, 0.007) butters at 15.90 ± 0.57 °C and 16.02 ± 0.67 °C, respectively. There was no significant difference between T-onset of GRS and CLV butters. The cooling profiles of each butter were characterized by two exothermic peaks (crystallization peaks) - first minor peak in energy followed by second major peak in energy. The 1st and 2nd peaks of crystallization were recorded at higher temperatures for TMR butters than that of pasture-derived butters. The GRS butters 1st peak of crystallization temperature occurred at 12.34 ± 0.40 °C which was lower than TMR (*P*, 0.11) at 14.25 ± 0.50 °C and CLV at 13.11 ± 1.39 °C. TMR 2nd peak of crystallization occurred at 9.26 ± 0.30 °C which was significantly higher than pasture butters (*P* = <0.001) at 7.07 ± 0.44 °C and 7.03 ± 0.17 °C for GRS and CLV, respectively. Enthalpy of crystallization for TMR butters was 28.39 ± 3.45 J/g which was higher (*P* > 0.05) than that of GRS at 26.06 ± 1.06 J/g and CLV at 25.92 ± 2.92 J/g. There was no significant difference in the final melting temperature (T-offset) of the butters, however, TMR T-offset was recorded at higher temperatures than GRS and CLV at 36.28 ± 0.24 °C, 35.27 ± 0.48 °C and 35.81 ± 1.29 °C, respectively.

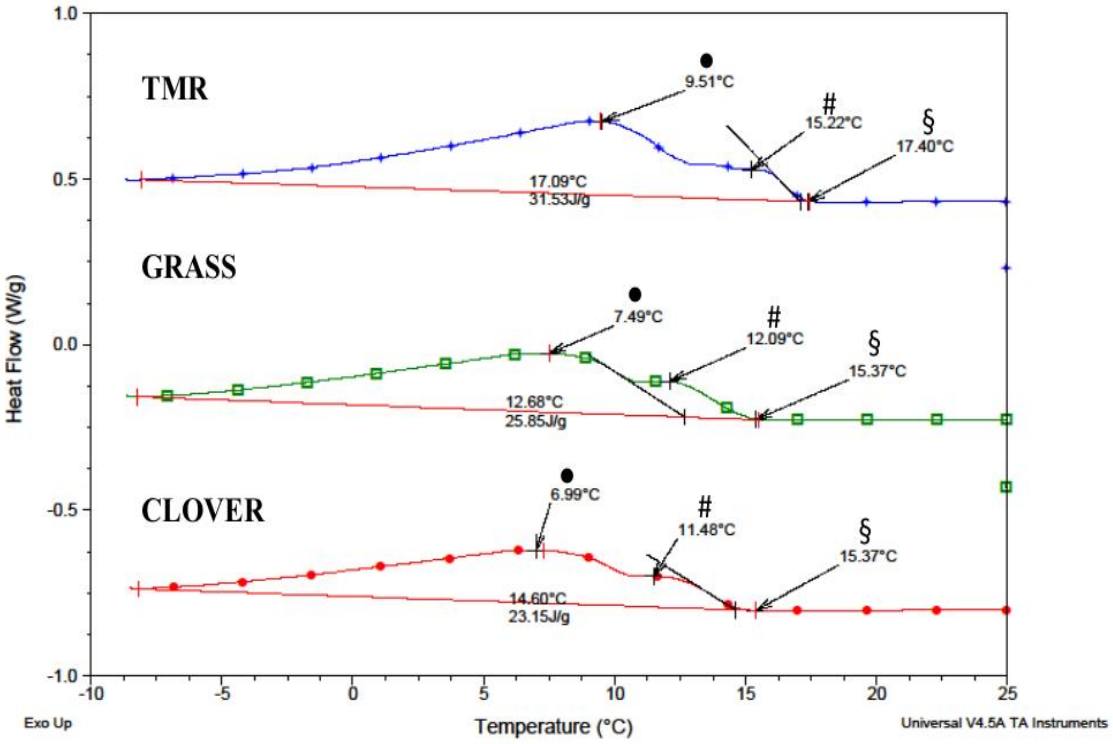


Figure 3.2. Relationship between the cow feeding system and the crystallization temperature of butter determined by differential scanning calorimetry (DSC). Example of DSC cooling chromatograms of butter derived from TMR, perennial ryegrass (Grass), and perennial ryegrass and white clover (Clover), indicating temperature at beginning of fat crystallization (T-onset; §) and first (#) and second (•) peak crystallization temperatures.

Table 3.2. Relationship between cow feeding system and crystallization and final melting temperature of butter determined by differential scanning calorimetry.

	Feeding System			*P*-value
	TMR	**GRS**	**CLV**	
T-Onset (°C)	17.24 ± 0.30	15.90 ± 0.57	16.02 ± 0.67	0.002
1st Peak Crystallisation (°C)	14.25 ± 0.50	12.34 ± 0.40	13.11 ± 1.39	0.013
2nd Peak Crystallisation (°C)	9.26 ± 0.30	7.07 ± 0.44	7.03 ± 0.17	0.001
Enthalpy of Crystallisation (J/g)	28.39 ± 3.45	26.06 ± 1.06	25.92 ± 2.92	0.291
T-offset (°C)	36.28 ± 0.24	35.27 ± 0.48	35.81 ± 1.29	0.178

3.4.4 Colour and trans β-Carotene Analysis

There was no significant difference in the L* and A* values of the butters at each of the sampling time-points. However, significant differences were recorded for the B* value of the butters from each of the feeding systems. Average (mean ± S.D.) B* value for GRS butter was greater than CLV (P = 0.025) and TMR (P = < 0.001) samples with scores of 42.06 ± 3.66, 38.87 ± 3.21 and 29.91 ± 2.26, respectively, indicating that pasture-derived butters are more yellow in colour than TMR which directly correlates with trans β-carotene results (Fig. 3.3). The CLV B* values were also significantly higher that TMR (P = < 0.001). Storage time also had a significant, effect on the butters A* (P = 0.002) and B* values (P = < 0.001). GRS butter's A* value increased over 6 months storage from -3.30 ± 0.45 at day 7 to -2.47 ± 0.20 at 6 months, indicating that the butter was becoming more red in colour over storage. The B* values for GRS and CLV butters also changed, dropping from 42.49 ± 0.98 to 36.14 ± 0.98 for GRS and 38.68 ± 0.97 to 34.05 ± 1.65 for CLV indicating butters were becoming more blue (pale) over storage at 4 °C.

The GRS butters had the highest trans β-carotene content at 5.16 ± 0.22 mg/ kg of butter, which was significantly higher than that of TMR (P < 0.001) and CLV (P = 0.019) content of 2.27 ± 0.13 mg/kg and 3.99 ± 0.46 mg/kg, respectively. There was a significantly positive correlation between the increase in B* scores of butters and their respective trans-β carotene content (r = 0.899, P < 0.001), (Fig. 3.3).

3.4.5 Texture Analysis

Refrigerated Temperature. At 5 °C, there was no significant (P = 0.058) difference in hardness of butters from different feeding systems. Hardness of each of the butters did increase significantly (P < 0.001) throughout the storage period. The TMR butter hardness increased by 19.44 N between M1 and M6, with measurements (mean ± S.D) of 37.95 ± 4.23 N and 57.39 ± 4.12 N, respectively. While CLV hardness increased by 15.95 N from M1 to M6 with measurements of 31.61 ± 4.40 N and 47.57 ± 7.42 N, respectively, and GRS hardness was the

least affected by storage increasing by 9.5 N from M1 to M6 with measurements of 33.09 ± 3.81 N and 42.60 ± 5.11N, respectively.

Room Temperature. At 20 °C, TMR butter appeared to be hardest throughout the 6-month storage period and was significantly (P = 0.001) harder than CLV and GRS. Hardness of each of the butters also increased significantly (P = < 0.001) over the storage period when measured at 20 °C. The TMR butter hardness increased by 5.82 N from M1 to M6 with measurements of 7.43 ± 1.22 N and 13.25 ± 0.60 N, respectively. GRS hardness increased by 7.11 N from M1 to M6 with measurements of 4.36 ± 1.11 N and 11.47 ± 1.21 N, respectively, while CLV was the softest butter at 20 °C; increasing by 7.02 N from M1 to M6 with measurements of 3.64 ± 0.14 N and 10.66 ± 0.51N, respectively.

3.4.6 Sensory Analysis

Panelist data scores (mean ± S.D.) for sensory analysis of butters are shown in Table 3.3. Butter from the GRS system scored significantly higher for the hedonic sensory descriptors - liking of appearance (P = 0.004) and liking of flavor (P = 0.030) compared to the TMR butter samples. Significant differences were recorded for ranking descriptive analysis between each of the butter samples. GRS butter scored significantly higher than TMR butter samples for colour (P = 0.007), diacetyl aroma (P = 0.015), diacetly flavor (P = 0.016) and cream flavor (P = 0.011). The CLV butter samples also scored significantly higher for texture (P = 0.02) in comparison to the TMR samples. The CLV and GRS butter samples were not significantly different for any of the other hedonic or ranking sensory descriptor attributes (Fig. 3.4).

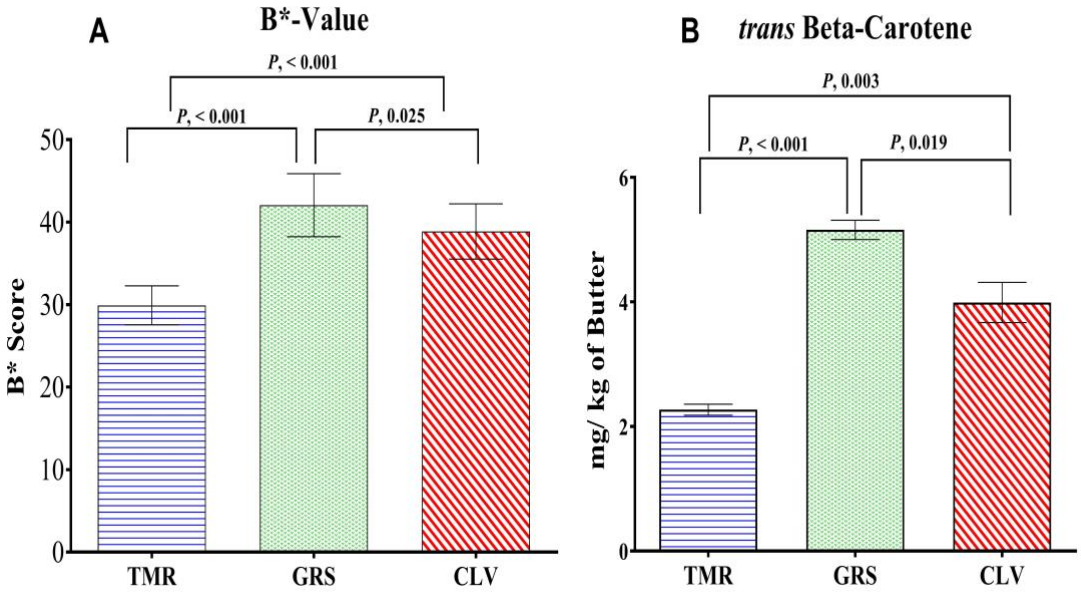


Figure 3.3. Relationship between cow feeding systems (TMR, perennial ryegrass (GRS), and perennial ryegrass and white clover (CLV)) and the color scores of butters in the blue–yellow axis (B*) and the trans-β-carotene content of butter. Trans-β-carotene and B* values appear to be positively correlated (r = 0.899); they are highest in pasture and lowest in TMR butters.

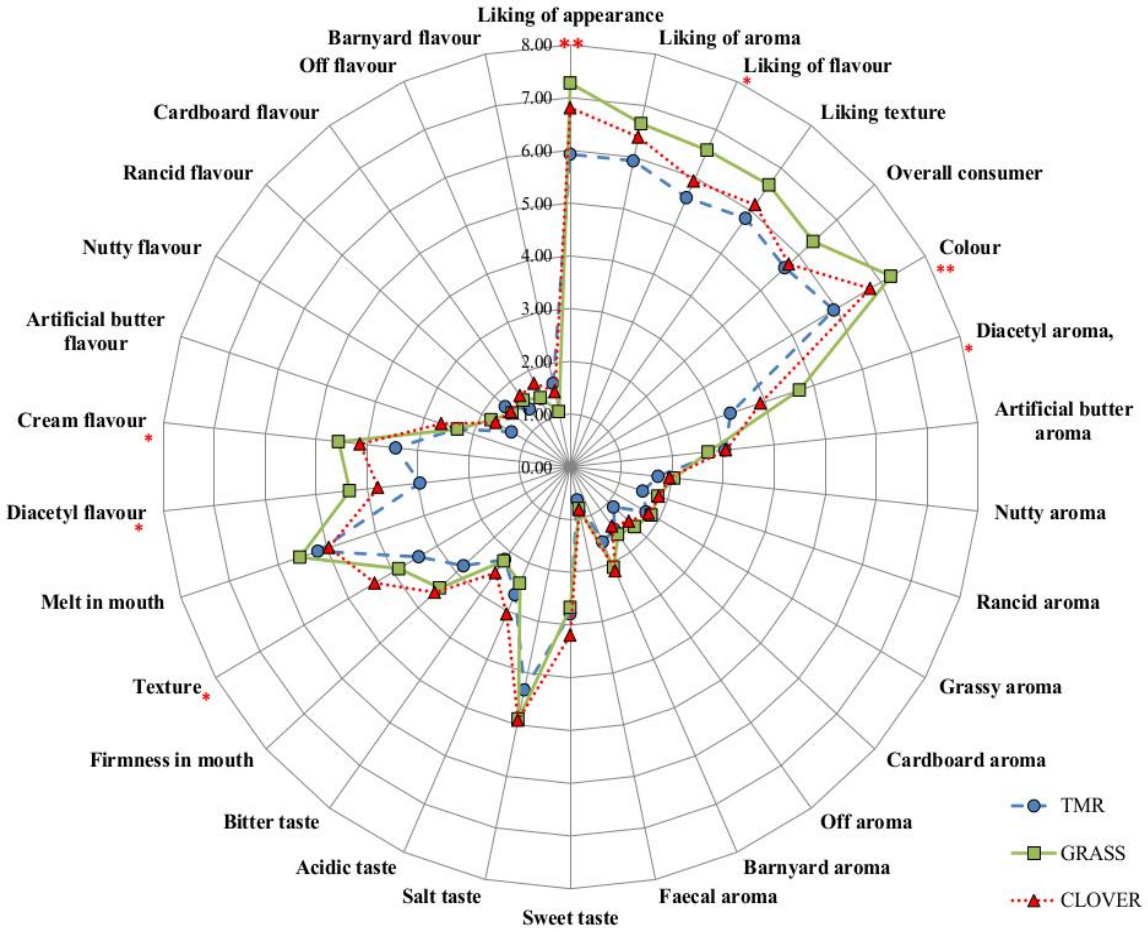


Figure 3.4. Sensory analysis of butter derived from different feeding systems of TMR, perennial ryegrass (Grass), and perennial ryegrass and white clover (Clover). Definition of sensory attributes available in Supplemental Table S1). Statistical analysis by one-way ANOVA, *P < 0.05, **P < 0.01.

Table 3.3. Relationship between cow feeding system and butter characteristics determined by sensory analysis

	Panellist's Scores 1-10			
Sensory Attribute	**TMR**	**GRS**	**CLV**	***P*-value**
	Hedonic			
Liking of appearance	5.93 ± 1.55	7.28 ± 1.55	6.81 ± 1.17	0.005
Liking of aroma	5.93 ± 1.65	6.65 ± 1.39	6.39 ± 1.17	0.197
Liking of flavour	5.59 ± 0.92	6.57 ± 1.70	5.93 ± 1.28	0.035
Liking texture	5.82 ± 1.42	6.61 ± 1.53	6.14 ± 1.08	0.130
Overall consumer Acceptability	5.64 ± 1.03	6.39 ± 1.54	5.74 ± 1.20	0.087
	Intensity			
Colour	5.94 ± 1.58	7.23 ± 1.56	6.76 ± 1.17	0.009
Diacetyl aroma	3.29 ± 1.68	4.71 ± 1.87	3.91 ± 1.68	0.020
Artificial butter aroma	3.04 ± 1.32	2.71 ± 1.58	3.06 ± 1.19	0.604
Nutty aroma	1.73 ± 1.06	2.04 ± 1.04	1.95 ± 0.87	0.520
Rancid aroma	1.49 ± 1.06	1.80 ± 1.13	1.82 ± 1.37	0.552
Grassy aroma	1.71 ± 0.99	1.83 ± 1.02	1.77 ± 0.87	0.909
Cardboard aroma	1.14 ± 0.93	1.69 ± 1.02	1.54 ± 1.19	0.164
Off aroma	1.47 ± 1.19	1.58 ± 1.13	1.39 ± 1.08	0.828
Barnyard aroma	1.56 ± 1.20	2.08 ± 1.20	2.16 ± 1.29	0.181
Faecal aroma	0.64 ± 0.68	0.81 ± 0.73	0.83 ± 0.81	0.630
Sweet taste	2.79 ± 1.21	2.67 ± 1.12	3.19 ± 1.26	0.274
Salt taste	4.33 ± 1.89	4.89 ± 2.05	4.92 ± 1.36	0.425
Acidic taste	2.65 ± 1.62	2.42 ± 1.40	3.06 ± 1.68	0.355
Bitter taste	2.17 ± 1.29	2.21 ± 1.23	2.48 ± 1.55	0.676
Firmness in mouth	2.80 ± 1.57	3.43 ± 1.33	3.56 ± 1.40	0.146
Texture	3.42 ± 1.12	3.87 ± 1.29	4.41 ± 1.44	0.029
Melt in mouth	5.18 ± 1.71	5.55 ± 1.65	4.96 ± 1.99	0.497
Diacetyl flavour	2.95 ± 1.64	4.33 ± 1.75	3.78 ± 1.79	0.021
Cream flavour	3.43 ± 1.44	4.55 ± 1.31	4.13 ± 1.27	0.015
Artificial butter flavour	2.33 ± 1.17	2.32 ± 1.33	2.65 ± 1.16	0.558
Nutty flavour	1.32 ± 0.94	1.78 ± 1.00	1.68 ± 1.05	0.235
Rancid flavour	1.71 ± 0.92	1.52 ± 1.14	1.56 ± 1.21	0.826
Cardboard flavour	1.35 ± 0.99	1.56 ± 1.17	1.67 ± 1.17	0.598
Off flavour	1.42 ± 1.02	1.43 ± 1.37	1.73 ± 1.62	0.655
Barnyard flavour	1.61 ± 1.24	1.07 ± 1.08	1.46 ± 1.14	0.243

3.4.7 Volatile Analysis

Volatile analysis of butters by GC-MS revealed 25 compounds in total within all of the butters (Table 3.4). These included six aldehydes (2-methyl butanol, pentanal, hexanal, heptanal, benzaldehyde and nonanal), five ketones (acetone, 2-butanone, 2-pentanone, 2-heptanone, 2-nonanone), two alcohols (1-pentanol, 2-methyl-1-butanol), three acids, (acetic acid, butanoic acid, hexanoic acid), five hydrocarbons (toluene, ethylbenzene, 3-ethyl toluene, m-xylene and o-xylene), three esters (ethyl acetate, butyl acetate and ethyl octanoate) and one terpene compound (β-pinene).

Higher intensities of acetone were detected in CLV butter samples than those of TMR ($P = 0.015$) and GRS ($P = 0.004$) butters. 2-Butanone was present at significantly ($P = 0.012$) higher intensity in TMR butters than GRS butters. The alcohol 1-pentanol was detected at significantly higher intensities in CLV butter samples than those of GRS ($P = 0.015$) and TMR ($P = 0.004$). The hydrocarbon toluene was significantly correlated with pasture derived butters, more than that of TMR ($P = < 0.001$) and was highest in CLV derived samples. Finally, β-pinene intensity was significantly lower in TMR samples than those of GRS and CLV ($P = 0.003$). The PLSR plot of butter volatiles also corresponds with these significant differences and shows clear separation of samples according to feeding system, where toluene appears to be significantly correlated with GRS and CLV derived samples (Fig. 3.5).

Table 3.4. Relationship between cow feeding regimen and butter volatile compounds, identified by GC-MS analysis

	CAS no.	R.T. (mins)	LRI	LRI*	*LRI (ref)	Odour Description	Average Area Values: TMR	GRS	CLV	SEM	*P*-value
Aldehydes											
2-Methyl butanal	96-17-3	5.8165	608	662	[32]	*Malty, powerful, cheese, green, dark chocolate,*	-	-	1.02×10^5	2.31×10^4	.051
Pentanal	110-62-3	6.3742	700	697	[33]	*Pungent, almond-like, chemical, malty, apple*	1.19×10^6	2.34×10^6	3.76×10^6	4.31×10^5	.117
Hexanal	66-25-1	8.6642	801	801	[34]	*Green, slightly fruity, lemon, herbal, grassy,*	1.01×10^5	9.69×10^4	3.00×10^5	6.38×10^4	.454
Heptanal	111-71-7	11.4019	902	901	[35]	*Slightly fruity (Balsam), fatty, oily, green, woody*	2.33×10^5	4.11×10^5	3.20×10^5	4.54×10^4	.205
Benzaldehyde	100-52-7	13.3059	969	960	[36]	*Bitter almond, sweet cherry*	1.57×10^6	-	-	4.44 × 105	.051
Nonanal	124-19-6	17.0154	1104	1106	[34]	*Green, citrus, fatty, floral*	6.44×10^5	4.56×10^5	3.30×10^5	5.57×10^4	.108
Ketones											
Acetone	67-64-1	4.0167	<500	496	[37]	*Earthy, strong fruity, wood pulp, hay*	1.03×10^7	7.76×10^6	1.68×10^7	1.14×10^6	**.005**
2-Butanone	78-93-3	4.802	583	593	[32]	*Buttery, sour milk, etheric*	4.26×10^6	-	6.49×10^5	8.03×10^5	**.013**
2-Pentanone	107-87-9	6.1244	659	687	[32]	*Orange peel, sweet, fruity*	-	1.29×10^5	-	2.36×10^4	.051
2-Heptanone	113-43-0	11.0388	889	891	[33]	*Blue cheese, spicy, roquefort*	1.53×10^5	1.13×10^5	9.01×10^4	3.05×10^4	.591
2-Nonanone	821-55-6	16.6312	1089	1094	[33]	*Malty, fruity, hot milk, smoked cheese*	3.04×10^5	1.18×10^5	1.24×10^5	5.65×10^4	.482
Alcohols											
1-Pentanol	71-41-0	7.8117	763	768	[35]	*Fruity, alcoholic, green, balsamic, fusel oil, woody*	1.24×10^5	5.28×10^5	1.44×10^6	1.93×10^5	**.006**
2-Methyl-1-butanol	137-32-6	7.8836	767	755	[35]	*Malty*	4.47×10^3	-	-	1.46×10^3	.368
Acids											
Acetic acid	64-19-7	5.6343	592	629	[38]	*Vinegar, peppers, green, fruity floral, sour*	4.42×10^5	2.70×10^5	1.06×10^5	9.75×10^4	.185
Butanoic acid	107-92-6	8.4244	790	814	[38]	*Sweaty, butter, cheese, strong, acid, facel, rancid,*	1.24×10^6	7.64× 10	3.93×10^5	3.13×10^5	.198
Hexanoic acid	142-62-1	13.2746	968	983	[36]	*Acidic, sweaty, cheesey, sharp, goaty, bad breath,*	1.05×10^4	6.36×10^5	-	1.88×10^5	.312
Hydrocarbons											
Toluene	108-88-3	7.9122	768	763	[37]	*Nutty, bitter, almond, plastic*	2.38×10^5	8.23×10^6	8.77×10^6	8.13×10^5	**.001**
Ethylbenzene	100-41-4	10.3568	863	851	[39]	*Heavy, floral*	2.67×10^6	1.73×10^6	2.02×10^6	5.78×10^5	.553
3-Ethyl toluene	620-14-4	13.392	972	969	NIST	*Turpentine-like*	1.89×10^5	9.01×10^4	1.96×10^5	5.51×10^4	.451
m-xylene	108-38-3	10.5981	872	875	[33]	*Sweet, aromatic*	4.64×10^6	3.00×10^6	4.04×10^6	1.03×10^6	.718
o-xylene	108-38-3	11.251	896	900	[33]	*Geranium*	4.05×10^6	2.37×10^6	1.36×10^6	7.92×10^5	.472

						Esters					
Ethyl acetate	141-78-6	5.0203	557	614	[35]	*Solvent, pineapple, fruity, fruity gum, apples*	-	-	1.39×10^{5}	3.30×10^{4}	.039
Butyl acetate	123-86-4	8.9579	812	812	[40]	*Pear, ethereal, green*	2.25×10^{6}	1.85×10^{6}	1.18×10^{6}	6.47×10^{5}	.990
Ethyl octanoate	106-32-1	19.2916	1191	1198	[35]	*Fruity, apple-like, green, fatty, orange, winey*	1.41×10^{4}	-	-	3.89×10^{3}	.051
						Terpenes					
β-Pinene	127-91-3	13.7283	984	980	[35]	*Herbaceous*	7.56×10^{4}	1.33×10^{5}	1.74×10^{5}	1.34×10^{4}	**.009**

3.5 Discussion

In this study, we investigated the effects of three widely practiced feeding systems on the characteristics, composition, quality and consumer perception of mid lactation sweet cream butter. Fatty acid methyl esters differing significantly in butters from each of the feeding systems followed similar trends to that of the raw milks from this study, previously reported [16]. These results highlight that only minor changes occur in milk FA triglyceride composition during the butter production process, which has been reported in the past [19, 41]. The atherogenecity (AI) and thrombogenecity (TI) indices outlined by Ulbricht and Southgate [1] are dietary risk indices for cardiovascular disease. Consumption of dairy products with lower AI and TI scores is therefore more favorable to consumer health than their high AI and TI score counterparts, with reported reductions in both total and LDL-cholesterol in humans with modified butter [42]. The TMR butters in this study had significantly greater TI scores than pasture butters, at 4.10, 3.56 and 3.21, respectively. Greater TI scores in TMR butter could be attributed to increased palmitic acid and *n*6 PUFA content in TMR butter compared to that of GRS and CLV butters which had significantly less palmitic acid, *n*6 fatty acids and significantly more *n*3 fatty acids. There was no significant difference in AI content of butters, however lower AI scores in fresh grass derived milks has also been reported in the past by Couvreur*, et al.* [7] when comparing the effects of TMR and 100 % fresh grass feeding systems. Through in-vitro and rodent model research, CLAc9t11 has been associated with the prevention of many lifestyle related disorders and metabolic syndromes by exerting potent physiological functions such as antihypertensive, anti-obesity, antidiabetic and anti-carcinogenic properties [43]. Dairy products derived from ruminants are a natural source of CLA, and feeding regimen is a major factor affecting the concentration of CLA in milk and dairy products [44, 45]. Several studies in the past have reported the positive linear response of CLA concentration in cows' milk to intake of fresh pasture [7]. Our study has demonstrated that butters derived from pasture-based systems contained greater than two fold

concentrations of CLA $_{c9t11}$ than that of TMR butters (1.71 and 1.35 g/100g of butter fat for GRS and CLV, respectively vs 0.58 g/100g of butter fat for TMR). However, this increase in CLA content is in fact lower than that reported by Mohammed*, et al.* [46] who compared the effects of grazing perennial rye grass and consumption of grass silage with CLA contents of 2.07 and 0.54 % of total FA respectively. Lerch*, et al.* [47] also demonstrated that the supplementation of a grass silage and hay based diet with extruded linseed oil and rapeseed oil had a beneficial effect on milk CLA concentration.

Free fatty acids are produced during hydrolytic oxidation and are typically used as a qualitative reference of milk, butter and other dairy products [48]. The FFA can also contribute to the sensory aspects of dairy products, with elevated levels of short chain FFA being responsible for rancidity [49]. There was no significant difference in the FFA values of each of the butters after 6 months' storage at 5 °C. This result is consistent with FFA values reported by Krause*, et al.* [50] who also reported that FFA values of butters remained relatively stable for up to 6 months before a slight increase in FFA was recorded.

Hardness and spreadability are inversely related characteristics of butters and potentially the most important aspects of texture, and rheological influence on consumer perception [51]. Hardness of pasture-derived butters was decreased at 20 °C compared to that of TMR. This could be attributed to differences in butter fatty acid content, with pasture butters having lower saturated FA content which appeared to be trending towards significance (P = 0.058) and having significantly lower concentrations of palmitic acid. The textural and rheological properties of milk fats are strongly dependent on the thermal and structural properties of the fats triglycerides [52]. Differences in butters fatty acid composition results in alterations of butter crystalisation and melting point. DSC analysis (Fig. 3.2) showed onset of crystalisation (change from a liquid to solid state) for TMR butters occurred at significantly higher temperatures than that of pasture which would contribute to increased hardness results at room temperature. In fact, Brunner [53] initially reported that 80 % of the variation in butter

texture can be attributed to differences in milk FAs. This also agrees with sensory ranking descriptive analysis scores where GRS and CLV scored higher for texture attribute ($P = 0.028$). Similar studies have reported significant differences in the spreadability index of butters from pasture versus TMR [7] although this trend was observed in this study, spreadability index scores were not significantly different between feedings systems. Over 6 months storage hardness of butters increased significantly which was also reported by Krause*, et al.* [50] and has been attributed to changes in the solidification of triglycerides during storage [54]..

β-Carotene content was highest in GRS butter and lowest in TMR butter. The β-carotene content of milk can vary depending on cows feeding system and is dependent on dietary supply of the pigment to the cow. β-Carotene supply is most abundant in fresh forages than its silage and concentrate counterparts as the ensiling process and processing of feeds for concentrates typically depletes or destroys many carotenoids, and as a result most concentrates fed to cows on a TMR system are low in β-carotene [55]. β-Carotene has many beneficial attributes such as being a precursor to Vitamin A, has been inversely associated with the risk of cancer and also has antioxidant properties particularly involved in preventing photo-oxidation of high fat dairy products [56, 57].

The carotenoid content also has a major effect on the colour of dairy products, particularly high fat dairy products such as butter, imparting a yellow colour. This yellow colour carries consumer perceptions of "fresh grass feeding" and thus trans β-carotene content of dairy products has been suggested in the past as a potential indicator for verification of products from grazing pasture systems [58]. This yellow colour can in turn be beneficial as a marketing attribute, however, yellow colour has also been reported in the past to be a negative feature when exporting to colour sensitive markets such as the middle east [59]. GRS and CLV derived butters had significantly higher B* values compared to that of TMR butters. Indeed higher B* values of butter was significantly correlated with higher trans-β carotene content (*r*,

0.899) further confirming the hypothesis by Prache, *et al.* [58] for the use of β-carotene as a verification method of pasture-derived dairy products.

Direct analysis of the headspace of butters provides a representative view of the volatiles relative to the olfactory receptors [12]. GC-MS analysis of our butters revealed twenty-five compounds present in butters from each feeding system, five of which differed significantly. Among the ketone compounds, acetone (earthy, strong fruity, wood pulp, hay) was significantly correlated with CLV-derived samples over that of GRS and TMR butters while 2-butanone (buttery, sour milk, etheric) was greatest in TMR derived samples. Acetone and 2-butanone have been reported in the past in milk and to originate from the cows feed [60]. 1-Pentanol (fruity, alcoholic, green, balsamic, fusel oil, woody) was greater in pasture derived samples than that of TMR, and CLV derived butters had significantly greater intensities of the primary alcohol than GRS and TMR samples. 1-Pentanol is derived from the aldehyde pentanal, and its concentrations correlate with those of its aldehyde source which was also greater in pasture derived samples and highest in CLV. Straight chain aldehydes such as pentanal have been reported to be derived from lipid degradation [61, 62]. Similar results were seen by Villeneuve, *et al.* [63] who reported increased 1-pentanol in pasture milks over that of hay-derived milks. The terpene compound β-pinene (herbaceous) intensity was significantly greater in pasture-derived samples than that of TMR, and greatest in CLV derived samples. Terpenes also originate from the feed and would have been transferred into the cows' milk. Toluene (nutty, bitter, almond, plastic) concentrations were significantly higher ($P = < 0.001$) in pasture derived butters than that of TMR butters, which was also reported by Villeneuve, *et al.* [63] who examined the effects on milk from timothy feeding of cows as hay, pasture or silage. Toluene in dairy products has been considered in the past as being a contaminant from the atmosphere or packaging [64]. However, toluene may more likely be present in pasture derived products as a product of β-carotene degradation [65], as area values

for toluene correlate with increased trans β-carotene concentrations of the butters from pasture feeding systems.

Multivariate data analysis using PLSR of butter FA triglyceride contents showed clear separation of butters by feeding system being utilized similar to the raw milks of this study previously reported [16]. Overall, this analysis demonstrates that the FA composition of butters from TMR diets and pasture based diets in this study were quite distinct, whereas the GRS and CLV pasture diets were much less differentiated. These data further indicate that FA profiling could potentially be used for verification of dairy products derived from the milk of cows consuming a fresh pasture only diet. This possibility is in agreement with Capuano*, et al.* [66] who concluded that FA profiling may be used for the verification of fresh grass feeding of cows.

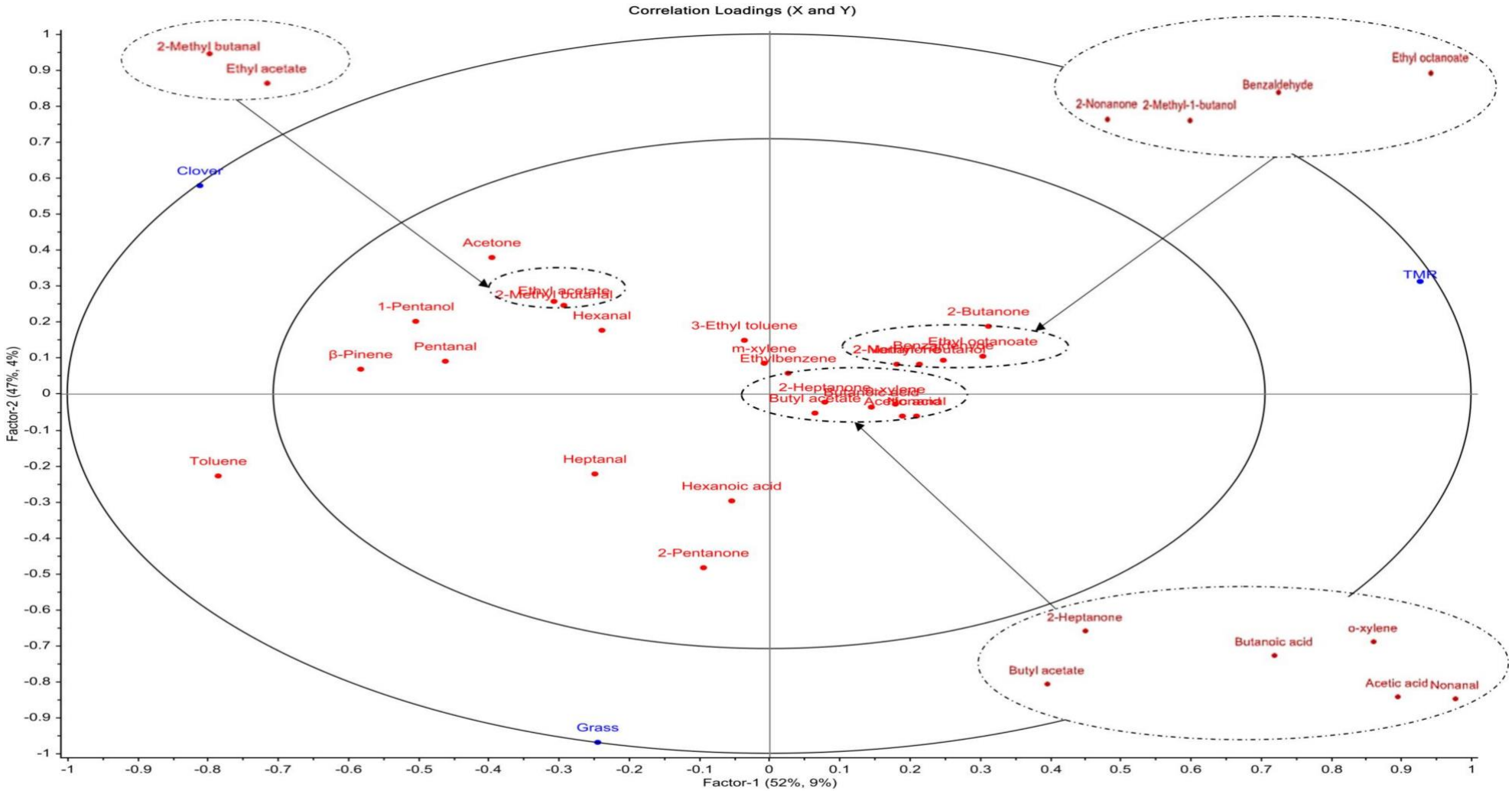


Figure 3.5. Multivariate data analysis partial least squares regression plot of volatile analysis of butters derived from different feeding systems of TMR, perennial ryegrass (Grass), and perennial ryegrass and white clover (Clover).

3.6 Conclusion

This study evaluated the effects of three widely practiced feeding systems of cows on the characteristics, quality and consumer perception of butter. Feeding system resulted in significant differences in FA compositions. Such alterations in the FA compositions contributed to significant differences in textural, thermal, sensory and volatile properties of butters. Pasture-derived (GRS and CLV) systems produced butters with improved nutritional aspects, including lower thrombogenecity scores and significantly higher concentrations of CLA_{c9t11} and β-carotene. Sensory panelist data revealed significantly higher scores for GRS derived butter in several attributes including "liking" of appearance, flavour and colour. Volatile analysis of butters by GC-MS revealed 25 different compounds from each of the butters, five of which differed significantly based on feeding regimen including acetone, 2-butanone, 1-pentenol, toluene and β-pinene. Toluene concentrations significantly correlated with pasture-derived butters over that of TMR. Finally, FA profiling coupled with multivariate data analysis again showed clear separation of butters derived from grazed pasture diets to that of TMR systems, offering further insight into the ability to verify such pasture-derived dairy products by FA profiling.

3.7 Acknowledgement

This publication has emanated from research conducted with the financial support of Science Foundation Ireland (SFI) under grant number SFI/12/RC/2273 and the Dairy Levy Fund administered by Dairy Research Ireland. Tom F. O'Callaghan is the recipient of a Teagasc Walsh Fellowship award. The valuable input of Elaine Patterson and David Mannion (Teagasc Moorepark, Ireland) is gratefully acknowledged. Technical assistance over the course of the experiment from Tim Coakley and John O'Reilly is greatly appreciated. The input of Dr. Sean Lacey (Department of Mathematics, Cork Institute of Technology, Ireland) for statistical analysis is very much appreciated. The authors sincerely thank the technical and farm staff at Moorepark for their excellent care of the experimental cows and assistance during the experiment.

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Supplementary Material

Supplementary Material: Table 3.1; Typical ingredient formulation (% as fed) and chemical composition (%) of total mixed ration concentrate.

TMR Ingredients	**% as fed**
Maize	13.00
Beet pulp molassed	15.50
Soyabean meal 48% CP	30.00
Maize distillers	12.00
ACID BUF	0.70
Maize/Beet Min Balancer	2.50
Salt	0.50
Barley (rolled)	15.00
Rapeseed meal	7.50
Megalac	3.30
Chemical Composition	**%**
Organic Matter	93.50 ± 0.94
Dry Matter	86.76 ± 0.75
Protein	23.73 ± 3.69
Fibre	7.77 ± 1.86
Starch	21.49 ± 1.93
Total Sugar	9.62 ± 0.35
Ash	6.50 ± 0.94
Moisture	13.24 ± 0.75
NCGD	83.35 ± 1.15

Supplementary Material: Table 3.2; Chemical composition (mean ± standard deviation) and nutritional content of silages from TMR diet (grass-silage and maize silage) collected weekly throughout lactation analysed by Near-infrared spectroscopy.

	g/ kg of DM	
	Grass Silage	**Maize Silage**
Dry Matter	389.37 ± 61.35	343.03 ± 43.45
Organic Matter (OM)	917.94 ± 7.45	972.53 ± 3.22
Crude protein	114.55 ± 12.55	68.97 ± 9.91
Starch	NA	285.37 ± 28.81
ADF	296.82 ± 23.40	NA
NDF	452.02 ± 39.31	434.80 ± 49.57
ASH	82.06 ± 6.75	27.47 ± 3.22
UFL (/kg DM)	0.93 ± 0.04	1.00 ± 0.02
PDIA (/kg DM)	24.55 ± 1.63	14.97 ± 2.20
PDIE (/kg DM)	68.07 ± 2.23	66.57 ± 3.14
PDIN (/kg DM)	72.52 ± 5.11	42.37 ± 6.05

Supplementary Material: Table 3.3; Chemical composition (mean ± standard deviation) and nutritional content of pasture systems forages (GRS and CLV) collected weekly throughout lactation, analysed by Near-infrared spectroscopy.

	g/ kg of DM	
	GRS	**CLV**
Organic Matter (OM)	928.00 ± 9.31	931.49 ± 7.18
OM Digestibility	764.43 ± 19.34	769.22 ± 18.97
Crude protein	210.90 ± 23.71	220.67 ± 14.05
ADF	218.89 ± 16.91	220.67 ± 14.05
NDF	427.62 ± 23.83	423.46 ± 18.94
ASH	72.00 ± 9.31	68.51 ± 7.18
UFL (/kg DM)	0.99 ± 0.03	1.00 ± 0.03
PDIA (/kg DM)	41.79 ± 3.03	44.80 ± 2.99
PDIE (/kg DM)	100.91 ± 3.38	104.48 ± 3.50
PDIN (/kg DM)	135.96 ± 15.73	151.67 ± 15.52

Supplementary Material: Table 3.4. Sensory terms for the affective and descriptive evaluation of whole butter

Attribute	Definition	Scale
	Hedonic	
Appearance-Liking	*The liking of appearance*	0 = extremely dislike10 = extremely like
Flavour-Liking	*The liking of flavour*	0 = extremely dislike10 = extremely like
Aroma-Liking	*The liking of aroma*	0 = extremely dislike10 = extremely like
Texture-Liking	*The liking of texture*	0 = extremely dislike10 = extremely like
Overall acceptability	*The acceptability of the product*	0 = extremely unacceptable10 = extremely acceptable
	Intensity	
Appearance-colour	*Appearance-Pale white to yellow*	0 = Pale, 10 = Yellow
Diacetyl aroma	*The smell associated with diacetyl, popcorn, butterscotch*	0 = none, 10 = extreme
Artificial Butter Aroma	*The smell associated with artificial butter*	0 = none, 10 = extreme
Nutty Aroma	*The smell associated with nuts*	0 = none, 10 = extreme
Rancid/Oxidised aroma	*The smell associated with oxidised dairy products*	0 = none, 10 = extreme
Grassy Aroma	*The smell associated with Grass*	0 = none, 10 = extreme
Cardboard aroma	*The smell associated with*	0 = none, 10 = extreme
Off-aroma	*Off-aroma (Rancid)*	0 = none, 10 = extreme
Barnyard aroma	*The smell associated with the farm, barnyard, ox tail*	0 = none, 10 = extreme
Feacal aroma	*The smell associated with manure*	0 = none, 10 = extreme
Sweet taste	*Fundamental taste sensation of which sucrose is typical*	0 = none, 10 = extreme
Salt Taste	*Fundamental taste sensation of which sodium chloride is typical*	0 = none, 10 = extreme
Acidic Taste	*Fundamental taste sensation of which lactic acid is typical*	0 = none, 10 = extreme
Bitter taste	*Fundamental taste sensation of which caffeine is typical*	0 = none, 10 = extreme
Smooth Texture	*Smoothness of texture in the mouth*	0 = none, 10 = extreme
Firmness	*Thick texture in the mouth*	0 = none, 10 = extreme
Melt in mouth	*Meltability in the mouth*	0 = none, 10 = extreme
Diacetyl flavour	*The flavour associated with diacetyl, popcorn, butterscotch*	0 = none, 10 = extreme
Cream flavour	*The flavour associated with creamy/milky products*	0 = none, 10 = extreme
Artificial Butter flavour	*The flavour associated with artificial butter*	0 = none, 10 = extreme
Rancid butter	*The flavour associated with rancid or oxidised butter*	0 = none, 10 = extreme
Nutty flavour	*The flavour associated with nuts, hazelnuts*	0 = none, 10 = extreme
Barnyard flavour	*The flavour associated with the farm, barnyard, ox tail*	0 = none, 10 = extreme
Cardboard flavour	*The flavour associated with wet cardboard, linseed oil like*	0 = none, 10 = extreme
Off-flavour	*Off-flavour (Rancid)*	0 = none, 10 = extreme

Chapter 4

Impact of pasture versus indoor feeding systems on quality characteristics, nutritional composition, sensory and volatile properties of full-fat Cheddar cheese

Published in; *Journal of Dairy Science*, 2017

DOI: 10.3168/jds.2016-12508

4.1 Abstract

The purpose of this study was to investigate the effects of pasture versus indoor total mixed ration (TMR) feeding systems on the chemical composition, quality characteristics and sensory properties of full fat Cheddar cheeses. Fifty-four multiparous and primiparous Friesian cows were divided into three groups (n=18) for an entire lactation. Group 1 was housed indoors and fed a TMR diet of grass silage, maize silage and concentrates, Group 2 was maintained outdoors on perennial ryegrass only pasture (GRS), while Group 3 was also maintained outdoors on perennial ryegrass/white clover pasture (CLV). Full fat Cheddar cheeses were manufactured in triplicate at pilot scale, from each feeding system in September 2015, and were examined over a 270 d ripening period at 8 °C. Pasture derived feeding systems were shown to produce Cheddar cheeses more yellow in colour than that of TMR, which was positively correlated with increased cheese β-carotene content. Feeding system had a significant effect on the fatty acid composition of the cheeses. The nutritional composition of Cheddar cheese was improved through pasture based feeding systems with significantly lower thrombogenicity index scores and a greater than two-fold increase in the concentration of vaccenic acid and the bioactive conjugated linoleic acid c9t11, while TMR derived cheeses had significantly higher palmitic acid content. Fatty acid profiling of cheeses coupled with multivariate analysis showed clear separation of Cheddar cheeses derived from pasture-based diets (GRS or CLV) from that of a TMR system. Such alterations in the fatty acid profile resulted in pasture derived cheeses having reduced hardness scores at room temperature. Feeding system and ripening time had a significant effect on the volatile profile of the Cheddar cheeses. Pasture derived cheeses had significantly higher concentrations of the hydrocarbon toluene, while TMR derived Cheddar cheese had significantly higher concentration of 2,3-butanediol. Ripening period resulted in significant alterations to cheeses volatile profile with increases in acid, alcohol, aldehyde, ester and terpene based volatile compounds. In conclusion, this study has demonstrated the benefits of pasture derived feeding systems for production of

Cheddar cheeses with enhanced nutritional and rheological quality compared with a TMR feeding system.

4.2 Introduction

Cheese has been targeted as a vital, added value, end product for the increased milk pool now available in Ireland following the abolition of European Union milk quota's in 2015 [1]. Many variables can affect the quality, composition and consumer acceptability of cheeses, including the manufacturing processes, initial composition, and the quality of raw milks used [2]. Cow feeding systems have long been identified as a major factor affecting the composition and quality of milk [3] and dairy products such as cheese [4, 5]. The feeding system utilised by farmers is principally dictated by the cow's nutritional requirements, climatic conditions and land availability. Irelands agricultural system is based around the use of pasture as a low cost primary feed source where cows are calved in the spring and maintained outdoors for the majority of their lactation [6-8], unlike farming systems where cows are maintained indoors year-round on a total mixed ration (TMR) diet of forage and concentrates as practised in the US, parts of Europe and parts of the southern hemisphere [9, 10]. Milk and dairy products are highly nutritious food items and a significant source of dietary proteins and fats. Conjugated linoleic acid (CLA) is a fatty acid (FA) bioactive isomer unique to rumen animal derived milks and meats, widely studied because of its many health benefiting attributes and interesting biological functions. Yang, *et al.* [11] reviewed the array of benefits associated with CLA which include impacting immune function, and protective affects against cancer, obesity, diabetes and atherosclerosis in animal and human cell line studies. Milk is also a source of omega 3 (*n*3) and omega 6 (*n*6) fatty acids, the ideal ratio of *n*3 to *n*6 fatty acids in the diet has been reported to be 1:1-4. However, changes in the western diet in recent years, with increased consumption of fat and vegetable oils rich in *n*6 FA have resulted in this ratio now being between 1:10 and 1:20 [12]. There is a consumer perception that dairy products from cows maintained outdoors consuming fresh grass is "more natural" than that of TMR farming systems [13]. This perception has become a major marketing scheme for countries such as Ireland and New Zealand, practicing fresh grass feeding, for promotion of dairy products. The inclusion of

pasture in the diets of lactating dairy cows has been shown to improve the nutritional composition of milks particularly in terms of C18:2 CLAc9t11 and the *n*3 FA. Couvreur*, et al.* [14] demonstrated a linear relationship between the proportion of fresh grass in the diet and CLA content of milks. Coakley*, et al.* [5] demonstrated that supplementation of cows on pasture with unsaturated FA resulted in significantly higher CLA than that of a TMR diet. A recent review of the topic has highlighted that the required consumption of CLA to observe health benefits range between 0.8 and 3.0 g/day, however the estimated current average human consumption in Europe, US and Canada is 0.21 g/d [15]. While consumption of milk with an improved nutritional composition can be very beneficial to the individual's overall diet, the amounts required to achieve recommended levels of these bioactive nutrients in certain cases may not be feasible. Cheese however*,* is a widely consumed food item, and as such, increased intake of beneficial nutrients per meal is possible. Therefore, cheeses with naturally improved nutritional composition offer an excellent opportunity to attain such desired levels of nutrients.

Typically, in Ireland, Cheddar cheese is ripened for 6-9 months before sale. During the production and ripening process, several microbial and biochemical processes such as glycolysis, lipolysis and proteolysis occur, contributing to the characteristics and acceptability of the final cheese product. Cheese flavour is highly dependent on these ripening related reactions, and compounds produced from the hydrolysis and metabolism of cheese macronutrients [16], which in turn are influenced by raw milk composition and quality. While research has investigated the effects of different cow feeding systems on the quality and composition of milk and dairy products*,* there are limited studies available comparing Irelands somewhat unique pasture only based systems, to more conventional TMR feeding systems, on the quality and characteristics of Cheddar cheeses.

The objective of this study was to investigate the effects of three feeding systems a TMR diet indoors, perennial ryegrass (*Lolium perenne* L.) outdoors (**GRS**), and perennial ryegrass/white

clover (*Trifolium repens* L.) outdoors (**CLV**) on the characteristics, nutritional composition, sensory and volatile properties of Cheddar cheeses throughout a nine-month ripening period.

4.3 Materials and Methods

4.3.1 Reagents

Hexane, heptane, formic acid, 25% sodium methoxide, valeric acid ($C_{5:0}$), undecylic acid ($C_{11:0}$) and margaric acid ($C_{17:0}$) were purchased from Sigma Aldrich (Dublin, Ireland). Diethyl ether was purchased from Fisher Scientific (Dublin, Ireland). Certified free fatty acid (FFA) standard (STD) mix containing $C_{4:0}$-$C_{22:0}$ free acids (GLC Reference standard 74 "Free acid"), trinonadecanoin acid ($C_{19:0}$) (part number: T-165) and a standard mix of conjugated linoleic acid (CLA) C18:2,c9t11 and C18:2,t10c12 (part number: UC-59M) were purchased from Nu-Chek-prep, Inc. (Minnesota, USA). Fatty acid methyl ester (FAME) triglyceride standard mix containing C4:0-C24:0 methyl esters (part no: 18919-1AMP) was purchased from Sigma Aldrich (Dublin, Ireland). 500 mg aminopropyl cartridges (part no. 12102041) were obtained from Agilent technologies (Little Island, Cork, Ireland).

4.3.2 Experimental Design

Experimental design for this study was the same as that previously described in studies which investigated the butters [17] and raw milks [18] from these feeding systems. Fifty-four spring calving Friesian cows were allocated to three groups (n=18) at the Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland. Groups were randomized based on milk yield, milk solids yield, calving date (mean calving date 19th February 2015) and lactation number. Group 1 was housed indoors and fed a TMR diet, Group 2 was maintained outdoors on perennial ryegrass only pasture (GRS) while Group 3 was also maintained outdoors on a perennial ryegrass/white clover pasture (CLV). For further information on the chemical and nutritional values of each of the diets see O'Callaghan*, et al.* [18]. Milking took place at 07:30 and 15:30 h daily. In order to obtain a representative sample

of milk, the cows in each of the three feeding systems were milked separately into designated 5000 L refrigerated tanks. The evening milk was stored at 4 °C overnight, to which the morning milk was then added, tanks were maintained at 4 °C and agitated prior to sample collection. Milk was collected from each of the groups in the trial for cheese manufacture on three separate occasions over a five-week period in September and early October 2015. Triplicate batches of Cheddar cheeses were produced from each feeding system, when cows were 196 ± 10 days in lactation on their respective diets, each of the Cheddar cheeses within each batch were manufactured on the same day.

4.3.3 Sample Collection and Cheddar Cheese Manufacture

Cheddar cheese making trials were performed in triplicate at pilot scale in Moorepark Technology Ltd. (MTL, Teagasc Moorepark, Fermoy Co. Cork, Ireland) with milk from each of the three herds in this study only. Cheese making trials were performed on the 3rd of September, 25th of September and 2nd of October, 2015. Approximately 550-600L of morning and evening milks were collected from each of the herds over a 3-day period as mentioned above. The milks from each herd were standardised to a protein:fat ratio of 0.95. Milks were pasteurised at 72°C for 15 s cooled to 31°C and pumped into 500 L stainless steel jacketed vats with automated variable-speed cutting and stirring equipment (APV Schweiz AG, Worb, Switzerland). A starter inoculum composed of *Lactococcus lactis* ssp *lactis*, *Lactococcus lactis* ssp *cremoris* and *Streptococcus thermophilus* [A2055] (Chr. Hansen Ireland Ltd, Co. Cork, Ireland), with an adjunct culture *Lactobacillus helveticus* [LHBo2] (Chr. Hansen Ireland Ltd, Co. Cork, Ireland) was used and the cultures were added as frozen pellets directly to the cheese-making vats. After a 50min ripening interval, Chymosin (Chymax plus, Chr. Hansen Ltd., Little Island, Cork) at a rate of 0.15 mL/100 Kg of milk, was diluted in 1:5 with deionised water and added to the cheesemilk. Cheeses were then manufactured as according to Fenelon, *et al.* [19]. Cheddar cheeses were vacuum packed and stored for ripening at 8°C. Cheddar cheese blocks (24 kg) from each batch for this study were ripened, over a 9-month (270 d) period. During this time

they were analysed for chemical composition, textural properties, FA composition, proteolysis, sensory properties and analyses of volatile compounds; pH, color and texture analyses were carried out on fresh samples, for remaining analyses samples were wrapped in aluminium foil, vacuum packed and stored at -80 °C and all other analyses were carried out together at the end of the ripening period.

4.3.4 Cheese Composition

Grated cheese samples were analysed in duplicate for salt content by a potentiometric method [20], fat content of cheese was analysed using the Röse–Gottlieb method [21] and the moisture content by oven-drying at 102ºC for 5h [22]. The pH of cheese was assessed three times at 90, 180 and 270 d ripening by blending 12mL of H_2O with 20g of grated cheese and measured using a standard pH meter (Mettler Toledo MP220, Mason Technology Ltd, Dublin, Ireland) [23]. Calcium content analysis was performed by inductively coupled plasma optical emission spectrometry following microwave assisted acid digestion. Trans-β-carotene was saponified using ethanolic potassium hydroxide solution for 16 h at room temperature and extracted once with EtOH:hexane (4:3 v/v) and two times with hexane. Trans β-Carotene analysis was performed by reverse phase high performance liquid chromatography (RP-HPLC) with an ultraviolet diode array detector (UV/DAD) at 452 nm. The calibration standards used were pure compounds from Sigma Aldrich (Dublin, Ireland), purity > 98 %. The purity of the standard for each calibration was determined by a series of spectrophotometric measurements (UV 340/455/483 nm). Both methods were performed by Eurofins Food Testing (Dublin, Ireland). All chemical analysis was performed after 90 d of ripening.

4.3.5 Proteolysis

4.3.5.1 %pH4.6-SN/TN. Primary proteolysis depicted by concentration of nitrogen soluble at pH 4.6 was determined in duplicate as described by Fenelon*, et al.* [24] using the macro-Kjeldahl method [25] and was expressed as a percentage of total nitrogen.

4.3.5.2 Free Amino Acids (FAA). Individual FAA were determined on the pH 4.6-SN extracts as described by Fenelon, *et al.* [19] using a Jeol JLC-500V AA analyser fitted with a Jeol Na^+ high performance cation exchange column (Jeol Ltd., Tokyo, Japan). The chromatographic analyses were conducted at pH 2.2 on samples after ripening for 90, 180 and 270 d.

4.3.6 Cheese Fatty Acid Analysis

Lipid extraction, FFA solid phase extraction and triglyceride analysis was carried out as described by O'Callaghan, *et al.* [17]; with the exception that prior to solvent extraction ~5 g of Cheddar cheese was ground with 10g of anhydrous sodium sulphate in a pestal and mortar, 0.3 mL of 2.5 M H_2SO_4 and 1 mL of internal free acid standard (ISTD) ($C_{5:0}$, $C_{11:0}$, $C_{17:0}$ at 1000 ppm in heptane) was added to the sample mixture.

4.3.6.1 Methyl Ester Derivatisation of Free Fatty Acids extract. The FFA extract (300 µL) was transferred into a capped (PTFE/white silicone cap, Agilent Technologies, Little Island, Cork) 2 mL amber GC vial (Agilent technologies) TMAH reagent (60 µL) was added to the sealed vial containing the FFA extract. This was vortexed at 3000 rpm for 1 min using a pulsed bidirectional spin of 5 s with a 1 s pause. Deionised water (300 µL) was added to the mixture and this was further vortexed for one minute at 1000 rpm. An aliquot of 100 µL of this aqueous layer was transferred to a sealed GC vial containing a 250 µL glass insert (Part no: 5181-8872, Agilent Technologies) and 2.0 µL was injected onto the GC for analysis. A glass insert was used to allow for such a small volume to be sampled correctly using a GC auto-sampler.

4.3.6.2 Instrument Conditions for Analysis of FFA's. The injector was held at 300 °C for the entire run and was operated in split/splitless mode using a split of 1:20. The inlet liner used was a wool packed liner (Part no: 8004-0118, Agilent Technologies, Little Island, Cork, Ireland). The column oven was held at 40 °C for 2 min and raised to 240 °C at 7.5 °C/min

and held for 7 min. The total runtime was 35.67 min. The FID was operated at 300 °C. The carrier gas was helium and was held at a constant flow of 1.2 mL/min.

Health indices of cheese triglyceride content, atherogenecity index (AI) and thrombogenicity index (TI), have been calculated as described by Ulbricht and Southgate [26];

$$AI = \frac{C12{:}0+(4\ x\ C14{:}0)+C16{:}0}{n6\ \text{PUFA}+n3\ \text{PUFA}+\text{MUFA}}$$

$$TI = \frac{C14{:}0+C16{:}0+C18{:}0}{(0.5\ \text{x MUFA})+(0.5\ x\ n6PUFA)+(3\ x\ n3\ PUFA)+(\frac{n3PUFA}{n6\ PUFA})}$$

4.3.7 Texture Profile Analysis

Six cube-shaped samples (2.5 cm) were cut from each cheese in each batch, wrapped tightly in aluminium foil and stored at refrigeration temperature (4 °C) overnight. Texture analysis was performed on cheese cubes at refrigerated temperatures after 90 and 270 d of ripening, and texture analysis was also carried out on cheese cubes at room temperature after 270 d of ripening. For refrigerated analysis each cube was taken from the refrigerator and immediately compressed in two successive bites, to 60 % of its original height at a rate of 60 mm/min on a TAHDi texture profile analyser (TPA) (Stable Micro Systems, Surrey, England) at room temperature. For analysis of cheese cubes at room temperature, cubes were left wrapped in tin foil in a temperature controlled room at 20 °C for three hours to acclimatize and were then analysed as outlined above. The following parameters were defined from the resultant force/ time curve, as hardness, chewiness, springiness, and cohesiveness as described and defined by Gunasekaran and Ak [27]

4.3.8 Color

Colour measurements were taken from the surface of Cheddar cheese blocks after 90, 180 and 270 d of ripening. Five replications of L* (lightness-darkness), a* (green-red) and b* (blue-yellow) values were taken at random locations across the surface of the cheeses using a Minolta Chroma-Meter CR-400 (Mason Technology Ltd, Dublin, Ireland).

4.3.9 Volatile Analysis of Cheeses

Volatile analysis of cheeses was performed after 90, 180 and 270 d of ripening using similar method as described by O'Callaghan*, et al.* [17] with 4g of grated cheese samples from each trial were analyzed in triplicate.

4.3.10 Sensory Analysis

4.3.10.1 Sensory Affective Evaluation. Twenty-four consumers were recruited in University College Cork, Ireland. Sensory acceptance testing was conducted using these untrained assessors [29, 30]. Age range of assessors was 21-48 years old. Selection criteria for consumers were availability and motivation to participate on all days of the experiment and that they were Cheddar cheese consumers. Assessors used the sensory Hedonic descriptors in Supplementary Table 4.1 for Cheddar cheeses from each of the three feeding systems, presented in triplicate (from each manufactured batch) over two time periods, 180 and 270 d ripening. Sensory analysis was carried out in panel booths conforming to International Standards [31]. All samples were blast frozen to -20 ˚C then stored at -20 ˚C until required. Samples were then held at refrigeration temperatures overnight (4 ºC), before monadic presentation to the naïve assessor panel at ambient temperatures (21 °C) and coded with a randomly selected 3-digit code. The cheese was immediately served to consumers monadically. Each assessor was provided with deionised water and instructed to cleanse their palates between tastings. Additionally, each assessor was asked to indicate their degree of liking on a 10 cm line scale ranging from 0 (extremely dislike) at the left to 10 (extremely like) at the right and rating subsequently scored in cm from left. The order of the presentation of all test samples was randomized to prevent first order and carryover effects and all samples were presented in triplicate.

4.3.10.2 Ranking Descriptive Analysis. Twenty-five panellists were recruited in University College Cork, Ireland. Age range of assessors was 22-48 years old. Selection criteria for panellists were availability and motivation to participate on all days of the experiment and that

they were Cheddar cheese consumers. Panellists used the sensory intensity descriptors described in Supplementary Table 4.1. Ranking descriptive analysis (RDA) [32, 33] was carried out in panel booths conforming to international standards [31] on the three Cheddar cheese samples to be tested over the two time points of ripening (180 and 270 d). All samples were stored at -20 ˚C until required. Samples were then held at refrigeration temperatures overnight (4 ºC), before being presented to the consumer panel at ambient temperatures (21 °C) and coded with a randomly selected three-digit code. The cheeses were immediately served to panellists simultaneously for separate time points. All analysis was conducted in triplicate significantly increasing the validity of results [29, 34].Therefore each sample was tested 75 times (25*3) for each hedonic attribute and the significant trends of the correlation of treatments and sensory variables are discussed. Each assessor was provided with deionised water and instructed to cleanse his/her palates between tastings. Additionally, each assessor was presented with triplicate samples (over separate sessions) and asked to assess the intensity of the attributes, according to a 10cm line scale ranging from 0 (none) at the left to 10 (extreme) at the right and rating subsequently scored in cm from left. The order of the presentation of all test samples was randomized to prevent first order and carryover effects. The panellists used in the RDA were trained in partial accordance with Richter*, et al.* [32]. Again, one training session was conducted to demonstrate the score sheet used in the RDA. In Richter*, et al.* [32] due to the number of attributes, the panel opted to evaluate the samples in two phases sequentially: one for analysis of appearance and aroma attributes and another for texture and flavor attributes. However, in the presented study the assessors opted to evaluate all attributes over a single session as they had previously participated in sensory descriptive analysis of cheddar cheese and were familiar with the lexicon presented. Again, each assessor was also presented with triplicate samples (over separate sessions) and asked to assess the intensity of the attributes. Ranking descriptive analysis has been successfully demonstrated for a number of other products including: Chocolate pudding [32] White pudding [35], Black Pudding [36],

Butter [17] and Mozzarella cheese [37],to mention just a few. Although not a replacement of QDA, the RDA approach uses a much reduced lexicon (19 attributes), compared to QDA, and identified the main sensory trends between treatments and sensory variables for the sample set analysed.

4.3.11 Statistical Analysis

Statistical analysis was performed using SPSS v18.0 (IBM Statistics Inc., Armonk, NY, USA). Datasets were analysed for normality using the Shapiro-Wilk's test and for homogeneity of variance using the Levene's test. Analysis which was carried out at only one time point and were normally distributed were analysed using one-way ANOVA with post hoc Tukey test. Datasets from analysis which was carried out at several time points throughout the study a Between- and Within-Subjects repeated measures ANOVA with post hoc Tukey test was used to compare Cheddar cheeses from herds on different feeding systems (TMR, GRS and CLV) (treatment) throughout 270 d ripening period (time). Pearsons correlation analysis between attributes was carried out in GraphPad Prism v7.01 for Windows (GraphPad Software, La Jolla California USA). Multivariate data analysis (partial least squares regression (PLSR) and principal component analysis (PCA) was applied to investigate relationships between triglyceride FA, sensory analysis and volatiles analysis data and the experimental treatments (different feeding systems) using The Unscrambler® X multivariate analysis program, v10.3 (CAMO ASA, Trondheim, Norway). *P*-values < 0.05 were considered significant.

Table 4.1: Mean (± standard deviation) composition of cheeses derived from different feeding systems; TMR, perennial ryegrass (GRS) and perennial ryegrass and white clover (CLV) feeding systems.

	TMR	GRS	CLV	Treatment P-Value
Protein (% wt/wt)	26.70 ± 0.10	26.65 ± 0.39	26.64 ± 0.37	0.98
Fat (% wt/wt)	30.95 ± 0.20	30.54 ± 0.25	31.28 ± 0.16	0.03
Moisture (% wt/wt)	35.81 ± 0.39	36.21 ± 0.60	35.93 ± 0.70	0.78
[1]F/DM (% wt/wt)	48.21 ± 0.40	47.88 ± 0.07	48.84 ± 0.78	0.24
[2]MNFS (% wt/wt)	51.85 ± 0.57	52.13 ± 0.67	52.29 ± 1.13	0.87
Salt (% wt/wt)	1.87 ± 0.07	1.83 ± 0.06	1.80 ± 0.07	0.56
[3]S/M (% wt/wt)	5.23 ± 0.25	5.05 ± 0.24	5.00 ± 0.18	0.61
Ash (% wt/wt)	3.95 ± 0.18	4.01 ± 0.08	3.82 ± 0.05	0.33
Calcium (% wt/wt)	0.84 ± 0.01	0.82 ± 0.02	0.81 ± 0.02	0.46
β-Carotene (mg/Kg of Cheese)	0.57 ± 0.05	1.40 ± 0.06	1.21 ± 0.03	< 0.001

[1]Fat in dry matter, [2]Moisture in non-fat substances, [3]salt in moisture

4.4 Results and Discussion

4.4.1 Cheese Composition

Results for chemical analyses of cheeses are shown in Table 4.1. The chemical composition of the cheeses (fat, protein, moisture) are similar to those previously described for a full fat Cheddar cheese [38, 39]. Feeding system had a significant effect on the β-carotene content of the cheeses; GRS derived Cheddar cheeses having the highest β-carotene content, which was significantly higher than CLV ($P = 0.02$). Both GRS and CLV-derived cheeses had a β-carotene concentration more than twice that of TMR cheese ($P = <0.001$). Feeding system did not have a significant effect on the pH of cheeses during ripening. There was a significant increase ($P = <0.001$) in cheese pH throughout ripening as expected, which increased from (mean ± standard deviation) 5.27 ± 0.04 after 90 d to 5.43 ± 0.06 after 270d ripening. Previous studies investigating the effects of feeding systems have also reported no differences in the chemical compositions of cheeses [5]. Increased β-carotene content of pasture derived cheeses is in agreement with β-carotene levels of the butters produced from theses diets [17]. The level of β-carotene in dairy products is highly dependent on the dietary supply of the pigment, which is most abundant in fresh pastures and is significantly depleted in conserved forages, and very low in maize or grain based diets resulting in whiter dairy products [40]. As a result, the β-carotene content of dairy products has previously been suggested as a potential biomarker for milk products from pasture based feeding systems [41].

4.4.2 Color

Feeding system and ripening period had a significant effect on the color of Cheddar cheeses, as shown in Figure 4.1. The TMR derived cheeses had significantly higher ($P = 0.002$) L* scores than GRS and CLV cheeses, at (mean ± standard deviation) 80.19 ± 1.51, 77.97 ± 1.63 and 77.89 ± 1.53, respectively. There was no significant difference between GRS and CLV L* values. The L* values of each of the cheeses increased significantly throughout ripening ($P = 0.001$). The GRS and CLV derived cheeses had the lowest a* values on average throughout

ripening at -4.31 ± 0.59 and -4.25 ± 0.51, respectively, which were significantly lower than TMR at -3.99 ± 0.52 (P = 0.12 and P = 0.03, respectively). The a* values of the cheeses increased significantly throughout ripening (P = 0.01). Feeding system had a significant effect on the b* value of cheeses, GRS and CLV cheeses had significantly higher b* values than that of TMR (P = <0.001) at 35.42 ± 1.78, 33.07 ± 1.45 and 25.23 ± 1.04, respectively. The b* values of cheeses decreased significantly throughout ripening (P = <0.001). The increases in L*, a*, and decrease of b* values throughout ripening indicated that each of the cheeses became paler in color as ripening progressed. Juric*, et al.* [42], also reported an increase in a* value and decrease in b* values of semi hard cheeses stored in modified atmosphere packaging over ripening which was attributed to light induced degradation of carotenoids and riboflavin. Overall, these results suggest that the TMR derived cheeses were paler in color compared to the pasture derived cheeses, and the GRS and CLV cheeses were more yellow in color than the TMR-derived product. The color of dairy products is highly dependent on their carotenoid content [41]. Indeed, the increased b* values of the cheeses were highly correlated to their β-carotene content (P = < 0.001, Pearsons r (r) 0.948) while the L* values of the cheeses were negatively correlated with the β-carotene content (P = 0.004; r -0.841). Coppa*, et al.* [43] reported similar alterations to the b* value of Cantal cheeses between cows fed a hay and concentrate based diet versus pasture based diets.

4.4.3 Proteolysis

%pH 4.6-SN/TN. Overall, feeding system did not affect the primary proteolysis of cheeses during ripening. However, a significant increase in %pH 4.6-SN/TN was recorded for all cheeses as ripening progressed. For all cheeses %pH 4.6-SN/TN increased from on average 12.31 ± 0.92 to 21.94 ± 0.43% between 90 and 270d ripening (P = < 0.001) (Supplementary Figure 4.1). The increasing trend in %pH 4.6-SN/TN over ripening is comparable to other Cheddar cheese studies [44-46]. The increase in %pH 4.6-SN/TN over ripening has been attributed to the breakdown of intact casein proteins primarily by the action of residual

chymosin, proteinase enzymes and the proteolytic activity of starter cultures [47]. Proteolysis during ripening of cheeses can have a significant effect on several cheese attributes including development of texture and flavor [19, 48], which in turn can affect the overall acceptability of the cheeses. It was, perhaps, unsurprising that feeding system did not have a significant effect on the level of primary proteolysis in Cheddar cheeses given the major differences from feeding regimen are associated with the fatty acid composition of milks in this study, rather than the proteins. Fenelon*, et al.* [19] reported a difference in Cheddar cheese proteolysis with increasing fat contents of Cheddar cheeses and this was primarily attributed to an increased protein content with concomitant decrease in fat content.

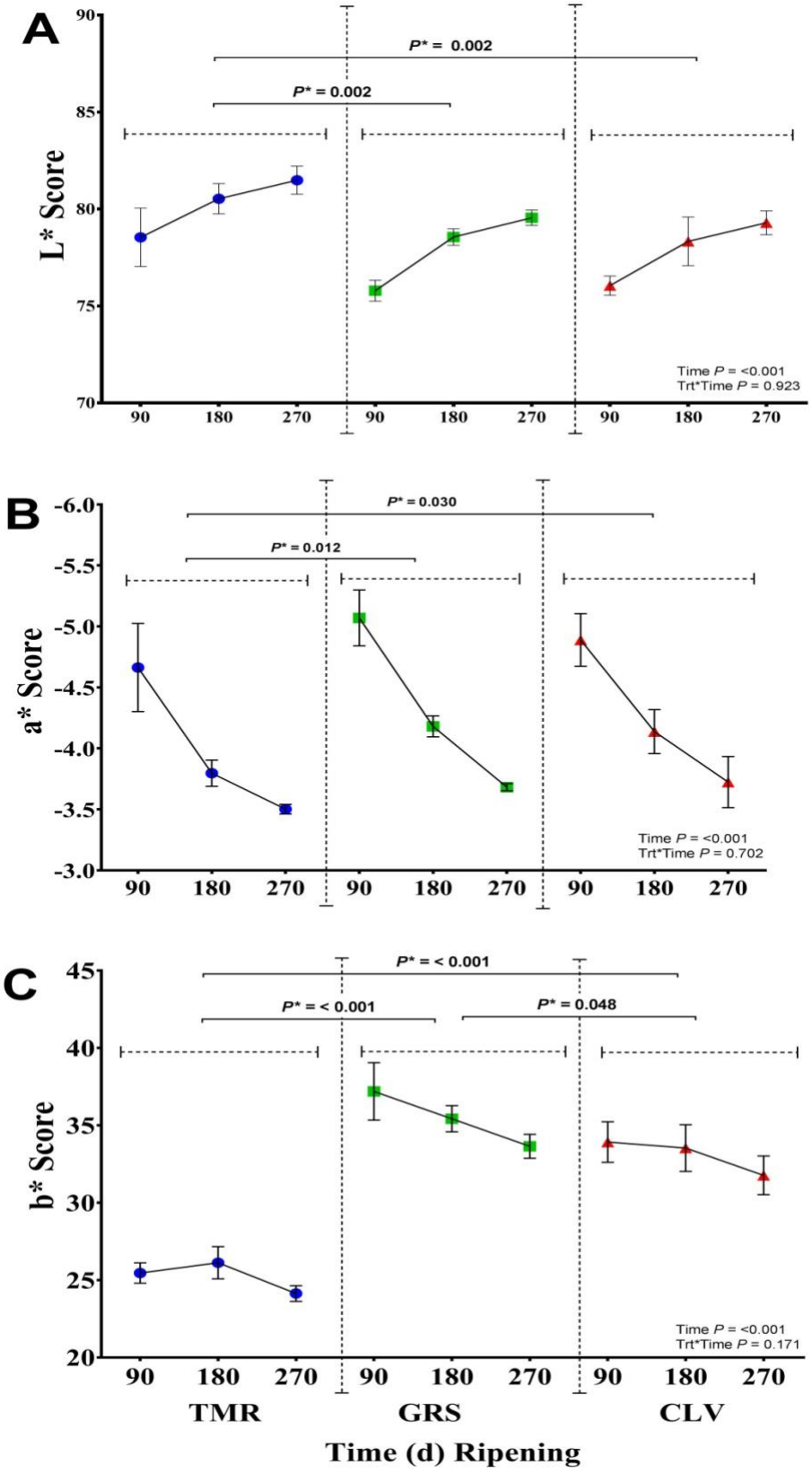


Figure 4.1: Colour measurements (mean ± standard deviation) of Cheddar cheeses derived from different feeding systems (TMR (●), GRS (■) and CLV (▲)) throughout ripening for 270d according to L*, a* and b* scale. P* denotes treatment (Trt) significant effects.

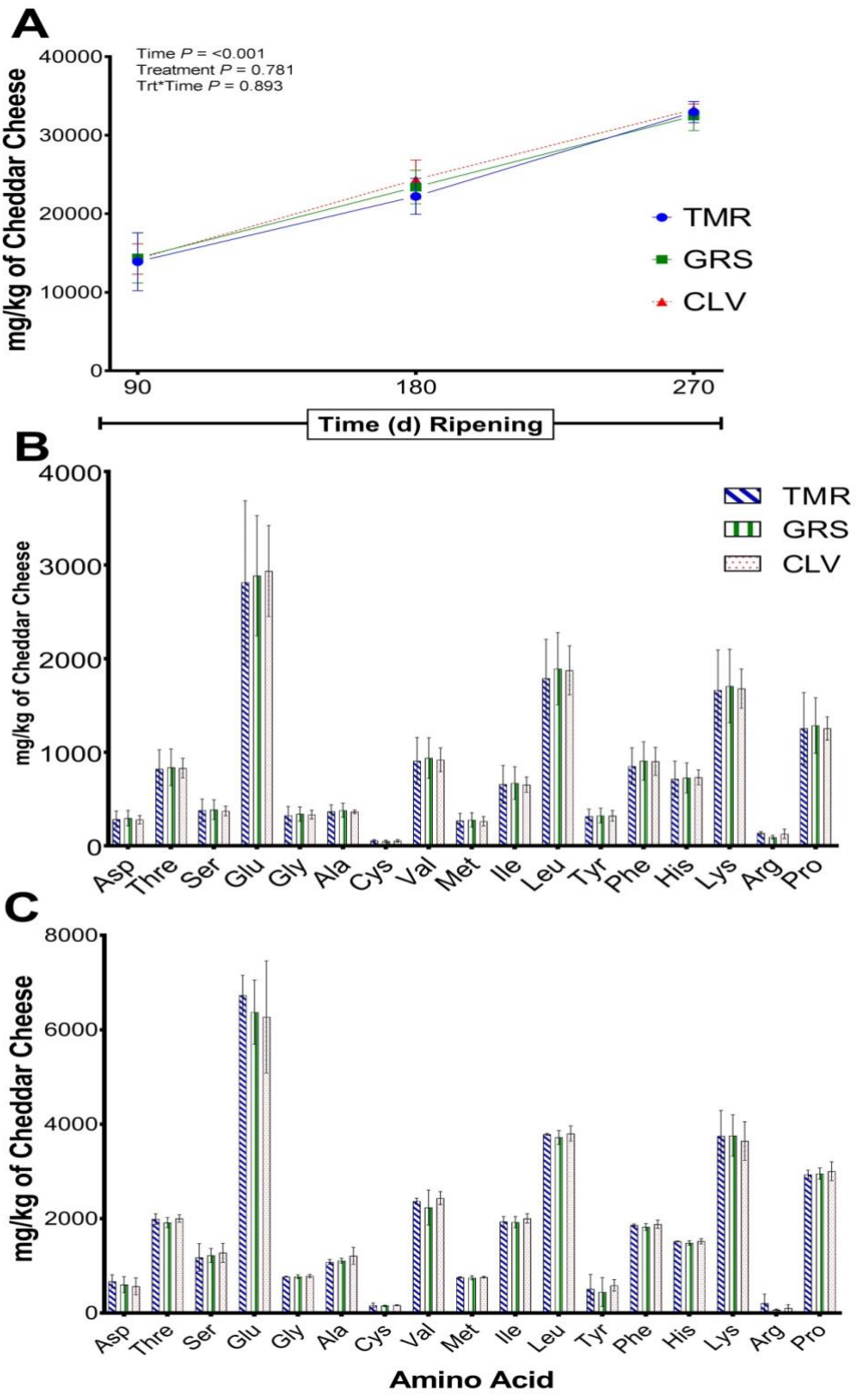


Figure 4.2: (A) Total free amino acids content in Cheddar cheeses derived from different cow feeding systems TMR (●), GRS (■) and CLV (▲) throughout ripening for 270d. Free amino acid profile of Cheddar cheeses at 90d (Figure 4.4B) ripening and 270d (Figure 4.4C) ripening (mean ± standard deviation)

Table 4.2: Mean fatty acid triglyceride (g/100g of cheese fat) contents of Cheddar cheeses derived from TMR, perennial ryegrass (GRS) and perennial ryegrass and white clover (CLV) feeding systems.

FA Triglycerides	TMR	GRS	CLV	Treatment
Butyric Acid (C4:0)	3.59 ± 0.15	3.58 ± 0.14	3.68 ± 0.14	0.768
Caproic Acid (C6:0)	1.96 ± 0.06	1.88 ± 0.04	1.93 ± 0.07	0.415
Caprylic Acid (C8:0)	1.09 ± 0.03	1.03 ± 0.02	1.07 ± 0.04	0.297
Capric Acid (C10:0)	2.41 ± 0.06	2.26 ± 0.05	2.35 ± 0.08	0.166
Undecanoic Acid (C11:0)	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.013
Lauric Acid (C12:0)	2.79 ± 0.06	2.63 ± 0.07	2.71 ± 0.09	0.152
Tridecanoic Acid (C13:0)	0.07 ± 0.00	0.06 ± 0.01	0.05 ± 0.00	0.014
Myristic Acid (C14:0)	8.72 ± 0.35	8.80 ± 0.22	7.55 ± 2.27	0.599
Myristoleic Acid (C14:1)	0.93 ± 0.01	1.01 ± 0.02	0.94 ± 0.03	0.021
Pentadecanoic Acid (C15:0)	0.83 ± 0.02	1.00 ± 0.03	0.94 ± 0.04	0.004
Palmitic Acid (C16:0)	26.62 ± 0.83	23.27 ± 0.66	22.80 ± 1.19	0.012
Palmitoleic Acid (C16:1)	1.43 ± 0.04	1.39 ± 0.04	1.29 ± 0.06	0.091
Heptadecanoic Acid (C17:0)	0.52 ± 0.02	0.61 ± 0.04	0.61 ± 0.02	0.042
Stearic Acid (C18:0)	6.15 ± 0.48	6.97 ± 0.92	7.37 ± 0.69	0.300
Vaccenic Acid (C18:1t11)	1.43 ± 0.11	3.23 ± 0.26	2.74 ± 0.18	< 0.001
Oleic Acid (C18:1n9c)	13.78 ± 0.53	14.23 ± 1.70	14.49 ± 1.17	0.843
Linolelaidic Acid (C18:2n6t)	0.15 ± 0.01	0.36 ± 0.03	0.42 ± 0.02	< 0.001
Linoleic Acid (C18:2n6c) (LA)	1.45 ± 0.09	0.49 ± 0.04	0.69 ± 0.03	< 0.001
α-Linolenic Acid (C18:3n3) (ALA)	0.26 ± 0.03	0.62 ± 0.09	0.98 ± 0.08	< 0.001
CLA (c9t11)	0.49 ± 0.02	1.45 ± 0.12	1.13 ± 0.06	< 0.001
CLA (c12t10)	0.11 ± 0.00	0.10 ± 0.01	0.10 ± 0.00	0.579
Eicosenoic Acid (C20:1)	0.02 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.003
Eicosatrienoic Acid (C20:3n6)	0.08 ± 0.01	0.01 ± 0.01	0.03 ± 0.00	0.001
Behenic Acid (C22:0)	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.309
Erucic Acid (C22:1n9)	0.05 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	< 0.001
Tricosanoic Acid (C23:0)	0.01 ± 0.01	0.10 ± 0.01	0.11 ± 0.01	< 0.001
Arachidonic Acid (C20:4n6)	0.00 ± 0.00	0.05 ± 0.01	0.06 ± 0.00	< 0.001
Saturated	54.82 ± 2.28	52.28 ± 2.18	52.66 ± 1.86	0.350
Unsaturated	20.16 ± 0.83	23.04 ± 2.31	22.98 ± 1.66	0.196
MUFA	17.63 ± 0.69	19.95 ± 2.04	19.57 ± 1.46	0.275
PUFA	2.54 ± 0.15	3.14 ± 0.29	3.47 ± 0.20	0.011
Short Chain C4-14	21.59 ± 0.81	21.30 ± 0.90	21.75 ± 0.75	0.791
Medium Chain C15-17	29.40 ± 0.99	26.28 ± 1.10	25.63 ± 1.31	0.017
Long Chain C18-24	24.00 ± 1.33	27.74 ± 3.18	28.25 ± 2.46	0.208
Omega 3 (n3)	0.26 ± 0.03	0.62 ± 0.09	0.98 ± 0.09	< 0.001
Omega 6 (n6)	1.68 ± 0.11	0.91 ± 0.09	1.19 ± 0.07	< 0.001
Omega 9 (n9)	13.83 ± 0.62	14.25 ± 1.78	14.52 ± 1.31	0.856
n3 / n6	0.16 ± 0.01	0.68 ± 0.03	0.82 ± 0.04	< 0.001
Hardness Index (C18:1n9c/C16:0)	0.52 ± 0.01	0.61 ± 0.08	0.64 ± 0.08	0.221
Atherogenecity Index (AI)	3.29 ± 0.04	2.87 ± 0.27	2.84 ± 0.23	0.119
Thrombogenic Index (TI)	3.92 ± 0.05	3.03 ± 0.24	2.78 ± 0.19	0.002

Statistical analysis by one way ANOVA with post hoc Tukey test.

*n*3; sum of α-linolenic acid (ALA), *n*6; sum of linolelaidic acid, linoleic acid (LA), γ-Linoleic Acid, eicosatrienoic acid and arachidonic acid; *n*9; sum of oleic acid and erucic acid.

Total and Free Amino Acid Analysis. The effects of feeding system on the total free amino acids (TFAA) throughout ripening is shown in Figure 4.2A. Feeding systems did not have a significant effect on the TFAA and individual FAA content throughout ripening. A significant increase in the TFAA (P = <0.001) during the 270 d of ripening was observed. Between 90 and 270d of ripening TFAA increased from 14,172 ± 2,490 to 32,893 ± 1179 mg/kg of cheese, respectively. The concentrations of individual FAA after 90 d and 270 d of ripening are shown in Figure 4.2B and Figure 4.2C respectively. Increased levels of FAA in cheeses throughout ripening has been attributed to the peptidase activity of lactic acid bacteria, particularly *Lactobacillus*. Hydrolysis of casein, during primary proteolysis results in the release of peptides which are subsequently hydrolyzed by intracellular peptidases of *Lactobacillus* [49, 50]. Free amino acids produced during this process have a significant effect on the subsequent development of flavor properties in cheeses [48].

4.4.4 Cheese Fatty Acid Composition

Table 4.2 shows the triglyceride composition of the cheeses after 90 d ripening. Feeding system had a significant effect on cheese triglyceride composition. Such differences between feeding systems followed similar trends to that of the raw milks from these diets previously reported [18]. Indeed, Coakley, *et al.* [5] and Lucas, *et al.* [51] reported that the cheese making process had little effect on the triglyceride composition of cheeses. Among the major FAs, TMR derived cheeses had a significantly higher content of palmitic acid ($C_{16:0}$) compared with GRS (P = 0.025) and CLV (P = 0.014) derived cheeses. The pasture derived cheeses had significantly higher vaccenic acid (VA) content ($C_{18:1t11}$) than that of TMR cheeses with a greater than 1.8-fold increase in VA (P = <0.001), while there was no significant difference in VA content of GRS and CLV cheeses. Pasture derived cheeses had significantly higher (P = <0.001) concentrations of linolaidic acid ($C_{18:2n6t}$) than TMR-derived cheese. Total mixed ration

Cheddar cheese had significantly higher contents of linoleic acid ($C_{18:2n6c}$) than that of CLV (P = <0.001) and GRS cheese (P = <0.001), and linoleic acid content of CLV-derived cheese was significantly higher than that of GRS (P = 0.034). The concentrations of CLAc9t11 was significantly higher in pasture derived cheese than TMR derived cheeses and was present at highest concentrations in GRS cheeses which was significantly higher than CLV (P = 0.015) and TMR (P = <0.001) cheese. The GRS and CLV had significantly higher α-linolenic acid and arachidonic acid contents compared to TMR. Similar to the milks from this study, pasture derived cheeses had greater than three times more α-linolenic acid than those from TMR cheese. Similar effects of TMR or pasture feeding systems on the α-linolenic acid content of milks have been reported in the past [52, 53]. Romanzin*, et al.* [54] reported that mountain pastures produced cheeses with increased α-linolenic acid content over indoor feeding systems. Increased α-linolenic acid content of CLV derived products over GRS (P = 0.005) could also be as a result of its higher intake and ruminal passage rates, in turn supplying a higher portion of the fatty acid for absorption [55].

Such differences in the FA contents of cheeses resulted in significant differences in the health indices of FAs. Thrombogenicity score for TMR-cheese was significantly higher than that of GRS (P = 0.006) and CLV (P = 0.002). The GRS and CLV feeding systems resulted in a 2.9 and 2.3-fold increase in CLAc9t11 concentrations, respectively, compared with the TMR feeding system.

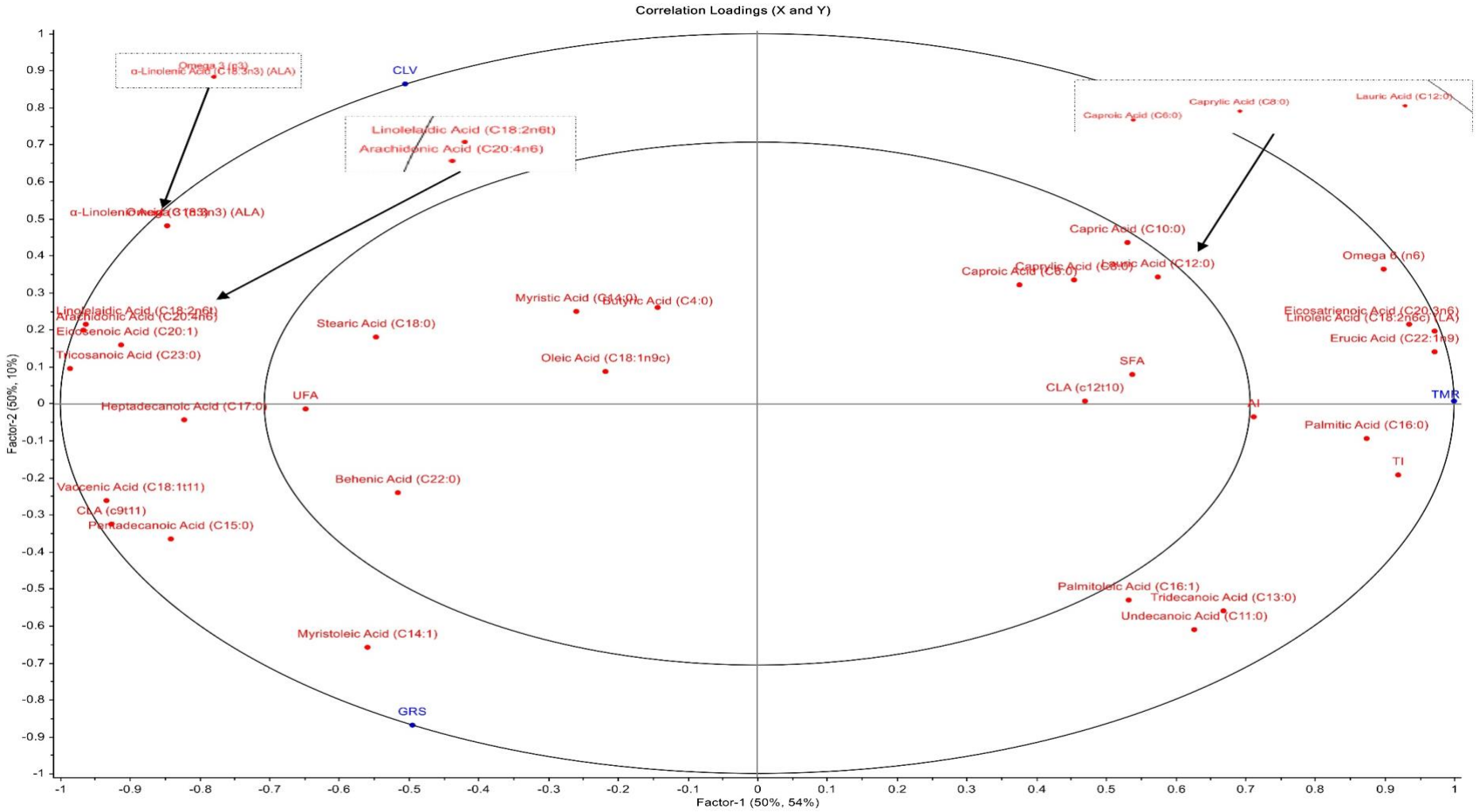


Figure 4.3: Partial least squares regression plot of Cheddar cheese fatty acid triglycerides contents after 90 days ripening from TMR, perennial ryegrass (GRS) and perennial ryegrass and white clover (CLV) feeding systems. CLA; conjugated linoleic acid, HI; Hardness Index, AI; Atherogenecity Index, TI; Thrombogenicity Index, SFA; Saturated Fatty Acids, UFA; Unsaturated Fatty Acids

Similar magnitude increase in CLAc9t11 was also reported by Romanzin*, et al.* [54], who compared the effect of mountain pastures versus indoor feeding systems on Montasio cheese. Although CLA concentration in TMR-derived cheese was similar to that reported by Coakley*, et al.* [5], in that study supplementation of pasture-fed cows with sunflower oil resulted in Cheddar cheese with a higher mean CLAc9t11 content than the pasture only cows used in this study. As mentioned, the consumption of CLA has been associated with several potential health benefits, as such Siurana and Calsamiglia [15] recommended an intake of between 0.8 and 3.2 g of CLA/d to attain such benefits based on animal models of therapeutic doses. Adjusting for the mean fat contents of cheeses in this study (Table 4.1), 100 g of Cheddar cheese from TMR feeding system would provide 0.15 g of CLAc9t11, 100 g of CLV cheese would provide 0.35 g of CLAc9t11 whereas 100 g of GRS derived Cheddar cheese from this study would provide 0.44 g of CLAc9t11. Therefore, the quantities needed to reach the minimum required CLA intake (0.8 g/day) would be much reduced from GRS and CLV cheeses than that of TMR cheese (mean ± std dev; 181.5 ± 14.5g, 226.5 ± 12.4 g and 531.5 ± 22.4 g of Cheddar cheese respectively).

Partial least squares regression analysis of triglyceride data from cheeses show complete separation of TMR and pasture cheeses based on their FA profile (see Figure 4.3). The GRS and CLV derived cheeses are separated to the left (negative) side of the plot and associated with content of CLAc9t11, vaccenic acid, arachidonic acid, linolaidic acid, α-linolenic acid, tricosanoic acid, pentadecanoic acid, eicosanoic acid and *n*3 FA content. TMR cheese is separated to the right (positive) side of the plot and is correlated with contents of palmitic acid, erucic acid, eicosatrienoic acid, tridecanoic acid, undecanoic acid, linoleic acid, higher atherogenicity and thrombogenicity scores and *n*6 FA content, which are in agreement with data in Table 4.2. Similar plots were reported for the butter and raw milks from these diets [17,

18] which further highlights the relevance of FA profile for distinguishing between pasture based and conventional TMR feeding systems.

The average FFA content of cheeses throughout ripening are shown in Table 4.3. Ripening period had a significant effect on the total FFA content of cheeses which increased, significantly on average, from ~982 mg/kg of cheese after 90 d ripening to ~1342 mg/kg FFA of cheese after 270 d ripening (Table 4.3). Hickey, *et al.* [45] concluded that starter bacteria are the major causative agents of lipolysis in Cheddar cheeses from pasteurised milk. While the FFAs produced via lipolysis can have a direct impact on cheese flavour, FFA are further metabolised to volatile compounds capable of impacting cheese flavour such as methyl ketones, lactones, esters and secondary alcohols [56].

4.4.5 Texture Analysis

Room Temperature. Feeding system had a significant effect ($P = < 0.05$) on the texture of cheeses at room temperature. Values are summarised in Table 4.4A. TMR cheeses were significantly harder than GRS ($P = 0.013$) and CLV ($P = 0.026$) chesses with hardness scores of 154.20 ± 10.08 N, 111.50 ± 4.17 N and 117.61 ± 13.68 N respectively. There was no significant difference in hardness of GRS and CLV cheeses. There was no significant effect of feeding system on the cohesiveness attribute of cheese textures at room temperature. The CLV-derived cheese had the lowest springiness values at 5.76 ± 0.13 mm which was significantly ($P = 0.017$) lower than that of GRS-derived cheese at 6.32 ± 0.15 mm. The TMR cheese had significantly higher ($P = 0.002$) chewiness values than those of GRS and CLV at 436.46 ± 19.93 J, 311.97 ± 22.48J and 277.39 ± 19.17 J respectively. At room temperature hardness of cheeses was significantly correlated with chewiness attribute ($P = 0,002$; $r = 0.879$).

Refrigerated Temperature. Feeding system did not have a significant effect on the hardness, cohesiveness and chewiness attributes of Cheddar cheeses at refrigerated temperatures. However, the springiness attribute of TMR was significantly higher ($P = 0.024$) than CLV

cheese. Significant differences in cheese texture were observed as ripening period progressed (Table 4.4B). The hardness of each of the cheeses decreased significantly ($P = 0.026$) between 90 and 270 d of ripening by ~19.14 %. The cohesiveness of cheeses dropped significantly ($P = <0.001$) between 90 d and 270 d of ripening by ~ 23.08 %. The springiness attribute of the cheeses was significantly ($P = <0.001$) reduced between 90 d and 270 d ripening by 12.28 %. Ripening time resulted in a 44.93 % reduction ($P = <0.001$) in the chewiness attribute of cheeses. At refrigerated temperature among the textural attributes, hardness was significantly correlated with chewiness ($P = <0.001$; $r = 0.897$). Cohesiveness was also significantly correlated with chewiness ($P = <0.001$; $r = 0.859$), and springiness was significantly correlated with the chewiness ($P = < 0.001$; $r = 0.906$) of cheeses. A decline in textural attributes of Cheddar cheeses during ripening has been widely reported and has been attributed to the levels of intact casein proteins as a result of primary proteolysis [38, 57, 58]. In the current study, levels of pH 4.6-SN/TN were significantly and negatively associated with each textural attribute; hardness ($P = 0.001$; $r = -0.720$), cohesiveness ($P = <0.001$; $r = -0.897$), and chewiness ($P = <0.001$; $r = -0.906$) throughout ripening.

Table 4.3: Mean free fatty acid (mg/Kg of cheese) contents of Cheddar cheeses derived from TMR, perennial ryegrass (GRS) and perennial ryegrass and white clover (CLV) feeding systems.

FFA	90 days			180 days			270 days			*P*-Value		
mg/kg of cheese	TMR	GRS	CLV	TMR	GRS	CLV	TMR	GRS	CLV	Treatment	Time	Trt x Time
C4:0	**29.72** ± 1.60	**32.32** ± 6.34	**45.12** ± 7.27	**42.22** ± 10.05	**40.78** ± 3.81	**78.64** ± 45.45	**57.26** ± 20.03	**54.06** ± 7.94	**105.11** ± 65.90	0.267	0.093	0.668
C6:0	**6.18** ± 4.45	**8.68** ± 3.39	**15.15** ± 3.44	**14.94** ± 3.32	**13.81** ± 1.52	**27.55** ± 16.86	**21.90** ± 7.75	**18.74** ± 2.35	**38.29** ± 23.67	0.244	0.049	0.747
C8:0	**8.20** ± 2.94	**9.87** ± 2.08	**15.00** ± 2.64	**13.09** ± 3.56	**10.74** ± 1.86	**24.01** ± 15.87	**18.20** ± 5.64	**14.90** ± 4.60	**31.48** ± 20.23	0.275	0.114	0.722
C10:0	**33.66** ± 4.30	**30.62** ± 7.17	**42.76** ± 0.26	**39.35** ± 1.11	**32.91** ± 2.26	**57.49** ± 24.89	**46.04** ± 7.96	**37.28** ± 3.25	**67.23** ± 31.12	0.203	0.122	0.686
C12:0	**45.19** ± 5.69	**38.91** ± 9.11	**49.41** ± 4.53	**49.80** ± 1.19	**41.11** ± 1.89	**65.49** ± 18.62	**56.31** ± 8.39	**48.11** ± 2.27	**75.53** ± 26.22	0.182	0.048	0.531
C14:0	**109.09** ± 13.92	**99.99** ± 26.07	**129.22** ± 11.94	**115.84** ± 3.31	**108.31** ± 6.51	**166.19** ± 39.54	**131.9** ± 12.39	**125.05** ± 6.72	**197.39** ± 62.74	0.110	0.044	0.453
C16:0	**371.30** ± 40.40	**317.76** ± 64.84	**370.22** ± 61.38	**405.78** ± 12.02	**337.57** ± 11.68	**427.54** ± 55.48	**428.88** ± 24.81	**364.16** ± 7.28	**451.58** ± 60.87	0.161	0.013	0.819
C18:0	**120.31** ± 13.39	**118.03** ± 25.51	**136.48** ± 11.80	**122.98** ± 6.07	**122.34** ± 5.69	**155.10** ± 29.44	**118.56** ± 9.48	**121.55** ± 11.85	**155.98** ± 28.76	0.181	0.433	0.741
C18:1	**168.71** ± 110.79	**211.91** ± 48.28	**274.80** ± 14.06	**265.15** ± 28.22	**235.40** ± 51.02	**401.97** ± 184.10	**315.40** ± 65.80	**282.06** ± 60.29	**466.55** ± 224.02	0.290	0.078	0.733
C18:2	**32.29** ± 5.05	**13.69** ± 3.27	**23.89** ± 1.04	**39.53** ± 5.34	**16.14** ± 4.46	**34.41** ± 12.71	**47.80** ± 12.15	**19.55** ± 4.98	**40.81** ± 16.81	0.045	0.011	0.713
C18:3	**2.32** ± 3.29	**11.68** ± 4.02	**24.51** ± 2.51	**5.27** ± 1.85	**13.25** ± 4.32	**37.75** ± 20.89	**6.43** ± 3.66	**16.87** ± 4.47	**46.23** ± 25.26	0.030	0.192	0.545
Total FFA	**926.97** ± 188.26	**893.46** ± 194.07	**1126.56** ± 62.27	**1113.95** ± 40.47	**972.36** ± 79.86	**1476.15** ± 458.27	**1248.40** ± 173.64	**1102.32** ± 105.25	**1676.18** ± 576.37	0.216	0.046	0.650

Overall, our results have shown that GRS and CLV derived milks yield cheeses that are less firm at room temperature than that of a TMR derived cheeses. Even though not statistically significant, the mean hardness values of TMR cheeses were higher than those from pasture at refrigerated temperatures. Similar results have been reported by Coppa*, et al.* [43] who investigated the effects of hay and concentrate or grazing based diets on texture of Cantal cheeses and reported pasture based cheeses being less firm than that of hay derived cheeses. The FA composition of the cheeses can have a significant effect on cheese texture, particularly at room temperature. Palmitic and oleic acids are the principal saturated and unsaturated FAs in dairy products with high and low melting points, respectively. The ratio of oleic acid to palmitic acid has previously been used as an index of hardness in dairy products [40]. Indeed, Coppa*, et al.* [43] reported the higher oleic to palmitic acid ratio in milk, was related to a less firm cheese. Our study reports similar results where the oleic to palmitic acid ratio was negatively correlated with cheese hardness ($P = 0.031$; $r = -0.714$) and chewiness ($P = 0.024$; $r = -0.735$) results. The increased hardness of TMR derived cheese could be attributed to the significantly higher concentrations of palmitic acid which has a higher melting point than that of unsaturated fatty acids resulting in a more solid fat at room temperature. Indeed, palmitic acid was significantly and positively correlated to hardness and chewiness attributes ($P = 0.005$; $r = 0.836$ and $P = 0{,}007$; $r = 0.816$ respectively). The increased CLA content of the pasture derived cheese was also negatively correlated with hardness ($P = 0.002$; $r = 0.877$), and chewiness ($P = 0.004$; $r = -0.849$).

Table 4.4: Textural properties of Cheddar cheese from TMR, perennial ryegrass (GRS) and perennial ryegrass and white clover (CLV) feeding systems at refrigerated and room temperature

A. Room Temperature (270 d)	TMR	GRS	CLV	Treatment *P*-Value
Hardness (N)	**154.20** ± 10.08	**111.50** ± 4.17	**117.61** ± 13.68	0.011
Cohesiveness	**0.46** ± 0.01	**0.44** ± 0.02	**0.41** ± 0.03	0.200
Springiness (mm)	**6.17** ± 0.14	**6.32** ± 0.15	**5.76** ± 0.13	0.018
Chewiness (J)	**436.46** ± 19.93	**311.97** ± 22.48	**277.39** ± 19.17	0.001

	90 days			270 days					
B. Refrigerated Temperature	**TMR**	**GRS**	**CLV**	**TMR**	**GRS**	**CLV**	**Treatment *P*-Value**	**Time *P*-Value**	**Trt × Time *P*-Value**
Hardness (N)	**249.77** ± 13.90	**219.37** ± 19.50	**220.46** ± 30.54	**188.78** ± 12.56	**190.92** ± 5.65	**177.92** ± 7.25	0.232	0.007	0.514
Cohesiveness	**0.54** ± 0.02	**0.56** ± 0.01	**0.54** ± 0.03	**0.42** ± 0.02	**0.41** ± 0.05	**0.43** ± 0.01	0.867	0.001	0.582
Springiness (mm)	**7.09** ± 0.13	**7.22** ± 0.13	**6.68** ± 0.27	**6.39** ± 0.15	**6.05** ± 0.18	**5.97** ± 0.07	0.024	0.001	0.190
Chewiness (J)	**952.56** ± 96.92	**872.66** ± 89.94	**786.76** ± 74.05	**507.14** ± 34.82	**468.68** ± 69.29	**462.65** ± 17.96	0.149	0.001	0.541

Statistical analysis determined by one way ANOVA (room temperature) and repeated measures ANOVA (refrigerated).

4.4.6 Volatiles Analysis of Cheddar Cheeses

Headspace analysis of cheeses in this study identified 39 volatile compounds which include 8 acids, 7 alcohols, 3 aldehydes, 2 benzene compounds, 4 esters, 9 ketones, 2 pyrazine compounds, 3 sulphur compounds and 1 terpene compound. Table 4.5 shows the mean volatile composition of cheeses throughout 270d ripening period. The GRS and CLV derived Cheddar cheese had significantly higher concentrations of toluene (*nutty, bitter, almond odour*) than that of TMR ($P = 0.028$ and $P = 0.015$ respectively). However, TMR derived Cheddar cheese had significantly higher concentrations of 2,3 butanediol (*fruity aroma, creamy buttery aroma*) than that of GRS ($P = 0.048$) and CLV ($P = 0.001$) derived cheeses, while the GRS derived cheese content of this compound was also significantly higher than that of CLV-derived cheese ($P = 0.003$). Toluene is believed to be present as a product of β-carotene oxidation [59] and 2,3-butanediol has been reported previously as a product of citrate metabolism [60].

Principal component analysis (PCA) of volatiles data throughout ripening (Figure 4.4) shows separation of the cheeses between stages of ripening. Ripening time appeared to have a significant effect on the volatile composition of the cheeses as expected. In particular, ripening period resulted in a significant increase in acids, alcohols, aldehydes, esters and terpene based compounds as shown in Table 4.5. Several volatile compounds appeared to be significantly correlated with changes in the chemical composition of cheeses throughout ripening (Table 4.6). The major attributes of cheese composition which appeared to affect the volatile profile of Cheddar cheeses were the levels of proteolysis, free amino acids and free fatty acids and all are reported to effect the volatile profile of Cheddar cheese. Review of the topic by Singh*, et al.* [61], discusses how catabolism of FFA contributes volatile compounds, such as methyl ketones, keto acids, and lactones, while proteolysis and FAA catabolism contributes amines, aldehydes, alcohols, pyruvate, ammonia and sulphur based volatile compounds to cheese flavor. Acidic compounds are typically derived from lipolysis, proteolysis and carbohydrate

metabolism. De Wit*, et al.* [62] reported that the principal volatile fatty acids present in cheese during ripening include acetic, propionic, butanoic, pentanoic and hexanoic acid. Ripening time in this study resulted in a significant increase in acidic compounds, acetic acid (*vinegar*), butanoic acid (*sweaty, butter, rancid*) hexanoic acid (*acidic, sweaty, cheesy*) heptanoic acid (*soapy, fatty*), octanoic acid (*pungent, waxy, cheesey*), nonanoic acid (*fatty, green, waxy*) and decanoic acid (*stale, butter, sour, fruit*). Similar increases in acidic compounds of Cheddar cheeses over ripening was reported by Gan*, et al.* [63]. Gan*, et al.* [63], also reported that heptanoic and hexanoic acid were significant in predicting the maturity of Cheddar cheese, similarly hexanoic acid (treatment*time $P = 0.031$) increased over ripening in this study. While the hexanoic acid content increased more in CLV than GRS ($P = 0.089$) and TMR ($P = 0.141$) cheeses over ripening there was no significant overall treatment effect between feeding systems ($P = 0.088$). Hexanoic acid is a common volatile compound found in cheese and is a direct result of lipolysis of milk fat [64], indeed the hexanoic content is highly correlated with the total FFA content of the cheeses ($P = <0.001$; $r = 0.891$), the significant treatment*time effect could be attributed to increased FFA in CLV cheeses. Heptanoic acid was not present in 90d samples, only present in CLV 180 samples and detected in all cheese samples after 270d ripening. Delta decalactone (*coconut-like, creamy, milk fat*) in each of the cheeses also increased significantly throughout ripening.

Table 4.5: Mean volatile compounds peak areas from Cheddar cheeses sampled after 90, 180 and 270 days ripening derived from TMR, perennial ryegrass (GRS) and perennial ryegrass and white clover (CLV) feeding systems

					90d Ripening			180d Ripening			270d Ripening				*P*-value		
	CAS	RT	LRI	Ref LRI	TMR	GRS	CLV	TMR	GRS	CLV	TMR	GRS	CLV	SEM	TRT*	Time	Trt × Time
Acids																	
Acetic acid	64-19-7	5.487	648	629	3.09E+05	7.35E+05	7.69E+05	1.16E+06	9.28E+05	1.84E+06	3.45E+06	2.66E+06	4.01E+06	4.15E+05	0.326	< 0.001	0.149
Butanoic acid	107-92-6	8.504	801	814	3.45E+06	3.08E+06	5.52E+06	5.69E+06	7.64E+06	1.16E+07	9.41E+06	6.99E+06	1.41E+07	1.15E+06	0.089	< 0.001	0.129
3 Methyl butanoic acid	503-74-2	9.579	842	848	1.12E+05	1.43E+04	5.62E+04	2.63E+05	3.70E+05	7.29E+04	1.91E+04	3.37E+04	8.00E+03	3.99E+04	0.700	0.082	0.560
Hexanoic acid	142-62-1	13.213	974	983	4.34E+05	4.30E+05	6.48E+05	7.35E+05	8.94E+05	3.20E+06	3.14E+06	1.69E+06	6.30E+06	6.18E+05	0.088	< 0.001	0.031
Heptanoic acid	111-14-8	15.604	1061	1080	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	2.87E+03	2.67E+03	4.94E+03	3.88E+03	6.28E+02	0.524	0.016	0.780
Octanoic acid	124-07-2	18.171	1157	1160	1.61E+04	2.09E+04	1.83E+04	3.39E+04	4.81E+04	1.70E+05	1.99E+05	1.13E+05	5.79E+05	5.68E+04	0.202	0.044	0.202
Nonanoic acid	112-05-0	20.579	1254	1276	0.00E+00	0.00E+00	0.00E+00	8.63E+02	9.29E+02	8.10E+03	1.18E+04	9.41E+03	1.02E+04	1.61E+03	0.201	< 0.001	0.533
Decanoic acid	334-48-5	22.879	1352	1379	7.89E+02	1.04E+03	0.00E+00	2.39E+03	3.19E+03	1.35E+04	1.29E+04	5.99E+03	2.80E+04	2.89E+03	0.260	0.017	0.155
Alcohols																	
Ethanol	64-17-5	3.813	475	426	1.56E+06	1.39E+06	1.59E+06	1.13E+06	7.43E+05	5.29E+05	1.13E+06	9.04E+05	9.02E+05	1.15E+05	0.327	0.355	0.980
1-Butanol	71-36-3	5.642	658	675	6.20E+03	0.00E+00	2.05E+04	0.00E+00	0.00E+00	0.00E+00	0.00E+00	5.98E+03	3.41E+04	3.79E+03	0.084	0.064	0.164
3-Methyl-1-butanol	123-51-3	6.946	731	733	3.16E+05	1.85E+05	2.76E+05	5.96E+05	1.28E+06	1.55E+05	6.19E+04	3.96E+05	8.96E+04	1.19E+05	0.107	0.107	0.244
2,3-Butanediol	513-85-9	9.204	828	802	1.39E+06	8.22E+05	4.13E+05	1.45E+06	6.32E+05	2.06E+05	4.42E+05	1.11E+06	5.88E+05	1.39E+05	0.001	0.984	0.315
Hexanol	111-27-3	10.246	867	868	3.31E+03	0.00E+00	2.07E+03	7.09E+03	3.36E+03	0.00E+00	1.67E+04	6.62E+03	1.81E+04	2.12E+03	0.087	0.002	0.346
2-Ethyl hexanol	104-76-7	14.663	1026	1031	1.38E+04	1.94E+04	2.00E+04	3.53E+04	6.88E+04	2.73E+04	3.80E+04	4.86E+04	4.00E+04	5.37E+03	0.144	0.068	0.591

Phenylethyl alcohol	60-12-8	17.146	1118	1112	1.55E+05	6.37E+04	7.23E+04	2.80E+05	5.61E+05	1.77E+05	8.18E+04	2.05E+05	2.67E+04	5.15E+04	0.237	0.180	0.708
Aldehydes																	
2 Methyl butanal	96-17-3	5.704	662	662	7.86E+05	6.22E+05	3.62E+05	4.94E+04	1.16E+05	5.05E+04	2.94E+04	3.57E+04	2.81E+04	9.14E+04	0.959	0.120	0.943
3 Methyl butanal	590-86-3	5.525	650	654/700	1.94E+04	4.67E+04	2.27E+04	7.05E+04	2.22E+05	7.48E+04	6.66E+04	6.27E+04	6.06E+04	1.88E+04	0.354	0.059	0.362
Nonanal	124-19-6	16.754	1103	1106	1.29E+04	1.14E+04	1.19E+04	1.95E+04	1.55E+04	1.59E+04	2.32E+04	2.16E+04	2.04E+04	1.38E+03	0.672	0.011	0.988
Benzene Compounds																	
Toluene	108-88-3	7.737	767	763	7.56E+04	2.75E+05	4.41E+05	5.78E+04	2.92E+05	3.58E+05	3.34E+04	2.87E+05	3.54E+05	4.66E+04	0.012	*0.009#*	*0.028#*
p-Xylene	106-42-3	10.379	872	867	1.56E+04	1.23E+04	1.82E+04	1.43E+04	1.72E+04	2.08E+04	1.54E+04	9.54E+03	2.96E+03	1.66E+03	0.887	0.046	0.110
Esters																	
Methyl butanoate	623-42-7	6.683	719	724	2.88E+04	1.17E+04	9.01E+03	2.79E+04	7.29E+04	1.44E+04	1.01E+04	3.44E+04	2.70E+04	6.25E+03	0.434	0.549	0.595
Butyl acetate	123-86-4	8.767	811	812	0.00E+00	0.00E+00	0.00E+00	1.48E+04	4.74E+04	0.00E+00	1.54E+05	1.68E+05	6.84E+04	2.12E+04	0.678	0.229	0.683
Ethyl hexanoate	123-66-0	13.804	995	1002	2.02E+03	1.36E+03	1.68E+03	8.10E+03	7.27E+03	1.11E+04	1.37E+04	1.17E+04	3.09E+04	2.88E+03	0.531	< 0.001	0.145
Ethyl ether	60-29-7	4.008	500	509	7.24E+05	9.47E+05	1.11E+06	5.15E+05	6.20E+05	3.64E+05	7.41E+05	5.34E+05	6.16E+05	7.20E+04	0.930	0.071	0.575
Ketones																	
Acetone	67-64-1	3.975	496	496	1.29E+06	1.08E+06	1.79E+06	1.03E+06	1.42E+06	1.88E+06	9.90E+05	8.23E+05	8.67E+05	1.21E+05	0.126	0.028	0.283
2-Butanone	78-93-3	4.771	599	593	1.14E+05	4.96E+04	1.34E+05	1.66E+06	2.36E+05	4.52E+05	2.67E+06	5.05E+05	2.16E+06	3.15E+05	0.492	0.058	0.380
2-Pentanone	107-87-9	6.004	682	687	2.94E+05	3.70E+04	3.50E+04	1.35E+05	1.87E+05	5.42E+05	1.49E+05	1.11E+05	9.89E+04	4.97E+04	0.630	0.400	0.427
Acetoin	513-86-0	6.771	723	709	5.78E+06	5.74E+06	7.15E+06	9.48E+06	6.88E+06	6.37E+06	6.53E+06	6.67E+06	8.76E+06	4.02E+05	0.769	0.924	0.632
2-Heptanone	110-43-0	10.817	888	891	4.93E+06	6.46E+05	5.78E+05	1.33E+06	3.61E+06	7.40E+06	2.48E+06	2.09E+06	2.26E+06	6.94E+05	0.760	0.571	0.381
Cyclohexanone	108-94-1	11.113	899	896	1.90E+04	0.00E+00	0.00E+00	1.66E+03	1.01E+05	6.45E+03	2.90E+04	1.05E+05	1.21E+05	1.61E+04	0.346	0.386	0.717
2-Nonanone	821-55-6	16.367	1088	1094	2.18E+06	7.73E+04	5.39E+04	1.67E+05	4.90E+05	8.88E+05	4.10E+05	2.36E+05	3.02E+05	2.10E+05	0.592	0.711	0.493

Undecan-2-one	112-12-9	21.383	1287	1294	1.26E+05	2.61E+03	1.87E+03	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.00E+00	1.31E+04	0.510	0.411	0.510
Lactone Compounds																	
Delta-Decalactone	705-86-2	26.117	1499	1506	1.26E+04	1.22E+04	1.10E+04	2.29E+04	2.02E+04	2.03E+04	2.87E+04	2.29E+04	2.55E+04	1.98E+03	0.104	< 0.001	0.336
Pyrazine Compounds																	
2,6-dimethyl-Pyrazine	108-50-9	11.575	916	912	1.02E+03	0.00E+00	4.19E+03	1.66E+04	1.54E+04	1.31E+04	3.34E+04	2.78E+04	6.18E+04	6.16E+03	0.957	0.109	0.902
trimethyl-Pyrazine	14667-55-1	14.038	1004	1014	0.00E+00	1.36E+03	2.32E+03	1.43E+04	1.42E+04	1.73E+04	4.16E+04	4.48E+04	8.56E+04	8.84E+03	0.976	0.013	0.990
Sulphur Compounds																	
Carbon disulfide	75-15-0	4.279	535	568	5.55E+06	4.11E+06	9.23E+06	3.58E+06	3.97E+06	7.16E+06	2.31E+06	3.45E+06	4.53E+06	6.71E+05	0.105	0.001	0.134
Dimethyl disulfide	624-92-0	7.221	743	739	3.26E+04	2.94E+04	2.13E+04	2.99E+04	2.40E+04	3.03E+04	5.55E+04	5.42E+04	4.65E+04	4.02E+03	0.502	0.081	0.564
Methional	3268-49-3	11.383	909	906	5.47E+02	8.94E+02	1.07E+03	2.84E+03	1.27E+03	6.20E+03	1.31E+04	9.50E+03	9.11E+03	1.47E+03	0.881	0.066	0.669
Terpenes																	
D-Limonene	5989-27-5	14.854	1033	1022	6.11E+03	1.74E+03	2.69E+03	6.67E+03	3.40E+03	5.29E+03	9.61E+03	4.87E+03	5.34E+03	7.37E+02	0.723	0.056	0.925

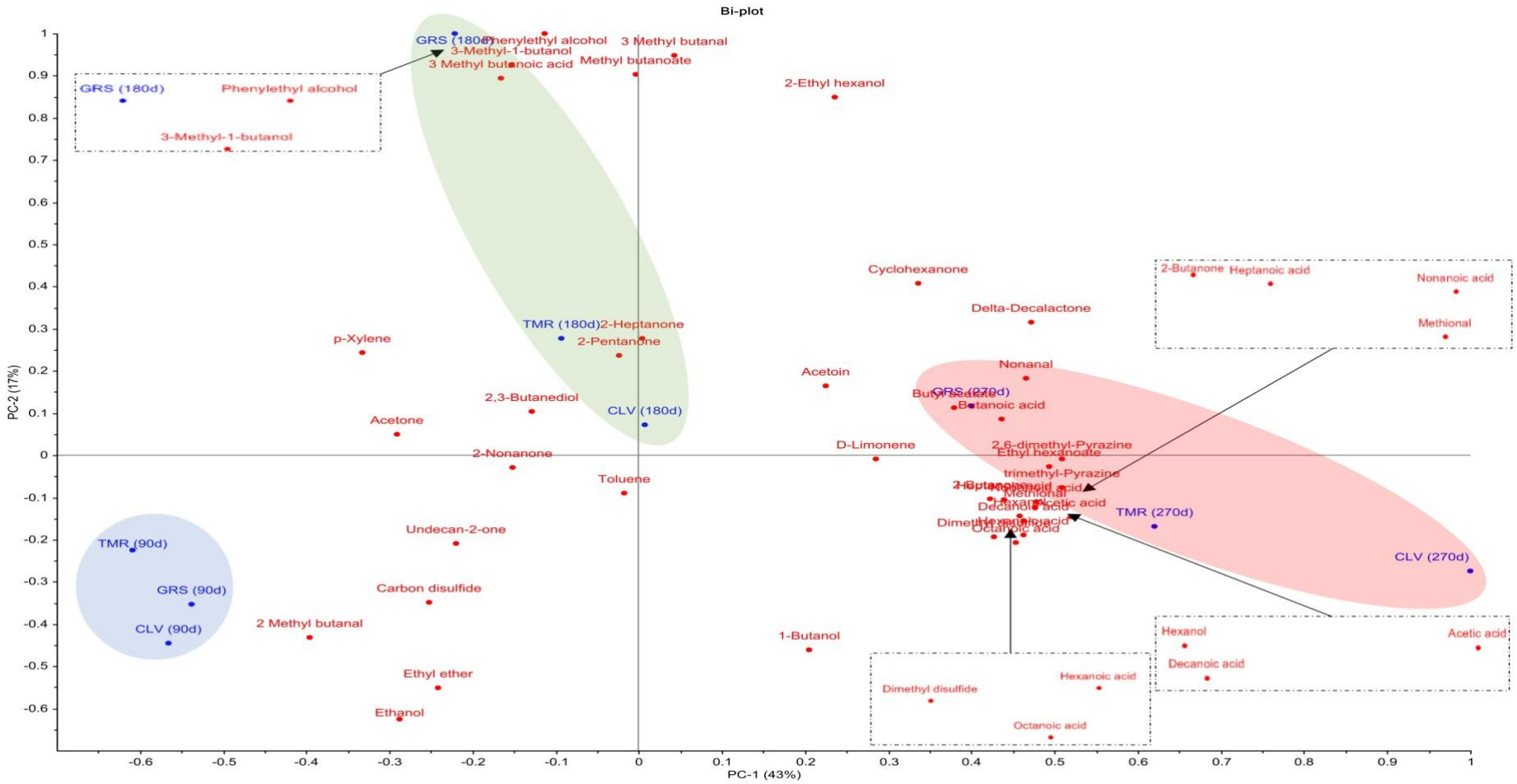


Figure 4.4: Principal component analysis of volatiles data of Cheddar cheeses from TMR, perennial ryegrass (GRS) and perennial ryegrass and white clover (CLV) feeding systems after 90, 180 and 270 days ripening.

Lactone compounds are derived from hydroxylated fatty acids produced from heating or through the action of lipases or by a one-step intermolecular trans-esterification reaction [16]. The terpene content of milks and dairy products are influenced by the feed source [65] although D-limonene was the only terpene compound detected in each of the cheeses, ripening time resulted in a significant increase in its concentrations. Aprea*, et al.* [66] also reported an increase in terpene content of Montasio cheeses over ripening which could be attributed to lactic acid bacteria ability to modify and biosynthesize terpenoids [67].

4.4.7 Affective and Ranking Sensory Descriptive Analysis of Cheddar Cheeses

Mean scores from sensory hedonic and ranking descriptive analysis of cheeses after 180 and 270 d ripening are summarised in Figure 4.5A and 4.5B respectively. The CLV derived cheeses after 180d ripening were significantly positively associated to liking of appearance ($P = 0.002$) and overall acceptability ($P = 0.048$). Following 180d ripening, the TMR-derived cheese was significantly positively associated with liking of texture ($P = 0.027$) and the scores for overall acceptability were positively associated to TMR cheeses ($P = 0.052$). The GRS-derived cheeses after 270 d ripening were significantly negatively associated to liking of appearance ($P = 0.013$) and the CLV-derived cheese after 270 d ripening was significantly negatively associated to liking of texture ($P = 0.018$).

GRS-derived cheese after 180 d ripening were significantly positively associated to colour ($P = <0.001$) but significantly negatively associated to sweet taste ($P = 0.020$). The CLV-derived cheeses after 180 d ripening was found to be significantly negatively associated to crumbly texture ($P = 0.026$). The TMR-derived cheese after 180d ripening were significantly negatively associated to colour ($P = <0.001$) but were significantly positively associated to fruity estery flavour ($P = 0.045$). The GRS derived cheese after 270 d ripening was also significantly positively associated to colour ($P = <0.001$) and salt taste ($P = 0.024$). The CLV derived cheese after 270 d ripening were significantly positively associated to colour ($P = 0.001$) but significantly negatively associated to sweet taste ($P = 0.013$). Finally, the TMR derived

cheese after 270 d ripening were significantly negatively associated to colour ($P = <0.001$) but significantly positively associated to sour taste ($P = 0.007$). Although there was no significant difference in FAA between feeding systems, PCA analysis of cheese FAA (Supplementary Figure 4.2) shows GRS 180 d clustering with phenylalanine and leucine which have been described to have bitter sensory characters, while CLV and TMR are clustering with threonine, glycine, serine and lysine which have been described as possessing sweet sensory characters [16]. Negative scores for TMR color could be as a result of the sensory panel being predominantly Irish participants, who would be more accustomed to the more yellow GRS and CLV dairy products, while the significantly higher concentrations of 2,3-butanediol in TMR could contribute to fruity estery flavour scores.

Table 4.6: Significantly positive Pearson correlation analysis coefficients of volatile compounds analysis and Cheddar cheese chemical analysis over ripening. Odour descriptors references: [1]Curioni and Bosset [64], [2]Avsar, *et al.* [68]

Volatile Compound	Odour Description	Pearson Correlation Analysis Volatiles - Chemical Analysis:
Acetic acid	Vinegar, peppers, green, fruity, sour[1]	TFAA (P = <0.001; r = 0.799), Proteolysis (P = <0.001; r = 0.762)
2-Pentanone	Orange peel, sweet, fruity[1]	TFFA (P = 0.031; r = 0.522)
Dimethyl disulphide	Sulphurous, cabbage-like, sour[1]	Proteolysis (P = 0.015; r = 0.576)
Toluene	Nutty, bitter, almond[1]	Cheese b* value (P = 0.001; r = 0.708)
Butanoic acid	Sweaty, cheese, faecal, rancid, toasted cheese[1]	TFFA (P = <0.001; r = 0.896)
2-Heptanone	Fruity, fatty, spicy, herbaceous[1]	TFFA (P = 0.018; r = 0.566)
Methional	Boiled or baked potato[1]	TFAA (P = 0.013; r = 0.587), Proteolysis (P = 0.046; r = 0.489)
Hexanoic acid	Bad breath, popcorn, sweaty, free fatty acid[1]	TFAA (P = 0.039; r = 0.504), TFFA (P = <0.001; r = 0.891)
Ethyl hexanoate	Fruity, malty, young cheese, mouldy, melon, sour fruit[1]	TFAA (P = 0.029r = 0.530), Proteolysis (P = 0.015; r = 0.578), TFFA (P = 0.024; r = 0.545)
2-Nonanone	Malty, fruity, hot milk, smoked cheese[1]	TFFA (P = 0.021; r = 0.556)
Octanoic acid	Sweat, fatty, rancid, pungent, cheese[1]	TFFA (P = <0.001; r = 0.832)
Decanoic acid	Stale, butter, grassy, fatty, sour fruit, milk[1]	TFFA (P = <0.001; r = 0.882)

Sensory scores for each of the Cheddar cheeses between 180 and 270 d of ripening could be related to previously discussed attributes of the cheeses. Pearson correlation matrix analysis revealed that the sensory scores for liking of texture was negatively correlated with the level of primary proteolysis ($P = 0.019$, $r = -0.545$). Colour scores from sensory panellists were negatively correlated with cheeses L* values ($P = 0.002$; $r = -0.681$) and positively correlated with cheeses b* value ($P = <0.001$; $r = 0.904$). Sensory scores for firmness in mouth over ripening were negatively correlated with the level of proteolysis ($P = 0.012$; $r = -0.578$) while crumbly texture scores were positively correlated with level of proteolysis ($P = 0.025$; $r = 0.525$). The level of FAAs was also negatively correlated with sensory scores for sweaty/sour aroma ($P = 0.002$; $r = -0.687$), sweet taste ($P = 0.002$; $r = -0.667$), sour taste ($P = 0.010$; $r = -0.591$), dairy sweet flavour ($P = 0.027$; $r = -0.520$), dairy fat flavour ($P = <0.001$; $r = -0.790$) and fruity estery flavour ($P = <0.001$; $r = -0.759$) scores. The FAA content of the cheeses was however positively correlated with salt taste scores for cheeses ($P = <0.001$; $r = 0.748$) from panellists after 180 and 270 d of ripening.

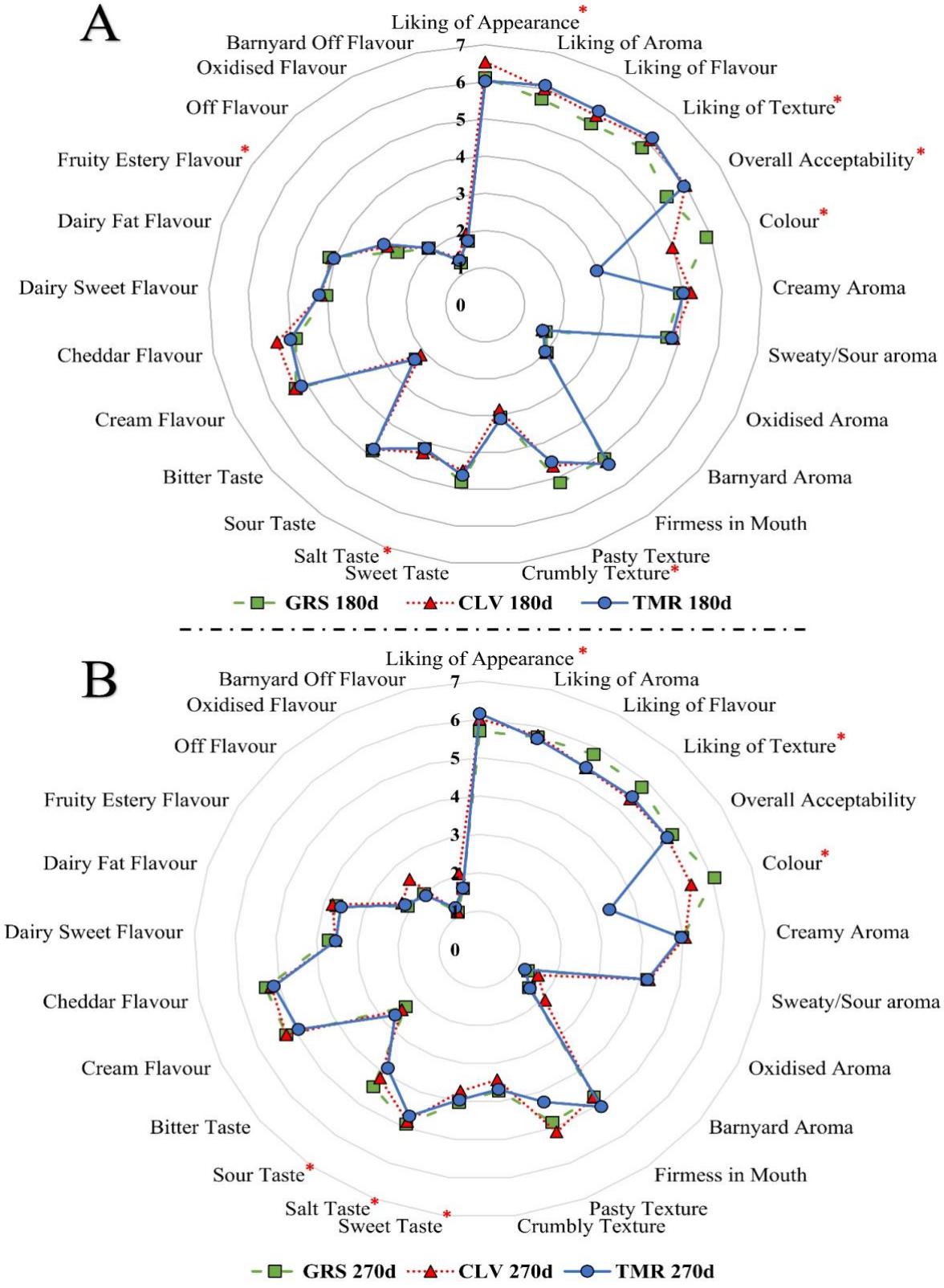


Figure 4.5: Mean sensory hedonic and ranking descriptive analysis scores of Cheddar cheeses derived from TMR, perennial ryegrass (GRS) and perennial ryegrass and white clover (CLV) feeding systems after 180d (A) and 270d (B) ripening.

Overall, these data indicate that feeding system has a significant effect on the organoleptic properties of Cheddar cheese, in particular, differences in β-carotene content of cheeses resulted in differences in colour, where pasture derived cheeses scored higher for colour intensity attribute. Levels of primary and secondary proteolysis are also correlated with alterations of several sensory attributes of the cheeses as mentioned previously. Interestingly, toluene was significantly and positively correlated with the color attribute scores ($P = 0.001$; $r = 0.724$) and the cheeses b* values ($P = 0.001$; $r = 0.708$) and was negatively correlated with cheeses L* values ($P = 0.020$; $r = -0.559$), which is in agreement with the previous study with the butters from these feeding systems where toluene was significantly correlated with pasture derived products rather than those derived from TMR feeding system [17]. Given this result across several products, toluene may be a potential compound for verification of pasture derived dairy products.

4.5 Conclusion

In this study, we have demonstrated that pasture derived feeding systems lead to production of Cheddar cheeses with a healthier fatty acid profile and are more yellow in color (as a result of increased β-carotene content) compared with TMR feeding system. The fatty acid profile of Cheddar cheese was enhanced through pasture based feeding systems with significantly lower thrombogenicity index scores and a greater than two-fold increase in the concentration of CLAc9t11 isomer and vaccenic acid, while TMR derived cheeses had significantly higher palmitic acid content. Pasture derived Cheddar cheese was shown to have significantly higher *n*3 fatty acid content while TMR cheese had significantly higher *n*6 fatty acid content. Such alterations in the fatty acid profile of the cheese resulted in pasture derived cheese having reduced hardness scores at room temperature. Feeding system and ripening time had a significant effect on the volatile and sensory profile of the Cheddar cheese. Fatty acid profiling of cheeses coupled with multivariate analysis showed clear separation of Cheddar cheese derived from pasture-based diets perennial ryegrass or perennial ryegrass/white clover from that of a TMR system.

4.6 Acknowledgements

This publication has emanated from research conducted with the financial support of Teagasc, Science Foundation Ireland (SFI) under Grant Number SFI/12/RC/2273 and the Dairy Levy Fund administered by Dairy Research Ireland. Tom F. O'Callaghan is the recipient of a Teagasc Walsh Fellowship. The valuable input of Elaine Patterson and Hope Faulkner is gratefully acknowledged. The authors sincerely thank the technical and farm staff at Moorepark for their excellent care of the experimental cows and assistance during the experiment.

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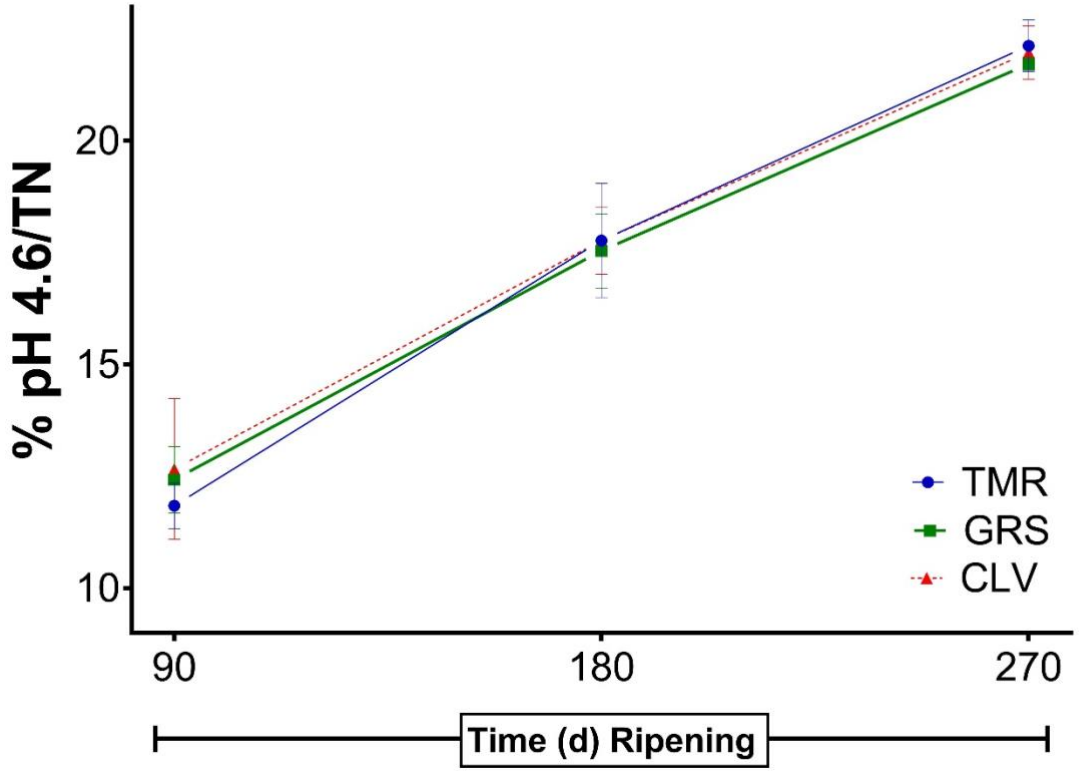
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Supplementary Material

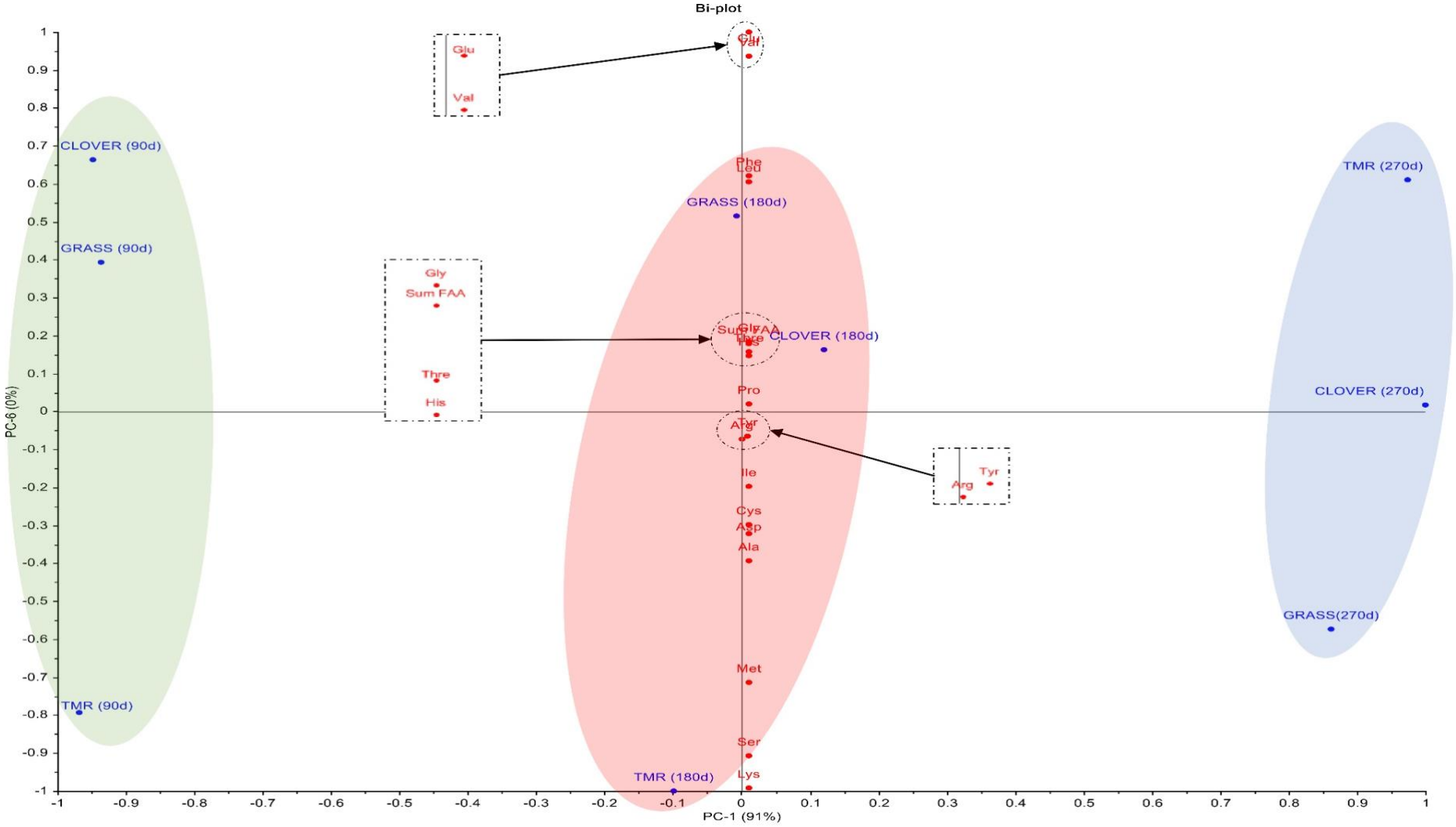
Supplementary Table 4.1: Sensory terms for the affective and descriptive evaluation of Cheddar cheese

Attribute	Definition	Scale
	Hedonic	
Appearance-Liking	The liking of appearance	0 = extremely dislike10 = extremely like
Flavour-Liking	The liking of flavour	0 = extremely dislike10 = extremely like
Aroma-Liking	The liking of aroma	0 = extremely dislike10 = extremely like
Texture-Liking	The liking of appearance	0 = extremely dislike10 = extremely like
Overall acceptability	The acceptability of the product	0 = extremely unacceptable 10 = extremely acceptable
	Intensity	
Appearance-colour	Appearance-Ivory to orange colour	0 = Ivory 10 = Orange
Creamy aroma	The smell associated with creamy/milky products	0 = none, 10 = extreme
Oxidised aroma	The smell associated with oxidised dairy products	0 = none, 10 = extreme
Barnyard aroma	The smell associated the farm, barnyard, ox tail	0 = none, 10 = extreme
Sweaty/sour aroma	The aromatics reminiscent of perspiration, foot odour. Sour, stale, slightly cheesy, moist, stained or odorous with sweat	0 = none, 10 = extreme
Firmness in the mouth	Firm texture in the mouth	0 = none, 10 = extreme
Crumbly	Crumbly texture in the mouth	0 = none, 10 = extreme
Pasty	Pasty texture in the mouth	0 = none, 10 = extreme
Sweet taste	Fundamental taste sensation of which sucrose is typical	0 = none, 10 = extreme
Salt taste	Fundamental taste sensation of which sodium chloride is typical	0 = none, 10 = extreme
Sour	Fundamental taste sensation of which lactic acid is typical	0 = none, 10 = extreme
Bitter taste	Fundamental taste sensation of which caffeine or quinine in soda water is typical	0 = none, 10 = extreme
Cheddar flavour	Intensity of Cheddar cheese flavour	0 = none, 10 = extreme
Cream flavour	The flavour associated with creamy/milky products	0 = none, 10 = extreme
Dairy sweet flavour	The flavours associated with sweetened cultured dairy products such as fruit yoghurt	0 = none, 10 = extreme
Dairy fat flavour	Intensity of fat flavour	0 = none, 10 = extreme
Off-flavour	Off-flavour (Rancid)	0 = none, 10 = extreme
Oxidised flavour	The flavour associated with rancid or oxidised products	0 = none, 10 = extreme
Barnyard flavour	The flavour associated with the farm, barnyard, ox tail	0 = none, 10 = extreme

Fruity/Estery flavour	The flavours associated with fatty acid ethyl esters	0 = none, 10 = extreme



Supplementary Figure 4.1: Ripening index. pH 4.6 soluble nitrogen (mean ± standard deviation) in Cheddar cheeses derived from different feeding systems TMR (●), GRS (■) and CLV (▲) expressed as a percentage of total nitrogen throughout 270 d ripening.



Supplementary Figure 4.2: Principal component analysis of free amino acids (FAAs) over 270d ripening of Cheddar cheeses derived from different feeding systems TMR, GRS and CLV.

Chapter 5

Impact of pasture versus TMR feeding systems on the bovine rumen microbiota

5.1 Abstract

The rumen microbiota is a complex ecosystem essential to the health of ruminants. As such increased understanding of its composition and variability will be beneficial to improve feed efficiency and animal performance metrics. The purpose of this study was to examine the effects of pasture feeding systems consisting of perennial ryegrass (GRS), perennial ryegrass and white clover (CLV) and a total mixed ration (TMR) system on the bovine rumen microbiota using 16s rRNA MiSeq sequencing. Rumen-fluid and solid fractions were collected from cannulated cows who were exposed to the feeding systems in early, mid and late lactation. The rumen microbiota was found to be remarkably complex. There was a clear separation between the liquid and solid fractions of the rumen with significant differences in the dominant phyla *Bacteroidetes* and *Firmicutes*. *Prevotella* was the most abundant genera in both liquid and solid fractions and its presence in the rumen could be beneficial in reducing methane emission. Significantly higher *Ruminococcus* and *Butyrivibrio* genera in the solid fraction is attributed to their fiber degrading potential. This study did not find any significant differences in the rumen microbiota between cows exposed to different diets, which is likely as a result of a shortened adaptation period (two weeks). As such, further work is required to fully understand the effects of these diets on the rumen microbiota and its functionality. Given that the majority of the genera in the rumen are still unknown, the use of full SHOTGUN metagenomic sequencing or meta-transcriptomics could provide valuable information on the effects of these diets on the microbiota and its functionality.

5.2 Introduction

The rumen microbiota is a remarkably diverse ecosystem consisting of bacteria, archaea, fungi and protozoa, essential to the health and production status of ruminants [1]. This complex microbiota is responsible for the fermentation and conversion of ingested plant materials and feed stuffs to energy and protein in the form of volatile fatty acids and microbial cell protein, and synthesis of B-vitamins to meet the requirements of the animal [2]. This ability to ferment and digest cellulolytic plant materials enables ruminants to utilize vast resources globally without directly competing with humans [3]. The gut microbiota is also responsible for the production of potent hormones which when taken up by the blood stream have the ability to affect and regulate not just local but also distal organs; as such the gut microbiota is itself considered a virtual endocrine organ [4]. The structure of rumen bacterial community has also been correlated with feed efficiency, milk yield and milk composition [5]. There are many factors that can affect the composition of the rumen microbiota which include diet [3, 6], feeding strategy [7], environment, age [8], host factors [9] and animal breed [10]. One of the best studied factors influencing the rumen microbiota is host diet [11], which also offers the simplest mechanism to beneficially alter the microbiota through provision of fermentation substrates to increase animal performance and production. Yang*, et al.* [12], demonstrated that the form in which the diet is provided, can have an effect on the rumen microbiota, as bacterial attachment levels linearly increased as the size of the feed particles decreased, which can be an important consideration for fibre degradation and bacterial flow to the duodenum. de Menezes*, et al.* [6], examined the effect of pasture and TMR diet on the microbiota and reported that while the bacterial and archaeal communities were affected, the protozoan community was mostly unaffected by diet. In particular higher abundance of *Fibrobacteraceae* was associated with the TMR solid fraction, while pasture resulted in increased abundance of propionate-producing *Veillonelaceae.* Alteration of the quality of hay and proportion of concentrates in the diet has resulted in significant differences at all taxonomic levels of rumen bacterial

communities. Feeding diets with sugar rich hay resulted in the genera *Ruminobacter* and *Fibrobacter* being suppressed while *Selenemonas* and *Prevotella* proliferated [13]. The inclusion of high levels of starch in the diet resulted in decreased abundance of the phyla *Fibrobacteraceae* and *Spirochaetes* [14]. As such the community of rumen bacteria that develops to digest a particular forage is quite specific [15]. Increasing the knowledge and understanding of the composition and variability of the rumen microbiota in dairy cows is essential to better understand its activity and the extent to which these factors affect animal performance [15, 16], and how to best exploit it through formulation of specialized feeding strategies for improved feed efficiency and milk yields [10], methane mitigation [6] and more efficient food production in the future [3].

The objective of this study was, to investigate the effects of three widely practiced feeding systems of, a total mixed ration (**TMR**) diet indoors, perennial ryegrass (*Lolium perenne* L.) outdoors (**GRS**), and perennial ryegrass/white clover (*Trifolium repens* L.) outdoors (**CLV**) in Ireland on the bovine rumen microbiota.

5.3 Materials and Methods

5.3.1 Experimental Design and Sample Collection

Three feeding systems were compared over a full lactation at the Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland. Herd 1 was housed indoors and fed a total mixed ration diet (**TMR**), herd 2 was maintained outdoors on perennial ryegrass only pasture (**GRS**) while herd 3 was also maintained outdoors on a perennial ryegrass/white clover pasture (**CLV**). On a dry matter basis (DM) the TMR diet consisted of 7.15 kg of grass silage, 7.15 kg of maize silage and 8.3 kg concentrates (see Table 5.1 and 5.2). Cows within the TMR system were fed at 08:30 h daily into electronically controlled Griffith Elder Mealmaster individual feed bins (Griffith Elder and Company Ltd, Suffolk, England) and feed was available *ad-libitum*. Pasture based cows consumed ~18 kg

DM/day measured by pre- and post-grazing sward heights daily using the rising plate meter (Jenquip, Fielding, New Zealand) while pre-grazing herbage mass was measured with an Etesia mower (Etesia UK Ltd, Warwick, UK). The CLV sward contained ~20 % white clover and was measured according to Egan*, et al.* [17] Compositional analysis of GRS and CLV swards are shown in Table 5.3.

Rumen sampling took place at 07:00 each morning. Nine ruminally cannulated, healthy, spring calving Friesian cows were allocated to three groups. This study was conducted in conjunction with that investigating the rumen metabolome. It has been previously described that 4 to 12 days is adequate time for animals to adapt to a diet [18], indeed for similar studies investigating the rumen metabolome an 11 day period was used [19]. With that in mind, for this study similar to chapter 6, three cows were randomly assigned to each diet for a two-week period, the first two weeks was an acclimatisation period after the end of the second week rumen-fluid and solids portions were collected on the morning of day 15. The cows were then rotated to a different feeding system and the two-week process was repeated. This sampling process was carried out in each stage of lactation (early, mid and late). Rumen samples were collected then filtered through cheese cloth into sterile containers, ~50mls of rumen fluid was stored in sterile falcon tubes and solids portions stored in sterile 100ml containers, frozen and stored at -80 °C. According to McCabe*, et al.* [20], 20g of frozen solid rumen material is considered representative.

5.3.2 Ethical Approval

Teagasc has both an Animal Welfare Body (AWB) and Animal Ethics Committee. The AWB are a legal requirement of Article 26 of Directive 2010/63/EU and Regulation 50 of S.I. No. 543 of 2012. The Health Products Regulatory Authority (HPRA) provides project authorization and the HPRA Licence number for this project is: AE19132/P019.

5.3.3 DNA Extraction and MiSeq Sequencing

All samples were individually ground to a fine powder under liquid nitrogen using a mortar and pestle. DNA was extracted using repeated bead beating plus column method according to Yu and Morrison [21].

The V3 - V4 regions of the 16S rRNA gene were amplified and adapter sequences chosen according to the 16S metagenomic sequencing library protocol for the Illumina MiSeq using the following 16S primer pair Forward - (5'CGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and Reverse - (5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAA CC).

5.3.4 Bioinformatical analysis

Libraries were sequenced on a MiSeq sequencer (Illumina) using 2x250 bp chemistry. The 64-bit versions of USEARCH 9.2 [22] and mothur [23] was used in combination with several in-house programs for bioinformatical analysis of the sequencing data. Following tag identification and trimming, all sequences from all samples were pooled. Paired end reads were merged, requiring at least 10 bp overlap and a merged read length between 300 and 500 bp in length. Sequences with ambiguous bases, without perfect match to the primers, or homopolymer length greater than 8 were discarded and primer sequences trimmed. Reads were quality filtered, discarding reads with more than 1 expected error and sequences strictly dereplicated, discarding clusters smaller than 5.

Sequences were clustered at 97 % sequence similarity, using the most abundant strictly dereplicated reads as centroids and discarding suspected chimeras based on internal comparison. Taxonomic assignment of OTUs was done using the method by Wang, *et al.* [24] with mothur's PDS version of the RDP training database v14. Following this, samples were

rarified to the lowest sequence number found in a sample ≥1000 (after in silico removal of contaminating OTUs).

5.3.5 Statistical Analysis

Univariate analysis was performed to discover potential differences between the feeding systems in the rumen-fluid and rumen-solid portions. General linear models (GLM) were built for each metabolite to determine whether the means of the three feeding systems (TMR, GRS and CLV) differ. The stage of lactation (early, mid and late) and the interaction with the diet were also included in the model. Data was log transformed (generalized logarithmic transformation in base 2) in order to reduce potential influential points and reduce the skew of the data. The type I error, due to multiple testing, was controlled by using the Benjamini and Hochberg false discovery rate (FDR) procedure. Two-sided *p*-values < 0.05 of the corrected p-values was used as the threshold to refuse the null hypothesis that the means of all the groups did not differ. Tukey's honestly significant difference (HSD) test was used as a post-hoc analysis to find which treatments were significantly different from each other ($p<0.05$) among the significant attributes.

Unsupervised hierarchical clustering analysis (HCA) was done to observe patterns in the data, and is shown as a heatmap. A supervised multivariate model was built using partial least squared discriminant analysis (PLS-DA) algorithm. In order to validate

Table 5.1: Typical ingredient formulation and chemical composition of TMR concentrate

Concentrate Ingredient Composition	% as fed
Maize	13.00
Beet pulp molassed	15.50
Soyabean meal 48% CP	30.00
Maize distillers	12.00
Acid buffer	0.70
Maize/Beet Min Balancer	2.50
Salt	0.50
Barley (rolled)	15.00
Rapeseed meal	7.50
Megalac	3.30
Chemical Analysis	/kg as fed
DM, g	87.49
UFL	1.02
UFV	0.99
Crude protein %	24.28
PDIN, g	169.26
PDIE, g	133.91
Starch %	18.10
Sugar %	6.92
Crude fibre %	6.10
Oil %	5.12
Ash %	7.99
Ca %	1.10
P %	0.62
Copper mg/kg DM	94.66
ME MJ / kg DM	11.16

Table 5.2: **Chemical composition (mean ± s.d.) and nutritional content of silages from TMR diet (grass silage and maize silage) collected weekly throughout lactation analysed by near-infrared spectroscopy.**

	Grass Silage	Maize Silage
DM (% of DM)	31.53 (± 11.71)	25.3 (±3.11)
CP (% DM Basis)	11.4 (± 1.44)	5.83 (± 1.04)
Starch (% DM Basis)	NA	20.84 (± 4.14)
ADF (% DM Basis)	27.16 (±2.03)	NA
NDF (% DM Basis)	40.66 (± 3.61)	50.97 (± 2.47)
ASH (% DM Basis)	8.83 (± 1.09)	2.95 (± 0.66)
UFL (/kg of DM)	0.99 (± 0.06)	0.88 (± 0.02)
PDIA (/kg of DM)	22.92 (± 6.30)	12.68 (± 2.26)
PDIE (/kg of DM)	69.40 (± 10.69)	61.27 (± 2.73)
PDIN (/kg of DM)	71.05 (± 6.36)	35.80 (± 6.37)

UFL = unité fourragère lait; PDIA = sum of the feed protein ruminally undegraded and truly digested in the small intestine; PDIE = sum of PDIA and the microbial true protein that is truly digested in the small intestine (PDIM) when energy is limiting; PDIN = sum of PDIA and PDIM when nitrogen is limiting. NA = not available.

Table 5.3: **Chemical composition and nutritional content (mean ± s.d.) of pasture system forages (GRS and CLV) collected weekly throughout lactation analysed by near-infrared spectroscopy.**

	GRS	CLV
OM digestibility	853.43 (± 50.24)	859.45 (± 41.14)
CP	234.45 (± 42.39)	248.93 (± 34.49)
ADF	286.30 (± 33.76)	288.46 (± 31.12)
NDF	387.97 (± 39.06)	362.21 (± 33.06)
Ash	62.37 (± 16.47)	58.23 (± 12.37)
UFL (/kg of DM)	0.97 (± 0.06)	0.97 (± 0.05)
PDIA (/kg of DM)	44.89 (± 5.71)	46.86 (± 4.76)
PDIE (/kg of DM)	103.31 (± 5.19)	105.97 (± 4.50)
PDIN (kg of DM)	151.99 (± 28.53)	161.71 (± 23.59)

UFL = unité fourragère lait; PDIA = sum of the feed protein ruminally undegraded and truly digested in the small intestine; PDIE = sum of PDIA and the microbial true protein that is truly digested in the small intestine (PDIM) when energy is limiting; PDIN = sum of PDIA and PDIM when nitrogen is limiting.

the model a permutation test with 2,000 repetitions was performed to check that the model differed from a random model. Also the R^2 and Q^2 parameters were obtained to obtain the performance of the model using a 10 fold cross-validation approach as well as the number of components to analyze. The variable importance plot (VIP) shows which variables have larger influence to the latent variables of the built model. Metaboanalyst [25] software and in-house R (R Core Team, version 3.4.2, 2016) code was used to perform the statistical analysis and produce the figures.

Paired t-test was used to examine differences between rumen liquid and rumen solid portions with an adjusted *p*-value of < 0.05 considered significant, t-test analysis was carried out using R.

5.4 Results and Discussion

5.4.1 Effect of cow diet

There was no significant effect of diet on the rumen microbiota as determined by 16s sequencing. Although diet has previously been reported to have a significant effect on the rumen microbiota, the lack of significant effects with adjusted *p*-values in this study could be attributed to the short time period (two weeks) cows were exposed to each of the diets. While such a time period has been shown to be sufficient to alter the rumen metabolome, it may not have been long enough to alter the macro-composition of the rumen microbiota but rather alters the functionality of the micro-organisms present. That said, a study by de Menezes*, et al.* [6], utilized a two week dietary period when comparing the rumen microbiota of TMR and pasture fed cows and reported significant differences in the microbiota using terminal restriction fragment length polymorphism and 454 16s sequencing. There are several differences between that study and the present one which may have caused the contradicting results, primarily, DNA extraction and analysis methodology, experimental design, and statistical analysis. Indeed McCann*, et al.* [3] highlighted how given the multitude of differences

in steps used for DNA extraction, it makes comparisons between studies challenging. Other previous studies analyzing the effect of cow diet on the rumen microbiota have used periods of between 21 days to one month of exposure to the diets prior to sample analysis [10, 12, 15, 26]. Therefore, it is noteworthy, that for future studies more in-depth microbiota analysis techniques such as SHOTGUN sequencing or meta-transcriptomics analysis would be more appropriate than 16S sequencing to demonstrate diet induced alterations to gene expression that are likely responsible for the changes in the rumen and milk metabolome described later (Chapter 6). SHOTGUN sequencing in particular generates true metagenomics sequences to predict the functional capability of a microbiota and given the high level of unknown organisms in this complex system could be particularly applicable.

Stage of lactation was shown to have a minor effect on the rumen microbiota in this study. Among the rumen-fluid portion time had a significant effect on the genera *Succiniclasticum* ($P = 0.019$), *Megasphaera* ($P = 0.038$), and an unknown genus of *Alphaproteobacteria* ($P = 0.001$) which was present in all samples at > 1 % of total abundances. Among the rumen-solid microbiota time had a significant effect on the genera *Succiniclasticum* ($P = 0.013$) and *Megasphaera* ($P = 0.020$). *Succiniclasticum* was significantly higher in the rumen liquid phase and was significantly higher in early and mid-lactation ($P = < 0.001$) than in late lactation samples. *Succiniclasticum* has previously been reported as one of the most abundant genera in the rumen [27, 28], and has been identified as an important bacteria due to its ability to convert succinate to propionate [29]. There was no significant difference in the rumen *Megasphaera* contents between solids and liquid fractions and rumen *Megasphaera* was higher ($P = <0.05$) in early lactation than that of mid and late lactation. *Megasphaera* has previously been reported as a genus associated with adaptation of the ruminal community to low pH [30]. In particular among the *Megasphaera* genera, *Megasphaera elsdensii*, has received much attention for its ability to utilize lactic acid and prevent its accumulation, thus preventing lactic acid acidosis, and may have potential use in

controlling rumen acidosis [31]. Nordlund, *et al.* [32] suggested that early lactation cows are at risk of experiencing sub-acute ruminal acidosis resulting from reduced absorptive capacity of rumen epithelium, poorly adapted rumen microbiota and rapid introduction to high-energy dense diets. Noel, *et al.* [11] and Jewell, *et al.* [33] also reported only small differences in the rumen bacterial communities over time. Noel, *et al.* [11] attributed the small changes in rumen microbiota over lactation to changing pasture feed quality throughout the seasons of a lactation and also the potential alterations as a result of the production phase of the cows. Interestingly the authors also highlighted that bacterial community structure returned close to the community structure associated with the same season in the yearly cycle and concluded that the rumen bacterial community in a production herd is remarkably stable over time [11].

5.4.2 Effect of rumen phase (solid vs liquid)

There was a significant difference in the microbiota of the liquid and solid portions of the rumen contents. Figure 5.1A demonstrates the clear separation of the rumen liquid and solid portions microbiota at phylum level using PLS-DA. Seventeen phyla were determined in each of the samples. The heatmap of liquid and solid portion phyla (Figure 5.1B) demonstrates that the phyla *Spirochaetes*, *Firmicutes*, *Euryacrhaeota*, *Fibrobacteres, Chloroflexi* and *Chloroplast* were more associated with the solids portion

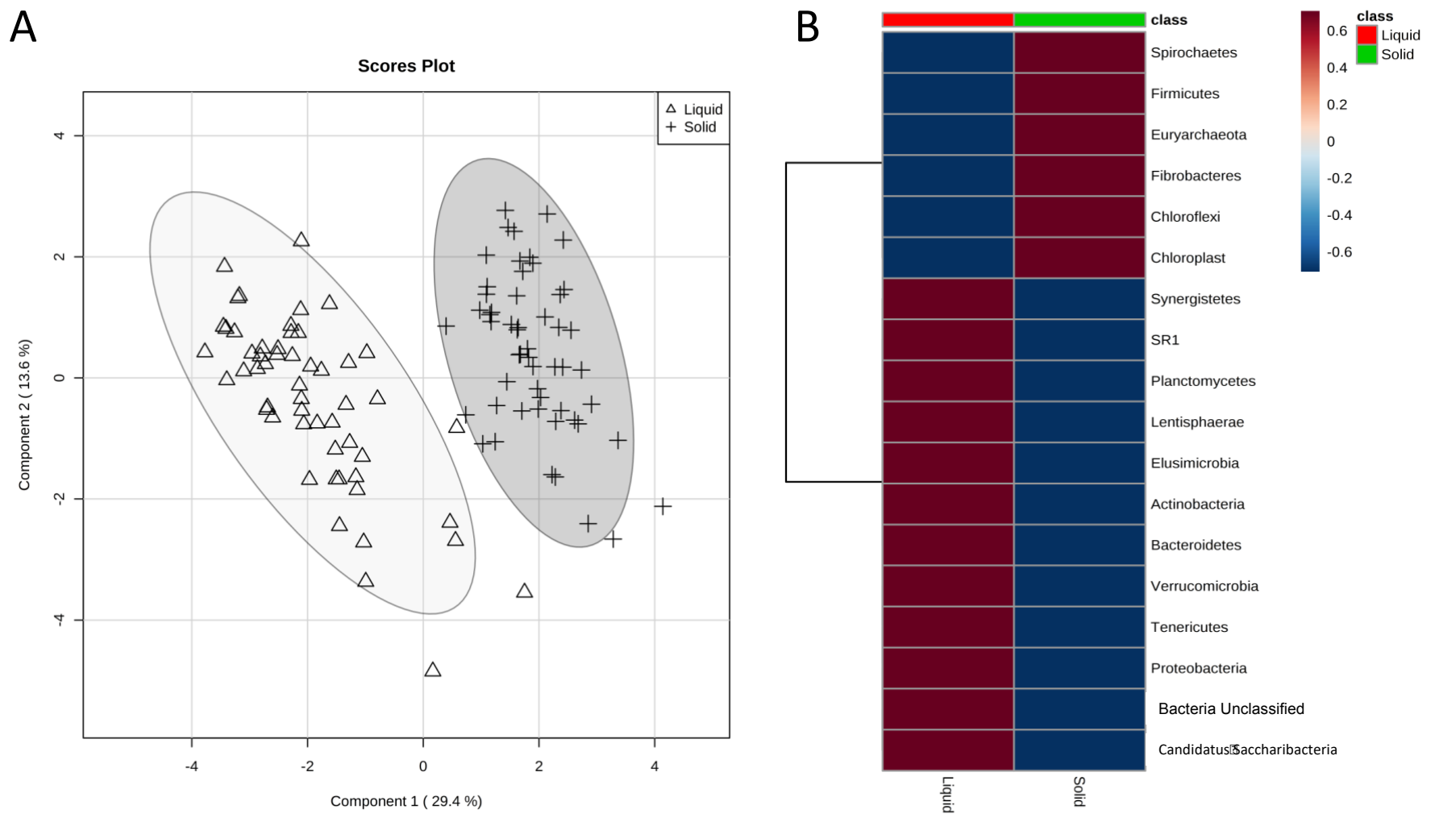


Figure 5.1: (A) Partial least square descriptive analysis (PLS-DA) of rumen solid and liquid portions microbiota at phylum level. P-value for the permutations test P < 0.01. (B) Hierarchal clustering analysis heatmap of solid and liquid rumen microbiota at phylum level.

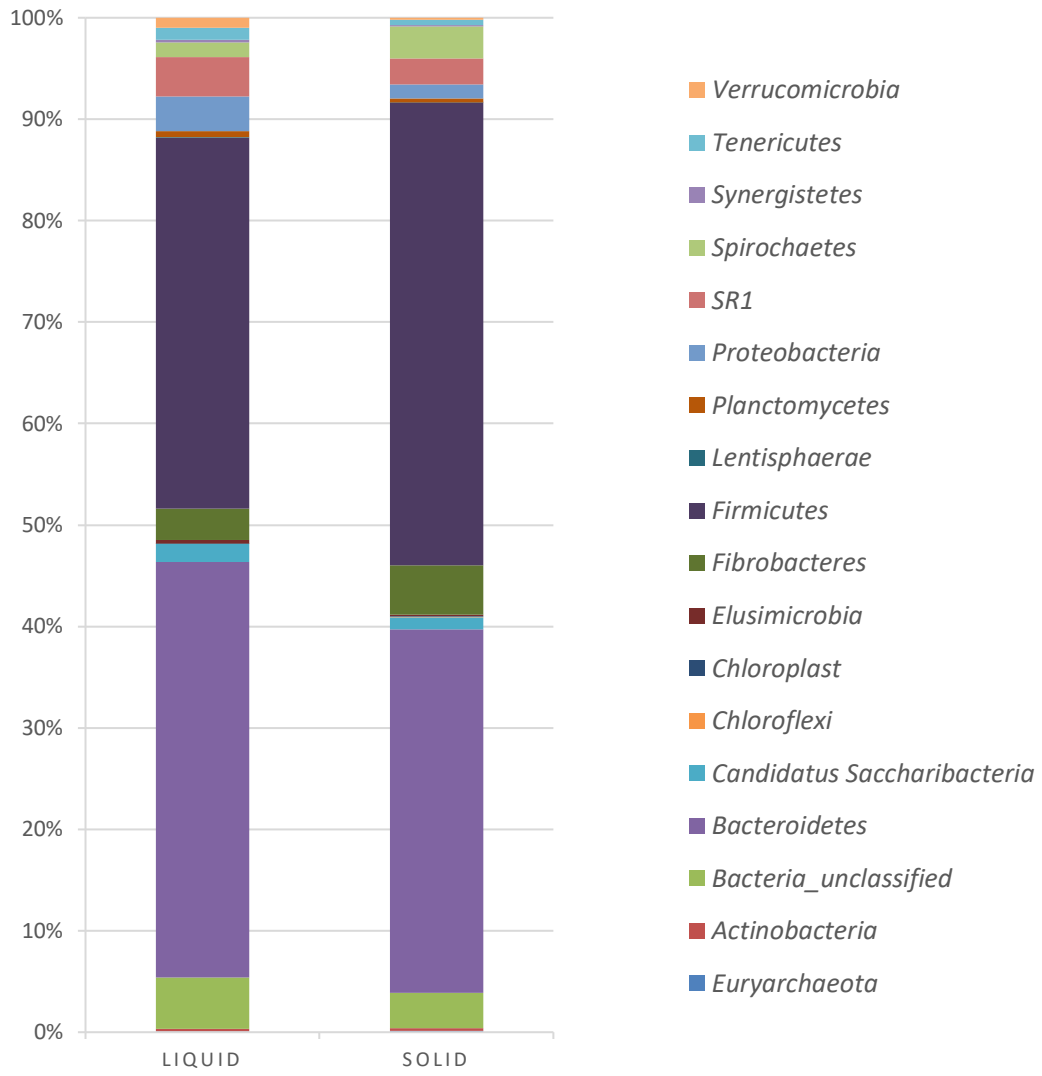


Figure 5.2: Bar chart demonstrating breakdown of abundances of different Phyla in solid and liquid portions of rumen microbiota.

while *Bacteroidetes, Synergistetes, SR1, Planctomycetes, Lentisphaerae, Elusimicrobia, Actinobacteria, Verrucomicrobia, Tenericutes, Proteobacteria* and *Candidatus Saccharibacteria* were associated with the fluid portion. Interestingly, increased levels of unclassified bacteria were also associated with the rumen fluid portion compared to the solid portion (5.07 % versus 3.44 % respectively). The average percentage of each phyla in liquid and solid portions are shown in Figure 5.2. *Firmicutes* were the most abundant phyla in the solid portion (45.62 %) followed by *Bacteroidetes* (35.84 %), and *Fibrobacteres* (4.90 %). Whereas *Bacteroidetes* were the most abundant phyla in the liquid portion (40.96 %) followed by *Firmicutes* (36.54 %), unclassified bacteria (5.07 %) and SR1 (3.91 %). *Bacteroidetes* and *Firmicutes* have been reported previously as being the dominant phyla in the rumen [1, 5, 6, 11, 15]. The significantly higher concentrations of *Firmicutes* in the solid portion and *Bacteroidetes* in the liquid portion has also been previously reported [11] and have been attributed to the biological functions associated with each phyla [1]. With that the bacteria attached to the ingested plant matter within the rumen are thought to be responsible for initial fiber degradation [34, 35].

There was clear separation between solid and liquid fractions at genus level, and Figure 5.3 and Figure 5.4 demonstrate the relative proportions of the most prevalent classified genera in liquid and solid portions which included *Prevotella* (23.58 % liquid, 16.93 % solid), *Succiniclasticum* (8.79 % fluid, 4.28 % solid), *Fibrobacter* (3.16 % fluid, 4.90% solid), *Ruminococcus* (2.23% fluid, 4.06% solid) and *Butyrivibrio* (0.74 % liquid, 1.64 % solid). Robert [26] also reported a similar core genera present in the rumen and noted that they likely contributed to the basic function of the rumen microbial ecosystem. Among the fully classified genera, *Anaeroplasma*, *Selenomonas*, *Prevotella*, *Elusimicrobium, Paraprevotella, Succiniclasticum* were significantly

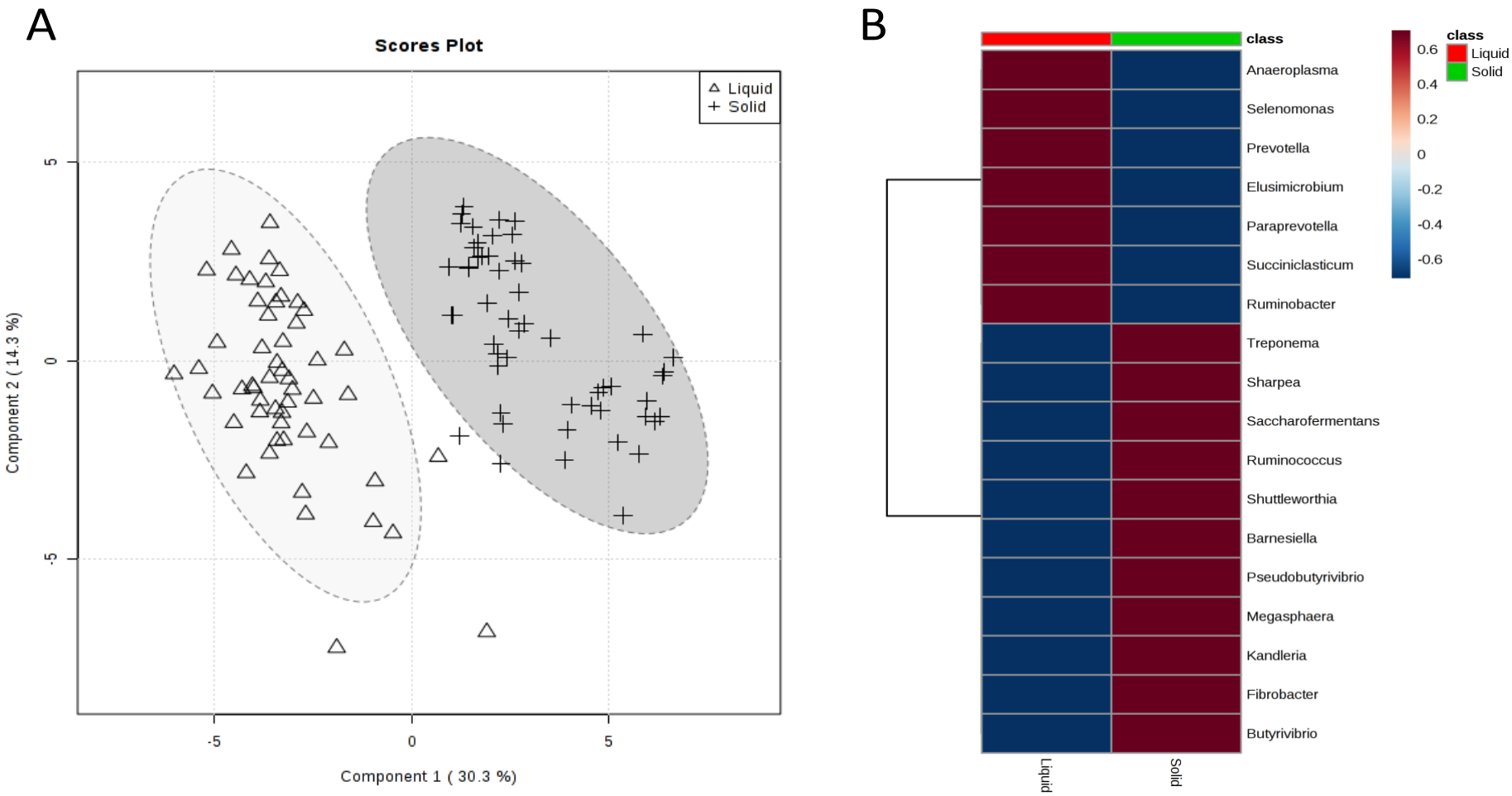


Figure 5.3: (A) Partial least square descriptive analysis (PLS-DA) of rumen solid and liquid portions microbiota at genus level demonstrating clear separation of microbiotas from each fraction. P-value for the permutations test P < 0.01. (B) Hierarchal clustering analysis heatmap of solid and liquid rumen microbiota at genera level. (Fully classified genera present at 1% abundance in at least 1 sample.)

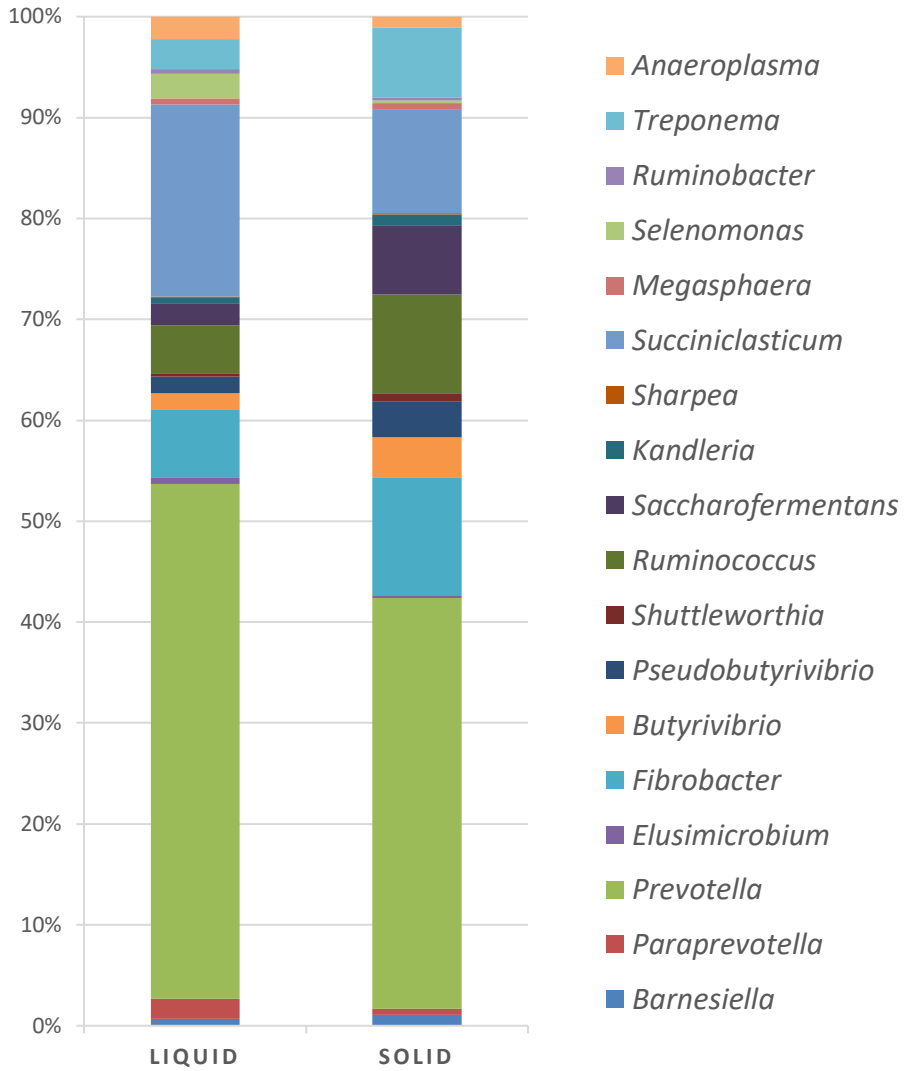


Figure 5.4: Bar chart demonstrating breakdown of abundances of different genera in solid and liquid portions of rumen microbiota.

(P = < 0.01) higher in liquid fraction, while the genera *Treponema, Sacchrofermentans, Ruminococcus, Shuttleworthia, Barnesiella, Pseudobutryvibrio, Fibrobacter, Butryvibrio* (*P* = <0.01) and *Kadleria* (*P* = 0.05) were significantly higher in the solid fraction. Significantly higher *Ruminococcus* and *Butyrivibrio* in the solid fraction was previously reported by Noel*, et al.* [11] and fits well with their known fiber degrading properties. In the past *Prevotella* sequences have been associated with either the fiber fraction of the rumen [13, 36] or the liquid fraction [37]; such differences between fractions could be affected by sampling procedures, filtering of the ruminal fluid and DNA extraction and analysis procedures [13]. *Prevotella* has previously been reported as the most abundant genera in the rumen [5, 15, 26, 38] and as such has been suggested to play a fundamental role in the rumen ecosystems [15]. de Menezes*, et al.* [6] reported significantly higher concentrations of *Prevotellaceae* and *Veillonellaceae* in cows fed perennial ryegrass compared to TMR diets and in a concurrent study to this using animals on the same diets, cows fed perennial ryegrass were demonstrated to produce up to 60 % less methane per cow or 11 % reduced methane emission on a dry matter intake basis [39]. *Prevotella* strains have been shown to be capable of producing propionate, thus increased levels of *Prevotella* in the rumen could therefore be beneficial in the goal to reduce agricultural levels of methane emission as they divert H_2 away from methanogenesis [3, 6]. However, increased proportions have also been shown to have a significant negative correlation with milk fat yield [5]

Our study has demonstrated a high bacterial diversity in the rumen of animals fed pasture or concentrate diets. Up to 173 genera were detected, however, 53 of these were unknown and only eighteen genera were fully classified and present at concentrations of at least 1 % in at least one sample. An interesting aspect of this data has highlighted that while there has been significant work carried out characterizing the rumen microbiota in the past there is still a considerable number of unclassified genera present; in this study on average 51.40 % of the

liquid portion and 56.05 % of abundances in the solid portion accounted for unknown genera. The most abundant unknown genera in the liquid and solid portion were derived from Bacteria (5.07 % fluid, 3.44 % solid), *Bacteroidetetes* (8.45 % fluid, 10.13 % solid), *Prevotellaceae* (4.53 % fluid, 5.78 % soild), *Clostridiales* (4.03 % fluid, 5.48 % solid), *Lachnospiraceae* (5.55 % fluid, 9.94 % solid), *Ruminococcaceae* (6.32 % fluid, 7.88 %) and *Firmicutes* (3.50 % fluid, 4.95 % solid). This level of unclassified and unknown organisms is potentially due to the highly anaerobic environment of the rumen and as such many of the organisms have previously been uncultivable, however with improving anaerobic techniques and methodology becoming available it's obvious there are still many novel genera in this complex ecosystem yet to be discovered.

5.5 Conclusions

This study examined the microbiota of dairy cows exposed to three different dietary regimens throughout lactation. In line with previous studies in this area the rumen microbiota was found to be remarkably complex and stable over time. There was a clear separation between the liquid and solid fractions of the rumen with significant differences in the dominant phyla *Bacteroidetes* and *Firmicutes*. *Prevotella* was the most abundant genera in both liquid and solid fractions and its presence in the rumen could be beneficial in reducing methane emission. Significantly higher proportions of *Ruminococcus* and *Butyrivibrio* genera in the solid fraction is attributed to their fiber degrading potential. This study did not report any significant differences in the rumen microbiota between cows exposed to different diets, while this is likely as a result of a shortened adaptation period, the extraction and analysis methodology cannot be ignored. As such, it is recommended that for future studies using similar experimental design more complex detailed analysis methods such as metagenomic SHOTGUN sequencing or meta-transcriptomics may prove more beneficial in highlighting diet induced microbiota and metabolic changes. Compiling the liquid and solid fractions prior to DNA extraction may also offer a clearer picture of the rumen microbiota as a whole and potentially alleviate discrepancies associated with extraction methodology. Although the rumen microbiota has been widely studied in the past the majority of this complex system is still unknown, therefore it is obvious there is still considerable amounts of novel genera in this ecosystem yet to be discovered.

5.6 Acknowledgements

This publication has emanated from research conducted with the financial support of Teagasc, Science Foundation Ireland (SFI) under Grant Number SFI/12/RC/2273 and the Dairy Levy Fund administered by Dairy Research Ireland. Tom F. O'Callaghan is the recipient of a Teagasc Walsh Fellowship. The authors sincerely thank the technical and farm staff at Moorepark for their excellent care of the experimental cows and assistance during the experiment.

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Chapter 6

Diet has a significant effect on the bovine rumen and milk metabolome: A comparison of pasture versus total mixed ration feeding systems

6.1 Abstract

The purpose of this study was to examine the effects of pasture feeding systems consisting of perennial ryegrass (GRS), perennial ryegrass and white clover (CLV) and TMR, on the rumen and milk metabolome and assess the potential to distinguish milks from different feeding systems by their respective metabolomes. Rumen-fluid was collected from cannulated cows under the different feeding systems in early, mid and late lactation and raw milk samples were collected from ten cows in mid lactation from each of the feeding systems. ^{1}H-NMR untargeted metabolomic analysis was performed on rumen-fluid and raw milk samples. Our results show that feeding system has a significant effect on both the rumen and milk metabolome. Increased concentrations of volatile fatty acids (such as acetic acid), an important source of energy for the cow, was detected in the rumen of TMR and CLV feeding systems. Pasture feeding resulted in significantly higher concentrations of isoacids in the rumen. Both CLV and GRS rumen were found to have increased concentrations of *p*-cresol, a product of microbiome metabolism. CLV feeding resulted in increased rumen concentrations of formate, a substrate compound for methanogenesis. TMR feeding resulted in significantly higher rumen choline content which can contribute to animal health and milk production, and succinate, a product of carbohydrate metabolism. Milk and rumen-fluids were shown to have varying levels of dimethyl sulfone in each feeding system and was found to be an important compound for distinguishing between diets. CLV feeding resulted in increased concentrations of milk urea, which can contribute to reduced milk protein quality through increased concentrations of non-protein nitrogen. Milks from pasture-based feeding systems were also shown to have significantly higher concentrations of hippuric acid, a compound previously suggested as a biomarker of pasture-derived milks. This study has highlighted that ^{1}H-NMR metabolomics coupled with multivariate analysis is capable of distinguishing both rumen-fluids and milk samples derived from cows on different feeding systems.

6.2 Introduction

Cows feeding systems differ across the globe, typically dictated by the regions climatic conditions and cow nutritional requirements. Traditionally, the natural diet of the dairy cow consisted of grazing fresh grasses and forages outdoors as is, typically, still the major practise in a minority of countries such as Ireland and New Zealand for a major portion of the cow's lactation. In recent decades, the intensification of the dairy industry has resulted in a shift to more conventional total mixed ration (TMR) feeding systems where cows are housed indoors year-round and fed a diet of grass/maize silages supplemented with high levels of concentrates; TMR feeding is typically practised in the US, parts of Europe, the middle east and Asia. Indeed, the United States Department of Agriculture reported that in 2014, <7 % of dairy operations in the US were grazing only based systems [1]. TMR feeding systems allow the farmer greater control over the cow's nutrition and also offers the animals protection from environmental extremes [2]. On the other hand, pasture feeding systems allow the animals access to fresh forages and enables them to perform more natural behaviours in a natural environment such as grazing [3].

However, in terms of the consumer, there is an increased demand for pasture-derived dairy products, resulting from consumer perceptions of a healthier more natural product and improved animal welfare compared to the more conventional indoor TMR feeding systems [4]. These perceptions appear to have some basis in fact. O'Callaghan*, et al.* [5] demonstrated that pasture derived milk has increased protein quality and an improved fatty acid profile with significantly higher CLA and Omega 3 fatty acids content than milk derived from a TMR feeding system throughout lactation. As such, "pasture-based" dairy has become a major part of dairy marketing schemes in countries which use them such as New Zealand and Ireland. However, there is limited information and essentially no method, currently available for the examination or verification of such pasture-derived dairy products and their source of primary production.

In addition to this, the cows rumen is an essential component for the animals overall health and productivity status, and should essentially be considered as a virtual endocrine organ. In particular outputs from the rumen's microbiota metabolism not only have local affects, but can also affect the status and function of distal organs and systems [6]. Metabolomics is the study of the metabolome which is formally defined as the collection of all small-molecule metabolites (endogenous or exogenous) that can be found in a living cell or living organism [7]. Quantitative nuclear magnetic resonance (NMR) has been successfully used in the past to investigate and characterise both the rumen [8] and milk [9] metabolomes. ^{1}H-NMR is as an attractive method for metabolomic analysis as it requires minimal sample preparation, low sample volumes, and is, by nature a non-targeted approach [10]. A study by Sundekilde*, et al.* [10] comprehensively reviewed the many applications of ^{1}H-NMR for milk analysis which include milk authentication and milk nutritional quality research.

Moreover, using ^{1}H-NMR spectroscopy Ametaj*, et al.* [11] revealed unhealthy alterations of the rumen metabolome with increasing proportions of cereal grain, and highlighted that different dietary systems of the cows could be distinguished based on the rumen metabolomic profile. Indeed, the effect of TMR feeding systems with increasing levels of cereal grain in the diet have been reported to result in unhealthy alterations to the rumen metabolome with increased concentrations of biogenic amines, methylamines and putrescine and increased rumen acidification [12]. However, there is limited information currently available comparing the effects of grazing pasture systems and TMR feeding systems on a cows ruminal fluid metabolome and raw milk metabolome.

With this in mind, the primary objective of this study was, to investigate the effects of three widely practiced feeding systems, consisting of a TMR diet indoors, perennial ryegrass (*Lolium perenne* L.) outdoors (GRS), and perennial ryegrass/white clover (*Trifolium repens* L.) outdoors (CLV) in Ireland on the cows ruminal fluid and milk metabolomes. A secondary

objective was to determine if it is was possible to distinguish pasture derived milk from TMR produced milk using NMR based metabolomics. Our results have indicated that this is possible.

6.3 Materials and Methods

6.3.1 Experimental Design and Sample Collection

Three feeding systems were compared over a full lactation at the Teagasc, Animal and Grassland Research and Innovation Centre, Moorepark, Fermoy, Co. Cork, Ireland. Herd 1 was housed indoors and fed a total mixed ration diet (TMR), herd 2 was maintained outdoors on perennial ryegrass only pasture (GRS) while herd 3 was also maintained outdoors on a perennial ryegrass/white clover pasture (CLV). On a dry matter basis (DM) the TMR diet consisted of 7.15 kg of grass silage, 7.15 kg of maize silage and 8.3 kg concentrates (see Table 6.1 and 6.2). Cows within the TMR system were fed at 08:30 h daily into electronically controlled Griffith Elder Mealmaster individual feed bins (Griffith Elder and Company Ltd, Suffolk, England) and feed was available *ad-libitum*. Pasture based cows consumed ~18 kg DM/day measured by pre- and post-grazing sward heights daily using the rising plate meter (Jenquip, Fielding, New Zealand) while pre-grazing herbage mass was measured with an Etesia mower (Etesia UK Ltd, Warwick, UK). The CLV sward contained ~20 % white clover and was measured according to Egan*, et al.* [13] Compositional analysis of GRS and CLV swards are shown in Table 6.3.

Table 6.1: Typical ingredient formulation and chemical composition of TMR concentrate

Concentrate Ingredient Composition	% as fed
Maize	13.00
Beet pulp molassed	15.50
Soyabean meal 48% CP	30.00
Maize distillers	12.00
Acid buffer	0.70
Maize/Beet Min Balancer	2.50
Salt	0.50
Barley (rolled)	15.00
Rapeseed meal	7.50
Megalac	3.30
Chemical Analysis	/kg as fed
DM, g	87.49
UFL	1.02
UFV	0.99
Crude protein %	24.28
PDIN, g	169.26
PDIE, g	133.91
Starch %	18.10
Sugar %	6.92
Crude fibre %	6.10
Oil %	5.12
Ash %	7.99
Ca %	1.10
P %	0.62
Copper mg/kg DM	94.66
ME MJ / kg DM	11.16

Table 6.2: Chemical composition (mean ± s.d.) and nutritional content of silages from TMR diet (grass silage and maize silage) collected weekly throughout lactation analysed by near-infrared spectroscopy.

	Grass Silage	Maize Silage
DM (% of DM)	31.53 (± 11.71)	25.3 (±3.11)
CP (% DM Basis)	11.4 (± 1.44)	5.83 (± 1.04)
Starch (% DM Basis)	NA	20.84 (± 4.14)
ADF (% DM Basis)	27.16 (±2.03)	NA
NDF (% DM Basis)	40.66 (± 3.61)	50.97 (± 2.47)
ASH (% DM Basis)	8.83 (± 1.09)	2.95 (± 0.66)
UFL (/kg of DM)	0.99 (± 0.06)	0.88 (± 0.02)
PDIA (/kg of DM)	22.92 (± 6.30)	12.68 (± 2.26)
PDIE (/kg of DM)	69.40 (± 10.69)	61.27 (± 2.73)
PDIN (/kg of DM)	71.05 (± 6.36)	35.80 (± 6.37)

UFL = unité fourragère lait; PDIA = sum of the feed protein ruminally undegraded and truly digested in the small intestine; PDIE = sum of PDIA and the microbial true protein that is truly digested in the small intestine (PDIM) when energy is limiting; PDIN = sum of PDIA and PDIM when nitrogen is limiting. NA = not available.

Table 6.3: Chemical composition and nutritional content (mean ± s.d.) of pasture system forages (GRS and CLV) collected weekly throughout lactation analysed by near-infrared spectroscopy

	GRS	CLV
OM digestibility	853.43 (± 50.24)	859.45 (± 41.14)
CP	234.45 (± 42.39)	248.93 (± 34.49)
ADF	286.30 (± 33.76)	288.46 (± 31.12)
NDF	387.97 (± 39.06)	362.21 (± 33.06)
Ash	62.37 (± 16.47)	58.23 (± 12.37)
UFL (/kg of DM)	0.97 (± 0.06)	0.97 (± 0.05)
PDIA (/kg of DM)	44.89 (± 5.71)	46.86 (± 4.76)
PDIE (/kg of DM)	103.31 (± 5.19)	105.97 (± 4.50)
PDIN (kg of DM)	151.99 (± 28.53)	161.71 (± 23.59)

UFL = unité fourragère lait; PDIA = sum of the feed protein ruminally undegraded and truly digested in the small intestine; PDIE = sum of PDIA and the microbial true protein that is truly digested in the small intestine (PDIM) when energy is limiting; PDIN = sum of PDIA and PDIM when nitrogen is limiting.

Rumen sampling took place at 07:00 each morning. Nine ruminally cannulated, healthy, spring calving Friesian cows were allocated to three groups (n=3). It has been previously described that 4 to 12 days is adequate time for animals to adapt to a diet [14], indeed for similar studies investigating the rumen metabolome an 11 day period was used [12]. With that in mind, for this study, three cows were randomly assigned to each diet for a two-week period, the first two weeks was an acclimatisation period after the end of the second week rumen-fluid was collected on the morning of day 15 and 16. The cows were then rotated to a different feeding system and the two-week acclimatization process was repeated. This sampling process was carried out in each stage of lactation (early, mid and late). Rumen samples were collected and then filtered through cheese cloth into sterile containers, and the rumen-fluid was stored at -80 °C prior to analysis.

For milk collection and analysis; thirty Friesian cows were divided into the three feedings systems (n=10) at the beginning of lactation using the experimental design as described previously [5, 15, 16].

Milks from ten individual cows in each of the groups was collected in July 2016, when cows were in the mid stage of lactation and peak production period. Milk from cows in each of the feeding systems was collected at morning and evening milking's and individual cows samples for each day were combined 1:1 (v:v) in a 50 ml falcon tube which was then vortexed, and stored at -80 °C. Milk collections were performed from each feeding system in triplicate one day apart (i.e. Monday, Wednesday and Friday) to provide a comprehensive sample set of milks at that particular time of lactation.

6.3.2 Feed Compositional Analysis

Feed samples were collected throughout lactation from the paddocks at the time of grazing. Grass silage samples were collected weekly. Samples were dried at 60 °C for 48 h, milled and stored prior to analysis. Samples were analysed using near infrared reflectance spectroscopy using a FOSS 6500 (FOSS Ireland Ltd, Dublin, Ireland). The UFL, PDIA, PDIE, PDIN have

been calculated according to the INRA feeding system equations [17]. Analysis of Maize silage was carried out by FBA Laboratories Ltd (Waterford, Ireland).

6.3.3 Ethical Approval

Teagasc has both an animal welfare body (AWB) and animal ethics committee. The AWB is a legal requirement of Article 26 of Directive 2010/63/EU and Regulation 50 of S.I. No. 543 of 2012. The Health Products Regulatory Authority (HPRA) provides project authorization and the HPRA Licence number for this project is: AE19132/P019.

6.3.4 NMR Sample Preparation

NMR analysis of rumen-fluids and raw milk samples was carried out at The Metabolomics Innovation Centre (TMIC), University of Alberta, Edmonton, Canada. Sample preparation for NMR analysis was performed using a similar method as described by Saleem*, et al.* [12] and Saleem*, et al.* [8]. Briefly, 3 kDa filters (Amicon Micoron YM-3; Sigma-Aldrich, St. Louis, MO) were washed five times using 500 µL HPLC water and centrifuged at 10,000 rpm for 10 mins. Samples were thawed at 4 °C overnight the day before analysis, once thawed rumen-fluid samples were vortexed and then centrifuged at 3,000 RPM for 5 min to sediment any particulate matter. 400 µL of sample fluid was filtered through washed 3 kDa filters at 11,000 rpm for 35 mins at 4 °C. 200 µL of filtrate was then added to 50 µL standard NMR buffer solution (5 mM DSS (disodium-2, 2-dimethyl-2-silapentane-5-sulphonate)), and 0.1 % NaN_3 in H_2O (Sigma–Aldrich, Mississauga, ON)) and transferred to standard NMR tubes. All ^{1}H-NMR spectra were collected on a 700 MHz Bruker NMR spectrometer equipped with a 5 mm cryoprobe. ^{1}H-NMR spectra were acquired at 25 °C using the first transient of the noesy-presaturation pulse sequence, which was chosen for its high degree of quantitative accuracy [18]. Spectra were collected with 128 transients using a 4 second (s) acquisition time, a 2 s relaxation delay and a 0.5 s mixing time.

6.3.5 NMR Compound Identification and Quantification

Prior to spectral analysis, all free induction decays (FIDs) were zero-filled to 64k data points and a line broadening of 0.5 Hz was applied. The methyl singlet of the added DSS served as

an internal standard for chemical shift referencing (set to 0 ppm) and for quantification. The resulting rumen ^{1}H-NMR spectra were processed and analysed using BAYESIL (http://www.bayesil.ca), a fully-automated NMR spectral profiling program. Milk ^{1}H-NMR spectra were processed and analysed using Chenomx NMR suite (v 8.1, Chenomx Inc, Edmonton, Alberta, Canada). Each spectrum was processed and analysed by at least two experienced NMR spectroscopists to minimize compound mis-identification and mis-quantification.

6.3.6 Statistical Analysis

The rumen and milk samples were analysed separately. For the rumen fluid, univariate statistical analysis was performed to discover potential differences between the feeding systems. General linear models (GLM) were built for each metabolite to determine whether the means of the three feeding systems (TMR, GRS and CLV) differ. The stage of lactation (early, mid and late) and the interaction with the diet were also included in the model. Data was log (base 2) transformed in order to reduce potential influential points and reduce the skew of the data.

For the milk study, a total of 30 cows were split randomly in three balanced different diet treatments (TMR, GRS and CLV). Metabolites were analyzed in the mid-lactation period for three consecutive days. For each cow the values of each repetition were averaged by the mean. One-way analysis of the variance (ANOVA) was performed by building a regression model for each metabolite to compare the mean values between the three different groups. Prior to the analysis, data was also log transformed. In both studies, the type I error, due to multiple testing, was controlled by using the Benjamini and Hochberg false discovery rate (FDR) procedure. Two-sided *P*-values < 0.05 of the corrected p-values was used as the threshold to refuse the null hypothesis that the means of all the groups did not differ. Tukey's honestly significant difference (HSD) test was used as a post-hoc analysis to find which treatments were significantly different from each other ($P < 0.05$) among the significant metabolites.

For both studies rumen-fluid and milk, multivariate analyses were performed. Unsupervised hierarchical clustering analysis (HCA) was done to observe patterns in the data, and is shown as a heatmap (Figure 6.1 and 6.3). A supervised multivariate model was built using partial least squared discriminant analysis (PLS-DA). In order to validate the model a permutation test with 2,000 repetitions was performed to check that the model differed from a random model. Also the R^2 and Q^2 parameters were obtained to obtain the performance of the model using a 10 fold cross-validation approach as well as the number of components to analyse. The variable importance plot (VIP) shows which variables have larger influence to the latent variables of the built model. Metaboanalyst [19] software and in-house R (R Core Team, version 3.4.2, 2016) code was used to perform the multivariate statistical analysis and produce the figures.

6.4 Results and Discussion

6.4.1 Rumen-fluid

NMR analysis of the rumen-fluids identified 63 metabolites present in each of the samples. Table 6.4 shows the average concentration of each of the metabolites throughout lactation, and the average metabolite concentration for each feeding system at the different stages of lactation is shown in Supplementary Table 6.1. The most abundant compounds present in the rumen-fluid metabolome were volatile/short chain fatty acids such as propionate, butyrate, acetic acid, valerate, isobutyric acid, and isovaleric acid. Similar trends were also reported by Saleem*, et al.* [8] who characterised the bovine rumen-fluid metabolome using a variety of analytical technologies.

The feeding system was shown to have a significant effect on the rumen metabolome as observed in the ANOVA test (diet *P*-values in Table 6.4). Such differences can be clearly observed in the clusters generated in the heatmap plot by the HCA analysis (Figure 1). It can be observed that the TMR group has higher levels of sugars, while the CLV group exhibits higher amino acids levels. The GRS group has a medium level of amino acids and high levels of nucleosides such as inosine and adenine. On the other hand, the multivariate PLS-DA

model (Figure 6.2) showed that it is possible to distinguish each of the feeding systems from each other using ^{1}H-NMR of the rumen fluid. While the GRS and CLV feeding systems appear to be more similar to each other (which is to be expected given the similarities of the diets) there is much clearer separation between the TMR and pasture based systems (Fig. 6.1). There are multiple rumen fluid metabolites that contribute to the separation between treatments in the PLS-DA model ($P < 0.001$ over 2,000 permutations). The metabolites with the largest contribution in distinguishing the feeding systems are shown in the variable importance plot (Supplementary Figure 6.1). The rumen fluid metabolites found to be most important for the observed separation were dimethyl sulfone, isopropanol, choline, maltose, methylamine, phenylacetate, uracil, nicotinate, isovaleric acid, cadaverine, isobutyric acid, *p*-hydroxyphenylacetic acid, 4-aminobutyrate, L-alanine and L-leucine.

Table 6.4: Average concentrations (Mean ± standard deviation) of rumen metabolites (μM) measured in the rumen of lactating dairy cows fed diets consisting of total mixed ration (TMR), perennial ryegrass (GRS) or perennial ryegrass and white clover (CLV) as determined by ^{1}H-NMR. Adjusted *p*-values from the ANOVA test for the feed system, lactation period and their interaction.

	Diet			*P*-Value		
Metabolite (μ*M*)	TMR	GRS	CLV	Diet	Time	Diet*Time
Dimethyl sulfone	3.83 (±2.43)	16.81 (±7.70)	33.38 (±12.49)	<0.01	0.03	0.21
Phenylacetate	199.58 (±56.74)	262.11 (±109.84)	381.02 (±191.25)	<0.01	<0.01	<0.01
Isovaleric acid	683.44 (±112.48)	701.31 (±135.43)	951.09 (±283.61)	<0.01	<0.01	<0.01
Isopropanol	18.16 (±3.86)	22.25 (±6.37)	42.18 (±27.01)	<0.01	<0.01	<0.01
Isobutyric acid	796.76 (±88.10)	811.95 (±103.96)	997.57 (±191.24)	<0.01	<0.01	<0.01
Uracil	200.49 (±91.29)	264.18 (±106.92)	297.61 (±107.14)	<0.01	<0.01	0.55
Nicotinate	25.77 (±7.67)	28.56 (±9.80)	35.93 (±9.57)	<0.01	<0.01	0.03
***p*-Cresol**	58.38 (±15.58)	65.95 (±24.67)	85.18 (±38.62)	<0.01	<0.01	<0.01
Choline	25.89 (±14.19)	14.68 (±8.23)	12.59 (±8.65)	<0.01	0.19	0.03
3-Phenylpropionate	745.91 (±112.81)	632.94 (±170.49)	634.19 (±124.84)	<0.01	<0.01	<0.01
O-Hydroxyphenylacetic acid	17.38 (±3.47)	13.08 (±3.78)	15.66 (±5.49)	<0.01	<0.01	0.03
L-Alanine	180.40 (±45.02)	189.58 (±55.46)	238.88 (±76.37)	<0.01	<0.01	0.04
L-Glutamic acid	293.98 (±71.48)	252.06 (±72.63)	350.58 (±97.44)	<0.01	0.02	0.06
Glycine	121.49 (±37.66)	119.16 (±40.23)	158.48 (±60.01)	0.01	<0.01	0.03
Inosine	11.53 (±6.29)	27.24 (±26.18)	22.14 (±18.14)	0.01	<0.01	0.03
Succinate	123.57 (±122.66)	74.56 (±51.26)	90.93 (±54.20)	0.01	<0.01	<0.01
Formate	118.49 (±4.16)	114.91 (±5.35)	118.09 (±2.89)	0.01	0.01	0.06
L-Leucine	89.73 (±19.51)	93.85 (±31.78)	119.58 (±38.95)	0.01	<0.01	0.21
Acetic acid	55295.87 (±7149.21)	54836.35 (±6828.43)	58322.59 (±3754.77)	0.01	<0.01	0.03
Acetone	8.67 (±3.16)	8.53 (±2.01)	12.03 (±6.44)	0.01	<0.01	0.07
Valine	93.59 (±23.59)	90.46 (±35.70)	128.79 (±65.36)	0.01	<0.01	0.11
Butyrate	12585.29 (±2066.81)	14245.39 (±2839.45)	14728.79 (±2514.33)	0.02	0.05	0.21
Benzoic acid	23.88 (±4.87)	26.31 (±7.47)	27.46 (±5.34)	0.02	<0.01	0.04
D-Maltose	71.21 (±66.10)	34.40 (±27.63)	33.35 (±26.23)	0.02	<0.01	0.18
Aspartate	153.24 (±54.47)	115.83 (±45.41)	148.01 (±79.63)	0.03	<0.01	<0.01
Tyrosine	44.27 (±12.59)	43.55 (±16.83)	56.05 (±20.71)	0.03	<0.01	0.21
***p*-Hydroxyphenylacetic acid**	17.86 (±4.95)	14.62 (±3.72)	13.77 (±4.27)	0.07	0.87	0.72
Isoleucine	84.44 (±23.65)	83.46 (±35.14)	105.76 (±38.25)	0.07	<0.01	0.27
L-Threonine	108.67 (±41.15)	85.44 (±32.09)	106.61 (±42.64)	0.08	<0.01	0.04
Valerate	1030.42 (±266.50)	1256.89 (±602.06)	1328.14 (±439.27)	0.08	0.01	0.04
L-Proline	84.33 (±34.94)	80.50 (±37.46)	108.33 (±63.16)	0.09	<0.01	0.09
L-Lysine	149.54 (±64.36)	173.82 (±77.85)	205.94 (±123.11)	0.14	<0.01	0.45
Methylamine	4.64 (±11.01)	11.39 (±16.79)	16.25 (±33.42)	0.25	0.15	0.04
3-Hydroxyphenylacetic acid	26.71 (±9.76)	22.41 (±9.00)	22.85 (±7.60)	0.25	<0.01	0.16
Methionine	33.63 (±9.78)	35.13 (±13.72)	40.76 (±12.37)	0.26	0.01	0.34
Acetoin	25.41 (±9.68)	21.36 (±6.28)	25.29 (±8.02)	0.26	0.10	0.68
Hypoxanthine	171.16 (±32.82)	159.21 (±56.02)	179.41 (±70.06)	0.27	<0.01	0.03
Dimethylglycine	14.28 (±21.99)	4.55 (±4.93)	7.24 (±13.14)	0.31	0.81	0.79
L-Phenylalanine	51.48 (±13.84)	50.64 (±19.03)	57.76 (±22.60)	0.32	<0.01	0.17

Table 6.4 continued

	Diet			*P*-Value		
Metabolite (μ*M*)	TMR	GRS	CLV	Diet	Time	Diet*Time
Adenosine	4.55 (±3.34)	6.78 (±5.51)	5.08 (±4.26)	0.35	0.16	0.49
4-aminobutyrate	36.39 (±18.73)	52.15 (±35.68)	48.77 (±20.07)	0.36	0.26	0.86
ryptophan	7.17 (±1.65)	7.39 (±3.02)	8.19 (±3.41)	0.36	<0.01	0.11
Ethanol	25.63 (±22.49)	59.29 (±109.16)	31.70 (±20.26)	0.37	0.66	0.28
Cadaverine	82.27 (±39.12)	96.51 (±76.25)	107.09 (±27.17)	0.43	0.97	0.16
Propionate	17119.26 (±3923.21)	18084.74 (±3647.63)	18140.78 (±2724.51)	0.43	0.01	0.04
***cis*-Aconitate**	5.58 (±3.40)	8.46 (±9.56)	7.59 (±5.55)	0.50	0.30	0.44
Trimethylamine	5.80 (±14.48)	1.85 (±1.38)	5.40 (±9.03)	0.51	0.37	0.13
Methanol	13.74 (±14.69)	9.69 (±1.28)	11.44 (±3.02)	0.51	0.81	0.44
Adenine	26.43 (±7.53)	26.12 (±12.30)	30.54 (±22.58)	0.51	<0.01	0.22
Betaine	6.91 (±10.81)	5.08 (±5.66)	3.80 (±1.87)	0.51	0.36	0.79
Creatine	8.69 (±5.98)	6.93 (±5.02)	6.93 (±3.56)	0.51	0.71	0.96
Glycerol	254.92 (±55.91)	242.33 (±34.69)	248.91 (±48.42)	0.51	<0.01	0.28
3-Hydroxybutyric acid	12.78 (±14.29)	8.68 (±5.09)	12.63 (±13.27)	0.51	0.13	0.28
Putrescine	58.93 (±21.87)	56.48 (±55.10)	45.56 (±16.64)	0.51	0.02	0.56
D-Glucose	520.63 (±287.13)	544.86 (±383.52)	602.03 (±536.85)	0.63	<0.01	0.03
Dimethylamine	4.25 (±7.97)	2.31 (±1.32)	4.80 (±9.51)	0.64	0.26	0.69
L-Histidine	32.96 (±11.46)	36.68 (±14.08)	34.49 (±15.58)	0.65	<0.01	0.32
2-hydroxyisovalerate	7.06 (±6.35)	6.17 (±4.99)	7.49 (±5.32)	0.78	0.81	0.21
Citric acid	7.46 (±6.93)	7.53 (±4.36)	6.62 (±2.72)	0.83	0.02	0.67
Uridine	7.40 (±8.28)	7.49 (±5.34)	8.81 (±8.03)	0.83	0.42	0.28
Beta Alanine	18.43 (±14.98)	18.24 (±16.25)	17.44 (±14.00)	0.94	<0.01	0.01
Ethanolamine	29.38 (±13.94)	28.26 (±13.02)	29.48 (±19.30)	0.94	<0.01	0.44
L-Lactic acid	30.66 (±22.16)	29.41 (±44.10)	27.49 (±18.07)	0.94	0.06	0.54

The TMR and CLV rumen-fluid had significantly ($P < 0.05$) higher acetic acid concentration than GRS. Among other volatile fatty acids (VFA's) detected in the rumen-fluid, CLV feeding was demonstrated to produce significantly higher concentrations of isovaleric acid and isobutyric acid. VFA's are produced in the rumen as end products of microbial fermentation of proteins and carbohydrates, and represent a major source of energy for the animals [20]. Overall, increased VFA levels are indicative of increased rumen fermentation. In particular, increased acetic acid could be as a result of increased protein consumption, which when broken down provides amino acids that can be fermented to branched chain organic acids [21]. Among the amino acids, L-alanine was significantly higher in CLV than both GRS and TMR. CLV had significantly higher concentration of glycine than both TMR and GRS. CLV also had significantly higher concentration of tyrosine than GRS. TMR and CLV had significantly higher concentrations of L-glutamic acid and aspartate. Increased levels of amino acids could potentially be attributed to increased digestible protein contents of feed providing a proteinaceous substrate for microbial degradation. Leucine and valine were significantly higher in CLV than GRS, which would contribute to increased presence of the isoacids (isobutyric and isovaleric acid) which are products derived from valine and leucine amino acids [20, 22]. This increase in the presence of isoacids is of interest as Andries*, et al.* [22] noted that from review of cattle experiments, it appeared that, in lactating cows, a nutritional supplement of isoacids may have a positive influence on milk production. This trend again would suggest even greater levels of rumen fermentation occurring in the CLV rumen which could be attributed to white clovers' characteristic high nutritive value, improved animal feed intakes and utilisation [23]. Indeed, increased milk yields have been reported with the inclusion of clover swards, as reported in comparison to perennial rye grass only swards [5, 24].

Each feeding system had a significant effect on the concentrations of dimethyl sulfone, with CLV > GRS > TMR (Figure 6.1). This compound was also the major compound responsible for the separation between the diets seen by PLS-DA (Figure 6.2, 6.4 and

supplementary Figure 6.1). Dimethyl sulphone in the rumen is produced by the catabolism of sulfur amino acids, in particular methionine, which is hydrolysed to dimethyl sulphide [25] and subsequently oxidised to dimethyl sulfone. Methionine has been previously reported in highest concentrations in pastures as opposed to silage and hay diets [26]. As such increased levels of dimethyl sulfone from pastures could be related to increased levels of digestible protein and is highest in CLV as a result of its increased sward digestibility.

TMR derived rumen-fluid had significantly higher concentrations of 3-phenylpropionate compared to that of the pasture derived rumen samples while, CLV feeding resulted in significantly higher concentrations of phenylacetate than both TMR and GRS. These compounds have been identified previously as important aromatic acids in ruminal fluid [12]. They are generated through the hydrogenation of plant phenolic compounds such as *p*-coumaric, ferulic, and caffeic acid by the ruminal micro-organisms, with subsequent dehydroxylation [27]. A concomitant study analysing the milks from these diets identified, through headspace analysis of volatile compounds of the feeds, that TMR had significantly higher content of phenols than that of pasture derived feeds [28] which could be potentially attributed to the diversity of the ration mix and inclusion of grass and maize silage and as Martin [29] stated phenols can be produced during ensilage. Ametaj*, et al.* [11] also stated that increased phenyl acetate present in the rumen-fluid could be a result of deamination of aromatic amino acids such as tyrosine which as mentioned was significantly higher in CLV.

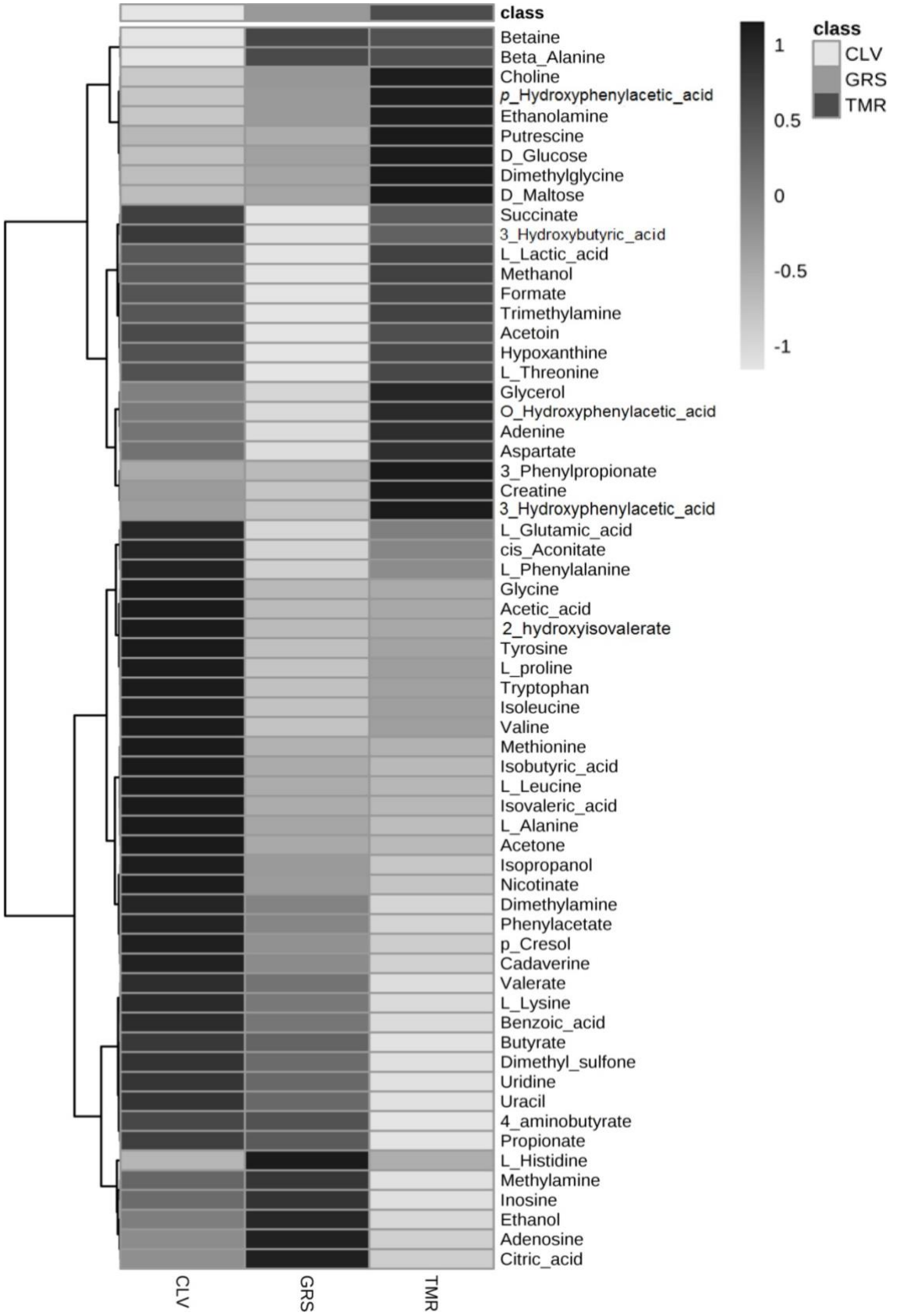


Figure 6.1: Hierarchical clustering analysis (heatmap) of average rumen-fluid metabolites from lactating dairy cows fed diets consisting of total mixed ration (TMR), perennial ryegrass (GRS) or perennial ryegrass and white clover (CLV) as determined by 1H-NMR

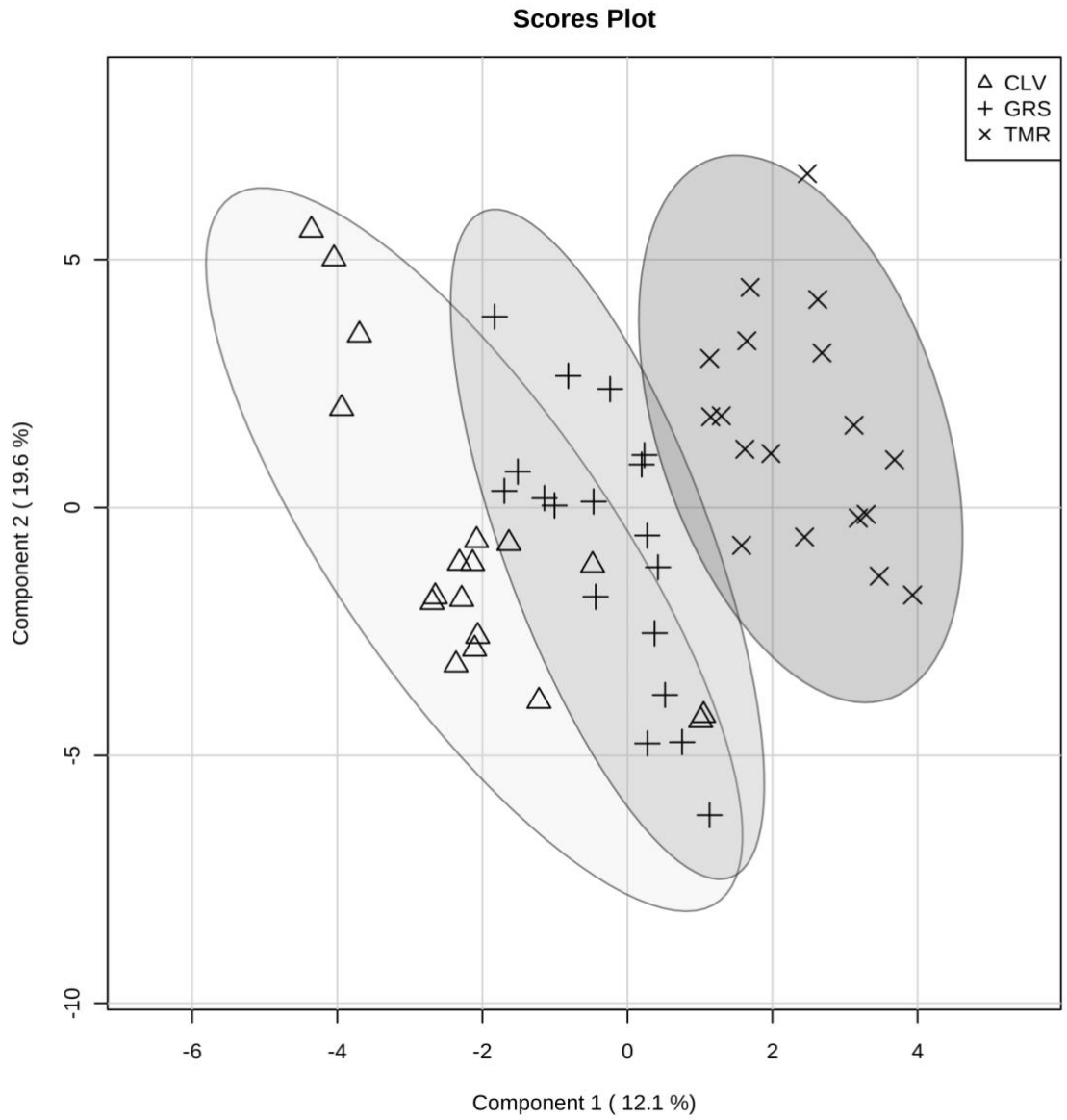


Figure 6.2: Partial least square discriminant analysis (PLS-DA) score plot of the rumen metabolome of lactating dairy cows fed diets consisting of total mixed ration (TMR), perennial ryegrass (GRS) or perennial ryegrass and white clover (CLV) as determined by 1H-NMR. The shaded ellipses represents the 95% confidence interval estimated from the scores.

The TMR resulted in significantly higher concentrations of the disaccharide D-maltose, compared to pasture feeding systems. This is to be expected considering the much higher starch content of the TMR diet (Table 6.1). Similar increasing trends in the concentration of D-maltose with increasing proportions of cereal grain in the diet have been previously reported [8, 11, 12].

Pasture feeding resulted in significantly higher content of uracil than that of TMR. Uracil has been reported previously in the rumen-fluid metabolome by Ametaj, *et al.* [11] and

recently by Zhang*, et al.* [30] who both reported contrary results to this study with an increase in uracil in high concentrate diets. Hypoxanthine, although not significant, was also highest in CLV. Both studies attributed the differences in uracil along with alanine and hypoxanthine (and xanthine which was not found in this study) in the rumen as products of bacterial degradation [31]. CLV had significantly higher concentrations of acetone and isopropanol than those of TMR and GRS. Bruss and Lopez [32] concluded through the use of in-vitro experiments that rumen microbial metabolism of acetone is the likely source of plasma isopropanol. Martin*, et al.* [33] also recently demonstrated that isopropanol can be produced through rumen hydrolysis of the methionine analogues, butanoic acid and its isopropyl ester. CLV rumen-fluid had significantly higher concentration of nicotinate than that of TMR and GRS. Nicotinate has been reported in the rumen-fluid metabolome previously [11, 12]; however, in these studies its concentration was not affected by diet but its presence was attributed to the ability of several bacterial species identified in rumen contents to synthesize nicotinate.

p-Cresol is a metabolite that has been receiving much attention in recent years in terms of its effects on the sensory quality of pasture derived milk and milk products. *p*-Cresol is a potent odorant and if present at excessive levels in products it provides "barny" or "animal-like" flavours [34] which have been described as undesirable traits to sensory palates not accustomed to pasture derived milk and products. Interestingly, CLV had significantly higher concentrations of *p*-cresol than that of TMR and GRS. Although not significantly different (*P.*>0.05) average *p*-cresol content of GRS was also considerably higher than that of TMR (65.95 µM vs 58.38 µM respectively). Interestingly the deviation of *p*-cresol concentrations throughout the study appeared to be considerably higher in pasture cows than that of TMR which is likely in response to changing pasture quality and intake over the season. Martin [29] concluded that *p*-cresol is a rumen metabolite of tyrosine produced through the deamination and decarboxylation reactions associated with the degradation of tryptophan and tyrosine

resulting in the formation of *p*-cresol [35]. Increased *p*-cresol could also be as a result of β-carotene degradation [36]. β-carotene is naturally higher in fresh pastures as the ensiling process and processing of feeds for concentrates typically depletes or destroys many carotenoids, and as a result most concentrates fed to cows on a TMR system are low in *β*-carotene [37]. As such *β*-carotene has been suggested as a biomarker of fresh pasture feeding in dairy products [38]. Finally, elevated levels of *p*-cresol in CLV could be as a result of formononetin, a constituent of clover species, which have been shown to be degraded in the rumen to produce *p*-cresol [39]. Overall, these alterations would further suggest that the feeding regimen is altering the fermentation processes in the rumen through alterations in microbiome functionality.

TMR feeding resulted in significantly higher choline content than pasture derived rumen-fluids. Choline is regarded as an important compound for health status and has been suggested to impact milk production [40]. Choline can be acquired in two major forms; through diet, although in ruminants dietary choline is extensively degraded by the rumen [41] and via endogenous synthesis by the phosphatidylethanolamine N-methyltransferase (PEMT) pathway which represents an important source of choline [42]. Choline is an important compound as it can act as a precursor for several metabolites such as acetylcholine (a neurotransmitter), and betaine (a source of labile methyl groups) and is required to make essential membrane phospholipids [43]. Phosphatidylcholine, in particular, is produced via the cytidine diphosphate (CDP) choline pathway [42] and is important for the removal of triacylglycerol from the liver. As such an absence of choline can result in fatty liver degeneration and choline is considered a lipotropic factor [44]. Higher choline availability achieved through feeding strategies utilising rumen protected choline have been shown to have a favourable effect on milk production. This could potentially be attributed to subsequent increased availability of methionine for milk synthesis, enhanced glucogenesis in the liver and general health improvement of the cows [44, 45].

The CLV feeding resulted in significantly higher content of formate than GRS, while TMR feeding resulted in significantly higher concentration of succinate than that of GRS. Formate and succinate have been described as important fermentation products of pure cultures of rumen bacteria [46, 47]. Succinate is an important extracellular intermediate in overall rumen fermentation, which is produced as a product of carbohydrate fermentation and is decarboxylated by a series of enzymes to form propionate. Propionate formation is essential to ruminants as a substrate for glucose synthesis [47]. Formate is metabolised rapidly in the rumen and can be an important source of hydrogen, which coupled with carbon dioxide appear to be chief substrates for methanogenisis [48]. Indeed, Lovley*, et al.* [49] reported that methanogenic bacteria have the potential to directly metabolize formate in the rumen to produce CH_4. This could in turn be disadvantageous in comparison to GRS, with potentially increased production of methane adding to overall greenhouse gas emissions. Further work is however required to confirm this.

Although not the main focus of this study, a change in the rumen metabolome was observed between different stages of lactation. PLS-DA (supplementary Figure 6.3) has shown that while mid and late lactation are very similar and cluster together, early lactation appears to be more distinguishable and separates from the other stages of the lactation cycle. Alterations of the rumen metabolome throughout lactation are to be expected as a result of changing energy requirements of the cow, and, the quality and availability of pastures. There was a significant (P = <0.01) Diet*Time interaction also seen for several metabolites including phenylacetate, isovaleric acid, isopropanol, isobutyric acid, *p*-cresol, 3-phenylpropionate, succinate and aspartate. These are likely as a result of changing energy requirements, intake and pasture quality during peak milk production mid-lactation period. However, major differences in the early lactation period observed in PLS-DA (Supplementary Figure 6.3) could potentially be as a result of negative energy balance experienced in cows at the onset and during early lactation. In early lactation, dietary intake is often unable to meet the demands of high

milk production. The cow therefore enters a period of negative energy balance, causing mobilization of body reserves to balance the deficit between food energy intake and milk energy production, which can result in alterations to metabolism [50].

6.4.2 Milk

^{1}H-NMR analysis of mid lactation milk samples identified 49 metabolites present in each of the samples the majority of which were also found to be present in the rumen-fluid portion. Table 6.5 shows the average concentration of each of the metabolites from milks from each of the feeding systems. The feeding system was shown to have a significant effect on the milk metabolome (Figure 6.3). Such differences are clearly visible using PLS-DA (Figure 6.4, P <0.005 on 2,000 permutations). These results demonstrated that it is possible to distinguish milks from each of the feeding systems from each other by their metabolomes using NMR based metabolomics (Figure 6.3 and 6.4). Similar to the rumen data, the milk from GRS and CLV appear to be more similar to each other although with a more obvious separation than that observed in the rumen. In addition there is a much clearer separation between the TMR and pasture based systems (Fig. 6.4). The PLS-DA also allowed the identification of the metabolites that were most important for the observed separation in milk samples as shown in Supplementary Figure 6.2, variables with VIP values > 1. The metabolites found to be most important for the observed separation were dimethyl sulfone, tyrosine, dimethylamine, L-Fucose, glucose-1-phosphate, acetone, hippuric acid, creatine phosphate, 3-hydroxybutyric acid and choline. Anova analysis and the multiple comparison tests of milk metabolites showed similar patterns to the rumen-fluid; CLV derived milk had significantly higher concentrations of acetone, while tyrosine concentrations were also greater in pasture derived milk than that of TMR. Each diet had a significant effect on the concentration of dimethyl sulfone with CLV>GRS>TMR. Dimethyl sulfone has previously been reported at significantly increased concentrations in milks derived from pastures [26, 51]. TMR derived milks had significantly higher concentration of dimethylamine than both pasture derived milks. Saleem, *et al.* [12]

reported the presence of dimethylamine in the rumen of cows fed increasing proportions of grain, and the presence of biogenic amines is related to dietary source and alteration to the rumen microbiota. Each feeding system also had a significant effect on the concentration of urea in milk with CLV>TMR>GRS. Urea is a typical constituent of milk and contributes a major portion to milk's non-protein nitrogen fraction. Indeed, levels of urea align with the non-protein nitrogen content of milks from these diets [5]. Urea is the metabolic end product of protein catabolism in the body [52]. As such, the concentration of milk urea nitrogen is influenced by dietary crude protein intake and digestibility. Harris*, et al.* [53] also reported that increased clover proportions in the diet resulted in increased urea concentrations. Crude protein is digested in the rumen producing ammonia which is taken up by the blood stream and transported to the liver where it is converted to urea which is then diffused into the milk and blood [54]. Indeed, milk urea levels are correlated with blood urea levels. Huhtanen*, et al.* [55] demonstrated that an increase in milk urea nitrogen is negatively associated with efficiency of nitrogen utilisation and positively associated with urea excretion. Reduced urea excretion would be advantageous in reducing the agricultural contribution to environmental pollution [54].

Table 6.5: Average concentrations (Mean ± standard deviation) of raw milk metabolites (μM) measured during mid lactation from cows fed diets consisting of total mixed ration (TMR), perennial ryegrass (GRS) or perennial ryegrass and white clover (CLV) as determined by 1H-NMR. Adjusted p-values from the ANOVA test for the feed system

Metabolites (μM)	TMR	GRS	CLV	*p*-value
Dimethyl sulfone	**11.67** (±1.59)	27.97 (±7.90)	46.41 (±12.72)	<0.01
Hippuric acid	112.76 (±33.00)	227.93 (±38.90)	165.93 (±32.77)	<0.01
Dimethylamine	14.60 (±2.77)	9.95 (±1.96)	9.40 (±2.30)	<0.01
Acetone	13.20 (±3.88)	11.66 (±1.22)	18.85 (±3.54)	<0.01
Urea	282.72 (±45.21)	221.68 (±102.60)	389.71 (±57.62)	<0.01
Tyrosine	4.71 (±0.96)	8.11 (±1.97)	8.65 (±2.51)	<0.01
Caprylic acid	12.92 (±6.01)	23.01 (±8.03)	14.23 (±6.69)	0.04
Uridine	16.07 (±4.91)	11.76 (±1.81)	11.78 (±2.37)	0.06
Ethanolamine	84.09 (±27.64)	125.81 (±39.77)	116.79 (±23.05)	0.07
L-proline	82.350 (±29.96)	55.55 (±12.68)	60.57 (±15.15)	0.07
Alpha-Lactose	109682.11 (±4835.98)	109179.54 (±4189.15)	103625.15 (±5267.57)	0.07
3-Hydroxybutyric acid	34.06 (±10.63)	28.18 (±2.92)	24.63 (±4.75)	0.07
Valine	11.27 (±3.98)	7.92 (±1.59)	8.55 (±2.14)	0.10
Acetic acid	71.81 (±41.67)	41.47 (±10.17)	47.88 (±12.36)	0.12
Butyrate	29.22 (±5.72)	43.93 (±16.09)	35.91 (±16.47)	0.23
Betaine	90.15 (±25.33)	72.22 (±16.17)	64.41 (±31.44)	0.23
Choline	214.22 (±58.37)	298.26 (±146.01)	310.54 (±77.69)	0.23
Formate	117.40 (±1.66)	116.44 (±1.93)	115.20 (±2.75)	0.23
L-Fucose	27.79 (±13.14)	21.03 (±14.33)	15.36 (±7.37)	0.23
Creatinine	50.69 (±7.42)	53.41 (±9.12)	45.12 (±8.40)	0.23
L-Alanine	29.20 (±5.91)	27.45 (±5.67)	23.82 (±5.36)	0.27
Phosphorylcholine	72.12 (±59.74)	30.83 (±34.97)	44.14 (±32.01)	0.27
Aspartate	28.86 (±11.37)	28.63 (±13.11)	20.35 (±7.08)	0.33
Succinate	23.20 (±6.60)	19.73 (±3.18)	23.06 (±3.75)	0.41
Isobutyric acid	15.87 (±20.47)	5.10 (±4.79)	14.45 (±15.29)	0.46
L-Leucine	4.64 (±1.55)	3.80 (±1.31)	3.81 (±0.83)	0.47
Glucose-1-phosphate	52.60 (±27.92)	86.70 (±128.89)	28.31 (±27.81)	0.47
D-Glucose	331.24 (±84.17)	340.62 (±65.95)	387.43 (±91.21)	0.47
L-Lactic acid	73.34 (±93.23)	47.31 (±26.19)	33.43 (±9.04)	0.49
***cis*-Aconitate**	35.20 (±4.97)	35.13 (±6.74)	32.12 (±3.51)	0.55
Glycerophosphocholine	617.18 (±83.87)	532.34 (±107.89)	531.67 (±240.19)	0.58
Isoleucine	5.15 (±1.64)	4.33 (±1.74)	4.20 (±1.53)	0.58
Propionate	5.71 (±7.02)	7.58 (±6.92)	3.84 (±2.27)	0.58
Pyruvic acid	38.03 (±8.02)	36.52 (±14.70)	31.75 (±8.44)	0.58
Capric acid	20.66 (±5.75)	23.59 (±7.41)	19.89 (±5.74)	0.58
Orotic acid	542.52 (±165.89)	581.43 (±134.08)	487.36 (±177.47)	0.59
***p*-Cresol**	13.87 (±11.56)	13.39 (±10.02)	9.36 (±2.04)	0.62
L-Carnitine	75.91 (±18.17)	81.36 (±20.84)	85.71 (±17.08)	0.64
L-Acetylcarnitine	45.97 (±7.39)	51.71 (±12.86)	49.66 (±12.65)	0.64
Creatine Phosphate	60.35 (±28.96)	49.33 (±52.66)	38.24 (±39.15)	0.64
D-Galactose	730.39 (±277.50)	901.88 (±624.82)	1023.29 (±936.59)	0.74
Valerate	6.50 (±3.19)	6.47 (±2.84)	5.44 (±2.61)	0.74
Methanol	27.43 (±15.84)	23.64 (±7.16)	29.85 (±19.30)	0.74
Oxoglutarate	117.93 (±17.22)	116.48 (±18.59)	111.39 (±15.62)	0.75
Citric acid	4595.41 (±448.18)	4602.04 (±617.46)	4453.36 (±225.49)	0.77
Fumaric acid	11.63 (±3.65)	11.20 (±4.36)	12.54 (±2.98)	0.77
Malic acid	92.79 (±29.23)	83.66 (±28.83)	91.15 (±26.69)	0.78
Creatine	436.42 (±79.99)	432.95 (±84.67)	427.70 (±115.63)	0.98
L-Glutamic acid	196.16 (±64.07)	188.62 (±77.08)	190.87 (±67.63)	0.98

Furthermore, increased levels of urea will also contribute to increased milk non-protein nitrogen content which would be disadvantageous to dairy manufacturers, whose current payment schemes are based on crude protein as opposed to true protein content and potential reduced product yields. Furthermore, levels of milk urea nitrogen at the time of insemination have been shown to have an effect on cows reproduction performance [56]. Pasture feeding resulted in milks with significantly higher hippuric acid content than that of TMR. Likewise, GRS hippuric acid was significantly higher than CLV. Hippuric acid has been identified as a constituent of the non-protein nitrogen fraction of milk [57]. Hippuric acid in milk has been attributed to the presence of caffeoylquinic compounds in forages and increased levels of hippuric acid in milks from pasture based feeding systems has previously been reported in milks from cows and goats [58, 59]. Increased levels of hippuric acid from pasture based feeding would also be in agreement with the results of Carpio*, et al.* [60], who using goats milks, suggested hippuric acid as a biomarker of feeding systems, where increased levels of hippuric acid represents a diet based mainly or exclusively on grazing pastures.

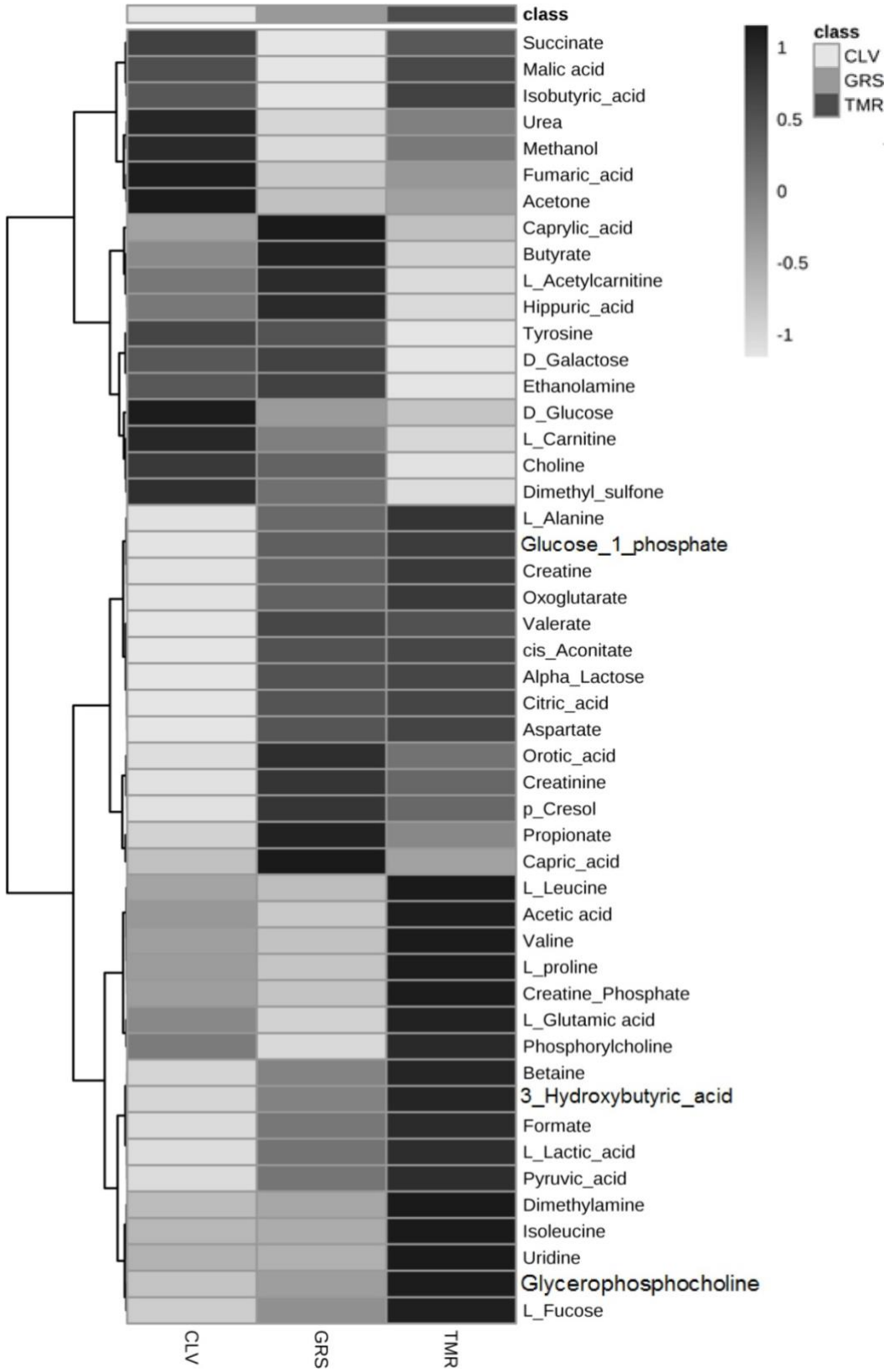


Figure 6.3: Hierarchical clustering analysis (heatmap) of average raw milk metabolites from mid-lactation cows fed diets consisting of total mixed ration (TMR), perennial ryegrass (GRS) or perennial ryegrass and white clover (CLV) as determined by 1H-NMR

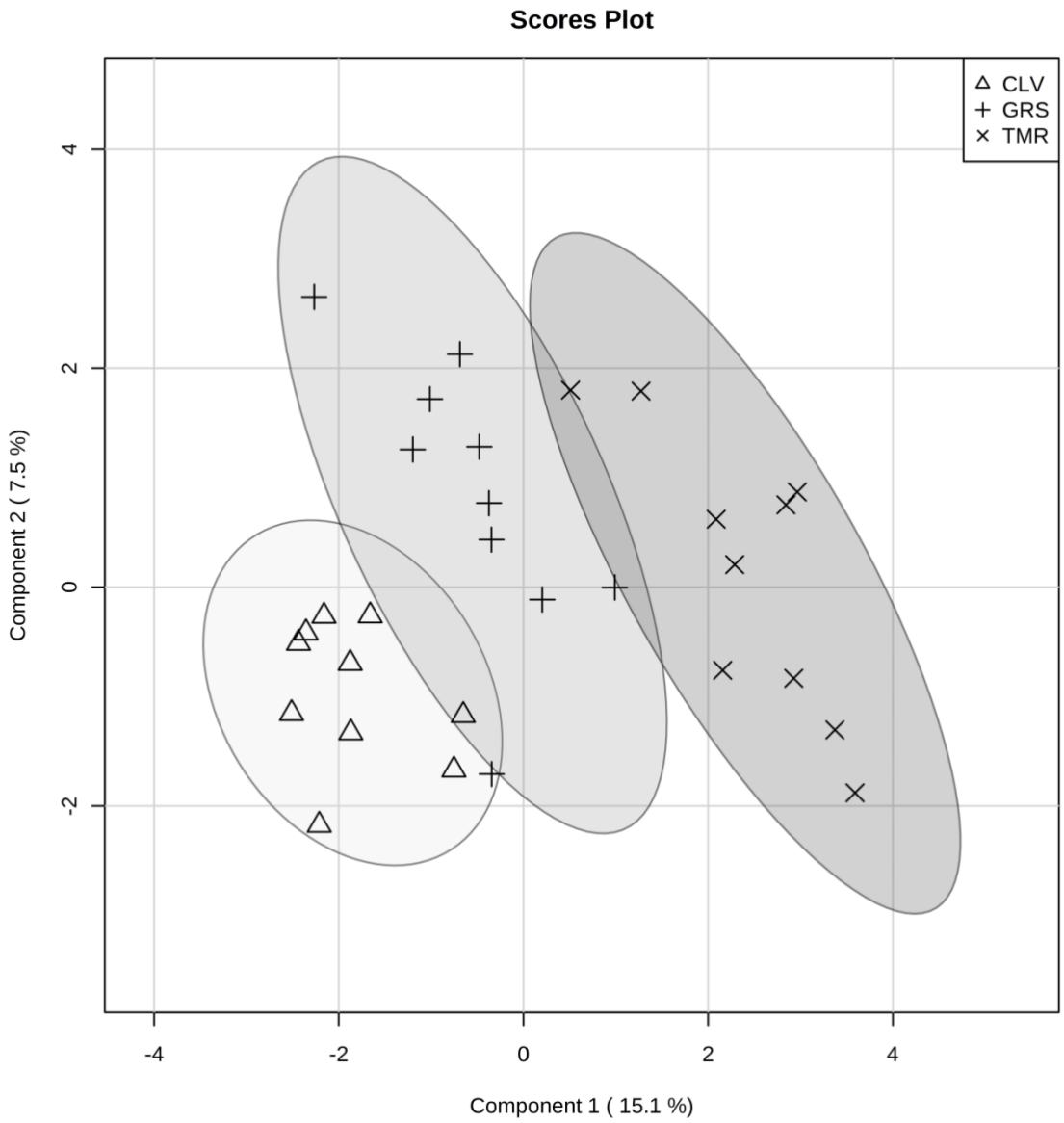


Figure 6.4: Partial least square discriminant analysis (PLS-DA) score plot of mid lactation raw milk metabolome of cows fed diets consisting of total mixed ration (TMR), perennial ryegrass (GRS) or perennial ryegrass and white clover (CLV) as determined by 1H-NMR. The shaded ellipses represents the 95% confidence interval estimated from the score

6.5 Conclusion

The type of feeding system used to nourish dairy cows was shown to have a significant effect on their rumen and milk metabolomes. Overall, the TMR group has higher levels of sugars (i.e maltose), while the CLV group has higher amino acids levels in the rumen. The GRS group has medium levels of rumen amino acids and high levels of nucleosides such as inosine and adenine. Among other compounds, increased concentrations of volatile fatty acids (i.e acetic acid), an important source of energy for the cow, were detected in the rumen of cows associated with TMR and CLV feeding systems. Pasture feeding systems resulted in significantly higher concentrations of rumen isoacids which can contribute to milk production. Both CLV and GRS rumen were found to have increased concentrations of *p*-cresol, a product of rumen metabolism. The CLV feeding system resulted in increased rumen concentrations of formate, a substrate compound for methanogenesis. The TMR feeding system resulted in significantly higher rumen choline content which can contribute to animal health and milk production, and succinate a product of carbohydrate metabolism. Milk and rumen-fluids were shown to have varying levels of dimethyl sulfone which was highest in pasture derived products. Such differences in the rumen metabolome would suggest alterations to the rumen microbiota activity as a result of the dietary source. When analysing milk we found that CLV and TMR feeding systems resulted in increased concentrations of milk urea, which can be indicative of nitrogen metabolism efficiency in the rumen and can negatively affect milk protein quality with increased non-protein nitrogen content. Milk from pasture based feeding systems was also shown to have significantly higher concentrations of hippuric acid, a compound which has previously been suggested to be a biomarker of pasture feeding systems in milk. Finally, this study has highlighted that ^{1}H-NMR is capable of distinguishing both rumen-fluids and milk samples based on feeding system. This observation suggests that NMR based metabolomics could be a potential tool for milk verification purposes in the future as "pasture" milk and dairy products become more popular with consumers.

6.6 Acknowledements

This publication has emanated from research conducted with the financial support of Teagasc, Science Foundation Ireland (SFI) under Grant Number SFI/12/RC/2273 and the Dairy Levy Fund administered by Dairy Research Ireland. Tom F. O'Callaghan is the recipient of a Teagasc Walsh Fellowship. Tom F O'Callaghan was the recipient of a Teagasc overseas training award to travel to TMIC at the University of Alberta, Edmonton, Canada. The authors sincerely thank the technical and farm staff at Moorepark for their excellent care of the experimental cows and assistance during the experiment.

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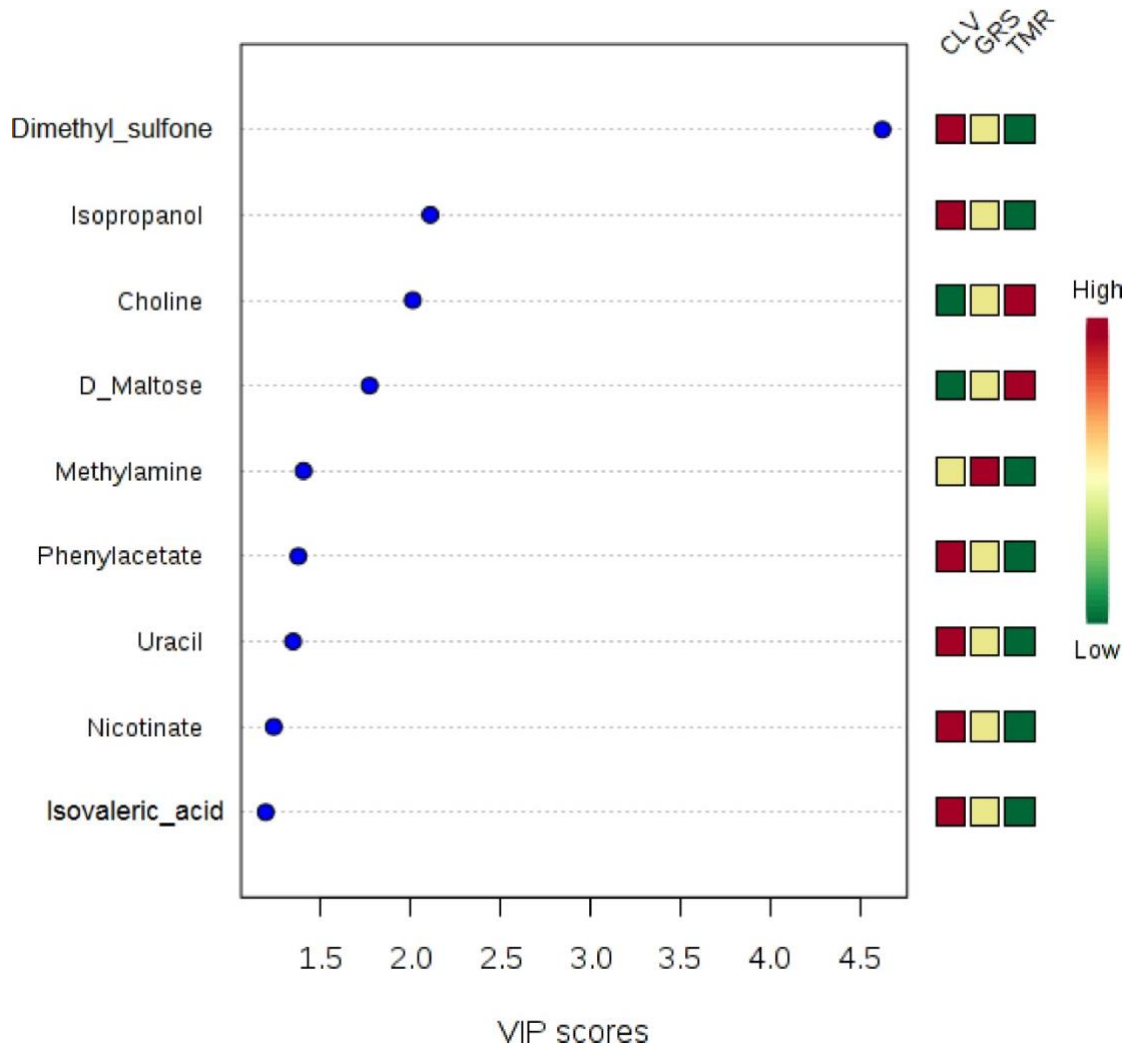
Supplementary Materials

Supplementary Table 1: Average concentrations of rumen metabolites (µM) measured in the rumen of lactating dairy cows fed diets consisting of total mixed ration (TMR), perennial ryegrass (GRS) or perennial ryegrass and white clover (CLV) throughout each stage of lactation early mid and late as determined by ^{1}H-NMR.

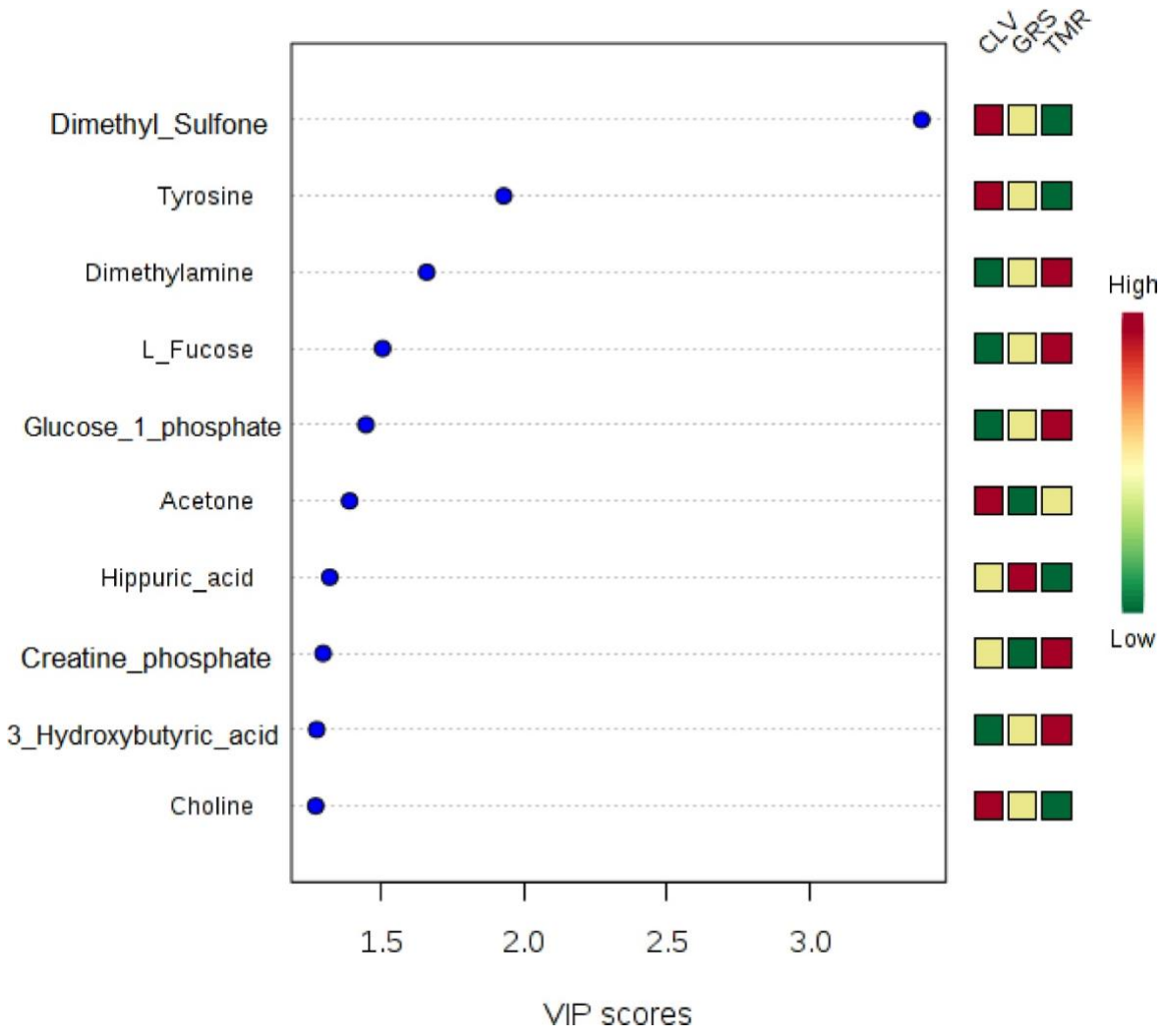
	Early-Lactation			Mid-Lactation			Late-Lactation			SEM
Compound (µ*M*)	TMR	GRS	CLV	TMR	GRS	CLV	TMR	GRS	CLV	
2-Hydroxyisovalerate	4.57	8.48	8.53	10.71	5.74	5.50	5.89	4.29	8.44	0.74
3-Hydroxybutyric acid	6.80	7.83	9.22	7.55	10.85	12.63	23.98	7.35	16.05	1.56
3-Hydroxyphenylacetic acid	23.01	27.90	22.28	36.44	26.53	27.88	20.68	12.80	18.39	1.20
3-Phenylpropionate	779.59	717.96	554.52	761.80	704.73	759.75	696.33	476.13	588.32	19.66
4-Aminobutyrate	22.71	49.42	36.95	36.29	50.38	56.43	50.16	56.67	52.93	3.56
Acetic acid	56,450.48	55,371.36	57,153.50	59,611.64	55,080.78	59,988.47	49,825.50	54,056.92	57,825.81	834.44
Acetoin	20.89	20.64	21.65	25.69	22.45	25.61	29.64	21.00	28.60	1.10
Acetone	6.14	8.42	6.62	9.86	8.84	14.48	10.00	8.34	15.01	0.61
Adenine	29.17	31.67	42.77	29.28	35.09	37.18	20.86	11.59	11.68	2.06
Adenosine	3.27	6.70	3.14	6.29	6.40	8.43	4.09	7.24	3.67	0.60
Aspartate	115.08	96.53	122.66	144.89	158.90	217.53	199.76	92.07	103.86	8.45
Benzoic acid	25.42	30.23	28.39	25.50	27.81	26.28	20.71	20.91	27.73	0.82
Beta Alanine	7.38	11.08	7.21	14.03	27.64	29.24	33.88	16.00	15.88	2.00
Betaine	9.76	4.38	3.98	7.73	8.03	4.07	3.23	2.83	3.35	0.96
Butyrate	11,884.27	15,544.54	13,741.89	13,676.94	14,568.74	16,162.84	12,194.68	12,622.88	14,281.63	352.66
Cadaverine	53.46	128.58	109.88	82.03	93.26	107.44	111.32	67.68	103.96	7.00
Choline	20.93	11.82	13.28	20.48	16.65	15.76	36.26	15.57	8.73	1.62
cis Aconitate	4.19	9.40	4.99	7.88	11.74	8.08	4.66	4.24	9.71	0.90
Citric acid	3.31	5.53	5.41	11.23	9.21	7.85	7.85	7.85	6.61	0.66
Creatine	7.71	6.76	7.08	9.90	8.13	6.92	8.46	5.90	6.79	0.66
D-Glucose	426.25	844.25	784.76	755.50	641.33	913.27	380.15	149.02	108.05	55.14
Dimethyl sulfone	6.04	19.23	37.43	3.03	14.60	36.46	2.43	16.59	26.23	2.00
Dimethylamine	1.68	3.14	2.08	8.06	1.82	9.18	3.02	1.97	3.13	0.96
Dimethylglycine	7.72	7.09	4.91	15.24	4.68	8.22	19.87	1.88	8.60	2.07

D-Maltose	124.21	58.00	43.83	31.74	29.01	39.19	57.68	16.20	17.03	6.30
Ethanol	12.53	112.58	27.92	21.71	37.59	42.91	42.65	27.70	24.28	8.88
Ethanolamine	28.92	32.07	34.83	37.34	37.35	43.58	21.87	15.35	10.03	2.07
Formate	115.52	114.01	116.01	121.81	113.23	119.66	118.16	117.49	118.62	0.60
Glycerol	209.23	220.06	219.03	286.20	270.45	294.14	269.32	236.48	233.55	6.28
Glycine	86.27	104.25	135.53	131.22	156.95	210.20	146.98	96.28	129.71	6.69
Hypoxanthine	161.40	169.98	213.58	190.70	203.38	213.12	161.39	104.28	111.55	7.38
Inosine	12.98	50.25	38.24	13.83	23.43	22.48	7.76	8.04	5.71	2.63
Isobutyric acid	808.14	745.82	775.18	818.37	859.50	1156.38	763.78	830.53	1061.15	21.81
Isoleucine	62.98	61.93	91.98	90.16	112.33	131.68	100.18	76.12	93.63	4.58
Isopropanol	16.71	23.59	26.03	19.85	26.48	63.61	17.92	16.67	36.89	2.57
Isovaleric acid	711.27	600.31	605.88	671.78	754.82	1187.49	667.28	748.80	1059.89	30.43
L-Glutamic acid	242.93	261.69	366.75	311.73	298.38	403.02	327.27	196.13	281.97	12.09
L-Alanine	139.00	189.28	211.52	206.31	231.38	306.74	195.89	148.08	198.38	8.72
L-Histidine	22.80	36.49	27.76	32.86	33.98	28.78	43.23	39.58	46.94	1.84
L-Lactic acid	14.46	20.63	18.28	26.22	18.61	36.30	51.30	48.99	27.90	4.02
L-Leucine	72.92	79.11	101.35	96.06	117.16	153.91	100.20	85.28	103.47	4.49
L-Lysine	143.95	170.81	176.06	202.61	240.94	323.59	102.05	109.71	118.18	12.56
L-Phenylalanine	42.16	43.90	50.09	58.58	68.03	78.82	53.72	40.00	44.38	2.53
L-Proline	77.17	67.54	95.11	86.45	107.08	161.58	89.36	66.88	68.31	6.43
L-Threonine	67.54	67.70	91.85	117.76	110.78	139.98	140.72	77.84	88.00	5.34
Methanol	10.76	9.68	11.35	10.78	10.37	13.90	19.68	9.01	9.08	1.17
Methionine	26.29	32.48	38.10	37.99	45.61	51.26	36.60	27.32	32.93	1.65
Methylamine	1.40	18.28	4.95	3.12	8.82	41.14	9.41	7.08	2.66	3.05
Nicotinate	24.98	35.88	42.51	31.36	30.60	37.12	20.97	19.21	28.18	1.33
O-Hydroxyphenylacetic acid	15.75	12.81	11.63	20.66	14.62	21.82	15.73	11.82	13.53	0.62
p-Cresol	55.29	43.48	37.74	57.63	75.38	110.54	62.23	78.99	107.27	4.00
Phenylacetate	202.23	159.84	157.23	194.39	318.03	536.45	202.11	308.47	449.38	20.18
p-Hydroxyphenylacetic acid	17.39	15.03	14.67	16.88	15.52	12.45	19.29	13.32	14.19	0.62

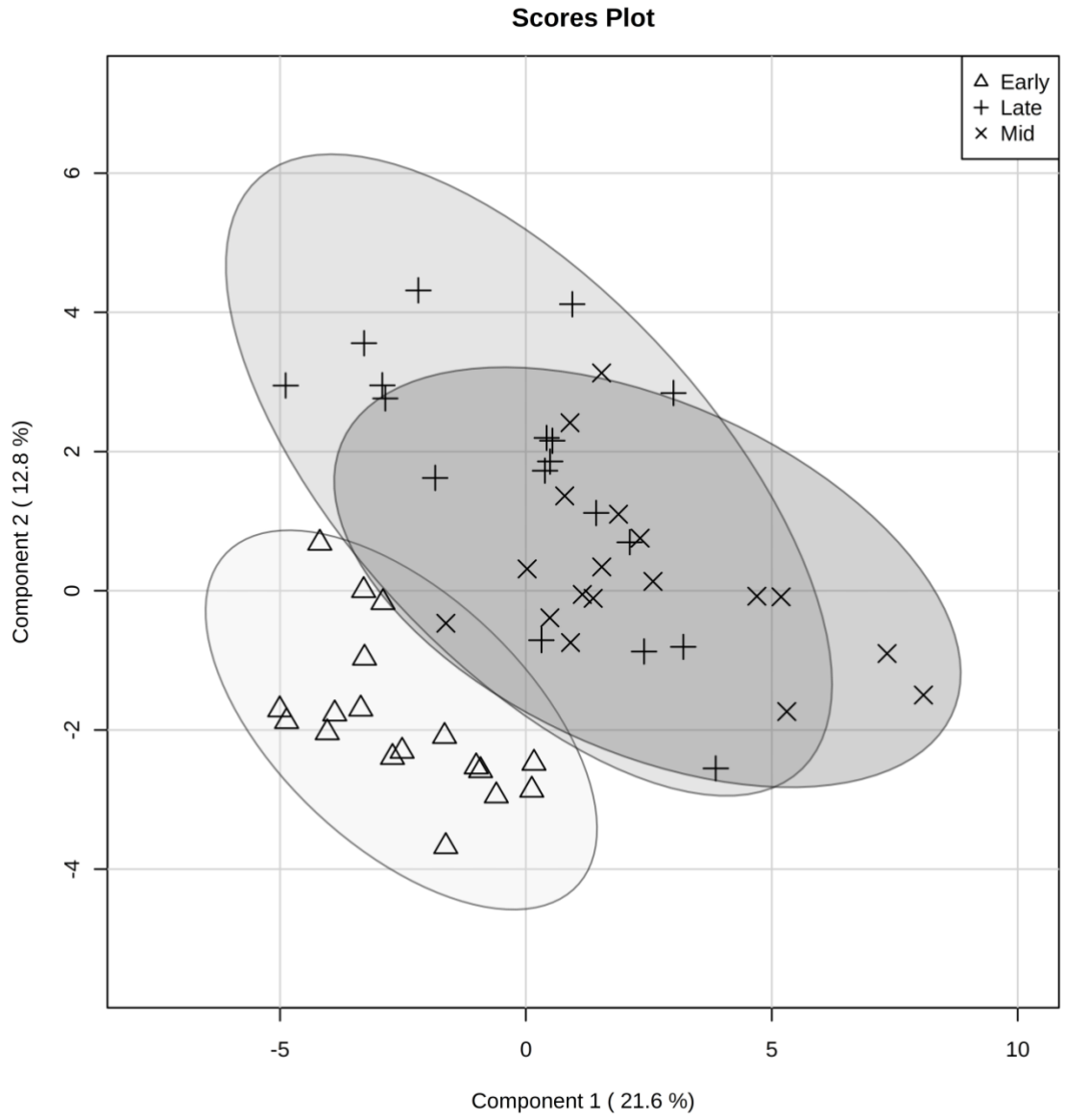
Propionate	17,764.88	20,803.31	18,280.66	19,344.51	17,120.38	17,982.38	14,248.39	16,330.55	18,159.29	463.30
Putrescine	64.55	89.79	59.37	65.59	53.28	48.96	46.63	26.36	28.37	4.77
Succinate	48.88	40.93	43.84	64.01	82.49	107.18	257.82	100.28	121.76	11.31
Trimethylamine	1.94	1.64	1.35	2.10	2.67	13.55	13.35	1.25	1.30	1.33
Tryptophan	6.21	7.33	8.28	7.97	9.41	10.69	7.34	5.42	5.59	0.37
Tyrosine	32.46	33.49	47.99	48.98	57.79	72.48	51.37	39.38	47.68	2.38
Uracil	225.92	287.28	350.22	285.07	364.46	373.89	90.48	140.81	168.73	14.57
Uridine	5.86	12.35	9.84	7.33	6.15	4.83	9.02	3.97	11.75	0.97
Valerate	1,020.82	1,715.66	1,141.45	1,201.26	1,212.65	1,611.75	869.19	842.36	1,231.22	62.85
Valine	71.25	71.60	92.41	106.05	118.54	191.78	103.48	81.25	102.18	6.42



Supplementary Figure 6.1: VIP compounds primarily responsible for separation of rumen-fluid metabolomes from cows fed diets consisting of total mixed ration (TMR), perennial ryegrass (GRS) or perennial ryegrass and white clover (CLV) as determined by PLS-DA.



Supplementary Figure 6.2: Variable importance plot (VIP) which shows the compounds primarily responsible for separation of raw milk metabolomes from cows fed diets consisting of total mixed ration (TMR), perennial ryegrass (GRS) or perennial ryegrass and white clover (CLV) for the PLS-DA model.



Supplementary Figure 6.3: Score plot of the partial least square descriptive analysis (PLS-DA) examining the effect of stage of lactation on the rumen metabolome of lactating dairy cows fed separate diets collected throughout each stage of lactation early, mid and late, as determined by ^{1}H-NMR.

Chapter 7

General Discussion and Conclusion

7.1 Scientific and industrial impact of the research

The agriculture and dairy industry forms a major part of Ireland's culture and heritage, while significantly contributing to the country's annual GDP. In 2016, the value of food and drink exports from Ireland was €11.15 bn, of which dairy and ingredients accounted for €3.38 bn, achieved through exports to 155 markets worldwide. The major markets for Irish dairy products include the UK, China, the Netherlands, Germany and the US; with cheese and butter being major products of export [1]. Ireland is one of the minority countries in the world with a suitable temperate climate to practise low input pasture based feeding systems for the majority of the cows lactation. The use and proper management of pasture based feeding systems is highly beneficial to the Irish farmer as it provides a relatively cheap form of nutrition for the production of high quality milk and dairy products, compared to high input expensive TMR systems which are heavily reliant on the use of concentrates and silages as opposed to fresh pasture. Coupled with this, in terms of the consumer, there is an increased demand for pasture-derived dairy products, resulting from consumer perceptions of a healthier and more natural product and improved animal welfare compared to the more conventional indoor TMR feeding systems [2]. This consumer perception is heavily exploited by manufacturers and organisations such as Ornua and Bord Bia who are responsible for the marketing and sale of Irish dairy to international markets, but with limited scientific information, especially from an Irish perspective, to advocate this. Furthermore, with the increasing prevalence of "pasture derived" labelling of products, there is essentially no methodology available for the verification of pasture derived dairy products. The purpose of this thesis was to address these gaps in knowledge and examine the effects of cow feeding systems in Ireland, namely a perennial ryegrass pasture system (GRS), a perennial ryegrass and white clover pasture system (CLV) and an indoor total mixed ration system (TMR) on the composition, quality and functionality of raw milk, dairy products (including sweet cream butter and Cheddar cheese) and the rumen microbiome.

7.2 Summary

The first study, as described in Chapter 2 examined the effects of bovine diet on the composition and quality of raw milk throughout an entire lactation. This study highlighted significant differences in the macro-nutrient composition of milks, with increased concentrations of protein and fat in pasture derived milks. Increased concentrations of these macronutrients are beneficial for manufacturers with increased product yields and efficiency in manufacture of protein products and butter. However, the CLV derived feeding system had significantly higher non protein nitrogen (NPN) content than that of GRS. This could be a concern for Irish manufacturers whose current payment schemes are based on a crude protein basis, with resultant losses in N and subsequent yield during manufacturing occurring via permeation of NPN during filtration processes for the manufacture of high protein ingredients. Furthermore, pasture derived milks were shown to have a more health beneficial fatty acid profile with increased concentrations of beneficial nutrients compared to that of TMR, including concentrations of omega 3 fatty acids, vaccenic acid and conjugated linoleic acid (CLA), while TMR derived milk had significantly higher concentrations of omega 6 fatty acids, palmitic acid and higher thrombogenic index. Such data offer initial evidence to endorse consumer perceptions of pasture derived milks as being nutritionally superior over milk produced from indoor feeding systems. Milk with naturally higher levels of omega 3 fatty acids along with other beneficial nutrients, when consumed on a regular basis, could improve omega 3 fatty acid status and potentially aid in counteracting the negative effect of the Western diet on human health [3].

Chapter 3 examined the effects of feeding system on quality aspects of sweet cream mid lactation butter. As such, this study demonstrated the effects of diet induced altered milk fat composition on the characteristics, quality, textural and sensory properties of butter. Pasture feeding (GRS or CLV) had a similar beneficial effect on the nutritional composition of butter fat as previously seen for raw whole milk (Chapter 2). However, alterations to the fatty acid

profiles also resulted in significant differences in butter textural properties. TMR butters were shown to have higher melting points, the temperature for onset of fat crystallisation, and as a result it was more solid at room temperature than butter derived from milk produced from pasture. These differences derived from alterations in the fatty acid composition, most notably increased levels of palmitic acid, which could be an important consideration for confectionary applications of butter. Notably, pasture feeding was shown to produce butters that were more yellow in colour as a result of increased concentrations of β-carotene. The yellow colour is characteristic of Irish butters and could be beneficial for the sale to certain markets carrying perceptions of fresh grass feeding, however it can also be disadvantageous when exporting to colour sensitive markets such as the Middle East [4]. Such changes also had an effect on the volatile and sensory profile of the butters as pasture derived butter scored significantly higher in attributes such as "liking" of appearance, colour and flavour.

Chapter 4 examined the effects of feeding system on Cheddar cheese quality. As such, this study demonstrated the effects of diet induced altered milk composition on the composition, quality, textural and sensory properties of Cheddar cheese throughout 270 d ripening. Pasture feeding was shown to have a beneficial effect on the nutritional composition of Cheddar cheese similar to milk and butter. However, given the increased levels of solids consumption of cheese as opposed to milk and butter per meal, significantly increased consumption of beneficial nutrients with this product is possible. Thus, this study demonstrated that less than half the amount of pasture derived cheese than that of TMR derived cheese is required in the daily diet to meet the minimum reported recommended CLA intake of 0.8 g/day [5]. Both feeding system and ripening time had an effect on the textural and volatile attributes of cheeses where the oleic to palmitic acid ratio was negatively correlated with cheese hardness, and increased ripening time induced proteolysis which resulted in a decline in cheese textural attributes that could have important implications for consumer acceptability. In each of the product streams (milk, butter and Cheddar cheese) fatty acid profiling coupled with

multivariate statistical analysis showed clear separation between pasture and TMR products offering insight into the ability to verify such pasture derived dairy products by fatty acid profiling.

Chapter 5 examined the effects of diet on the rumen microbiota. While no significant effect of diet on the microbiota was observed, this is likely as a result of the shortened adaptation time. This study did highlight that the rumen microbiota is remarkably diverse and the majority of its composition in both the liquid and solid fractions is still undiscovered. Thus, it emphasized the need for future exploratory culturing and screening experiments to further understand this complex ecosystem and attain a greater understanding of rumen functionality and metabolism.

Chapter 6 examined the implications of cow diet on the rumen fluid and milk metabolome. Unlike microbiota composition, as reported in Chapter 5, there were significant effects of diet on the rumen metabolome from each of the feeding systems which would suggest alterations to the rumen microbiota activity as a result of the dietary source. The CLV and TMR rumen fluid samples were shown to have increased levels of volatile fatty acids (VFA) i.e. acetic acid. As VFA contribute energy to the cow this could contribute to explaining the increased milk yields of TMR and CLV feeding systems reported in Chapter 1. Pasture feeding systems were demonstrated to have increased concentrations of isoacids which can also contribute to milk production. While perennial rye grass pastures have previously been shown to produce less methane than that of TMR [6], CLV feeding resulted in increased concentration of rumen formate, a substrate for methanogenesis. With regards to the milk metabolome, CLV derived milk was shown to contain significantly higher concentrations of urea, which would be indicative of decreased efficiency of N utilisation [7], but would also account for the increased levels of NPN in CLV derived milk described in Chapter 2. This study has highlighted that ^{1}H-NMR is capable of distinguishing both rumen-fluids and milk samples based on feeding

system, suggesting that NMR based metabolomics have potential as a tool for milk verification purposes in the future as "pasture" milk and dairy products become more popular with consumers.

7.3 Looking to the future

Since the 1960's the consumption of milk and fat containing dairy products has been criticized. This criticism is as a result of its high concentrations of saturated fatty acids, some of which have been shown to increase serum LDL cholesterol concentration, which can be linked with increased risk of coronary heart disease (CHD). As such in the 1980's the US Department of Agriculture and the UK both released dietary guidelines recommending a reduction in the consumption of fat and saturated fat in the diet [8]. More recent research however has found contradicting results on this topic. A meta-analysis of prospective epidemiologic studies concluded that there is no significant evidence suggesting that dietary saturated fat is associated with an increased risk of CHD or cardiovascular disease (CVD) [9]. Furthermore, there is growing evidence that saturated fatty acids in the context of dairy foods, particularly fermented dairy products, have neutral or inverse associations with CVD and that the replacement of saturated fatty acids in the diet with carbohydrates may have had a detrimental effect [10]. Chapters 2, 3 and 4 of this thesis have highlighted that pasture derived dairy products are naturally enriched with significantly higher concentrations of beneficial nutrients such as omega 3 fatty acids, CLA, ALA, vaccenic acid, lower levels of omega 6 fatty acids and palmitic acid similar to trends reported by Kelly*, et al.* [11], Couvreur*, et al.* [12] and Stanton*, et al.* [13] previously. Therefore the next logical step in this research and in the argument for benefits of pasture versus conventional indoor TMR feeding systems derived dairy, would be to confirm the health effects, ideally in humans, and also by conducting animal trials to investigate mechanistic aspect, using pigs or mice. Such models should include animals consuming dairy fat containing products such as Cheddar cheese from pasture and TMR systems and tracking a variety of health attributes and indices such as hypertension, glucose intolerance, obesity,

dyslipidemia, serum LDL cholesterol and total cholesterol:HDL. Such studies could yield some valuable and meaningful data and could aid in the further promotion of pasture derived products. The application of more in-depth and emerging technologies to the milk and dairy products from pasture based diets could also further highlight significant differences in their nutritional composition. This study focused on a particular selection of major triglycerides within the fat fraction, which accounted for approximately 70% of dairy fat in chapters two, three and four, however, there are a large number of other fatty acids in milk not analysed for in this study as well as other portions of milk fat including phospholipids and cholesterol which could be of interest. While traditional GC-FID has been a standard method for the analysis of fatty acids, two dimensional GC x GC could be beneficial in further elucidating differences in the fatty acid profile of milks and products. A recent study by Bergamaschi and Bittante [14] using two-dimensional GC yielded a detailed and informative FA profile on 11 dairy products from cows grazing summer highland pastures. A similar approach investigating the effects of pasture and TMR diets could be useful in further understanding the effect of diet on the fatty acid profile of milks and dairy products. Chapters 3 and 4 of this thesis examining butter and Cheddar cheese respectively, and a study by Faulkner*, et al.* [15] examining the milks from these diets, demonstrated that products from pasture based systems had a distinguishable volatile profile to that of TMR. The future application of gas chromatography analysis with olfactometric detection (GC-O) could be very beneficial in further understanding the contribution of each volatile compound to these products sensory characteristics. Furthermore, the use of international sensory panels to examine the sensory pallet of major markets such as the US or China will be an important next step to fully understand the suitability of pasture derived products to such regions consumer, and gain a greater understanding of favourable or undesirable sensory characteristics.

Chapter 6 demonstrated that cows diet had a significant effect on the rumen metabolome suggesting an alteration to the rumen microbiota, although no differences in composition of

the microbiota, per se, were noted (Chapter 5). Further in depth examination of rumen function using full SHOTGUN sequencing or Meta-transcriptomics would provide an in-depth understanding of the effects of diet on the rumen microbiota function, animal health and performance. The use of strictly anaerobic culture techniques to further classify the dominant undiscovered fraction of the rumen microbiota may uncover novel species of bacteria with potentially beneficial functional properties and characteristics as well as aid in the further understanding of ruminant metabolism.

7.4 Final Conclusions

This work has contributed scientific data to the fields of nutrition and dairy chemistry providing valuable information of interest to dairy producers, manufactures, marketers, academia and consumers alike. In conclusion this work has demonstrated:

1) Pasture based feeding has a beneficial impact on the nutritional composition of milk and dairy products at both macro nutrient and fatty acid levels.
2) Diet induced alterations to milk results in butter and cheese products with significantly altered textural, volatile and sensory properties.
3) Fatty acid profiling is capable of distinguishing between pasture and TMR derived products offering insight into the ability to verify such pasture derived dairy products.
4) Irish sensory panellists demonstrated a preference for dairy products derived from pasture based diets over TMR feeding system.
5) Pasture derived dairy products are more yellow in colour than those derived from TMR feeding system, as a result of increased levels of β-carotene in the milk from the former compared to the latter animal feeding system.
6) Several compounds have been identified as being significantly higher in pasture derived products and show potential to be biomarkers of feeding systems including, CLA, β-carotene, toluene, and hippuric acid.
7) Feeding system resulted in significant alteration to the rumen and milk metabolome, but did not result in differences in microbiota composition.
8) NMR based metabolomics offer potential as a tool for differentiating milk derived from pasture based feeding system over TMR, based on differences in the milk metabolome.

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